Advances in CLINICAL CHEMISTRY

VOLUME 44

Edited by

Gregory S. Makowski

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PREFACE

Volume 44 of *Advances in Clinical Chemistry* contains a series of chapters of diverse interest for clinical laboratory scientists.

In this volume, the role of caspases as key effector molecules in apoptosis is explored with respect to their impact at multiple points in the evolution of myocardial infarction. Their use as potential targets for therapeutic intervention provides an attractive alternative for pharmacological modulation of this disease process. Advances in biochemical identification of antibody targets such as deamidated gliadin in celiac disease are also presented in this volume. Immunoassays that target the cellular receptor for urokinase type plasminogen activator may provide an important analytical tool in the prognosis of cancer in patients. A critical review of proteomic studies is also highlighted in this volume with emphasis on study design, data organization, formatting, and mining. The pathogenesis of paraneoplastic syndromes is explored with respect to its heterogeneous nature and its potential role in early identification of cancer. An interesting review of the biochemistry of angiogenesis is presented. Markers associated with process may provide unique insight in assessment of human disease. Bikunin, a urinary trypsin protease inhibitor, is explored with respect to its role as a biomarker in a variety of disease states, including infection, cancer, and kidney and vascular disease. Finally, advances in gene expression assays are reviewed in this volume with illustrative examples in the fields of cancer and neuroscience. These assays provide a unique view of pathophysiological processes in breast cancer, Huntington's disease, and schizophrenia.

I extend my appreciation to each contributor of Volume 44 and thank colleagues who participated in the peer review process. I also extend my sincere thanks to my editorial liaison at Elsevier, Ms. Pat Gonzalez for her continued support.

I hope the second volume of 2007 will be enjoyed and stimulate thoughts. I actively invite comments of the readership so that the high quality of chapters for the *Advances in Clinical Chemistry* series will be maintained.

In keeping with the tradition of the series, I would like to dedicate Volume 44 to my father Edmund.

GREGORY S. MAKOWSKI

CASPASES IN MYOCARDIAL INFARCTION

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1. Abstract

The discovery of apoptosis sheds a new light on the role of cell death in myocardial infarction and other cardiovascular diseases. There is mounting evidence that apoptosis plays an important role at multiple points in the evolution of myocardial infarction, and comprises not only cardiomyocytes but also inflammatory cells, as well as cells of granulation tissue and

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fibrous tissue.It appears that apoptosis contributes to cardiomyocyte loss in the border zone and in remote myocardium in the early phase, as well as months after myocardial infarction, thus playing a role in remodeling and development of heart failure after myocardial infarction.

Apoptosis, being a highly regulated process, is a potential target for therapeutic intervention. Caspases are the key effector molecules in apoptosis, and are therefore a particularly attractive target for pharmacological modulation of apoptosis. Although several potential therapeutic agents have been tested in animal models of ischemia/reperfusion heart injury with some success, nearly none of the specific antiapoptotic agents have reached the stage of clinical research.

2. Introduction

Cardiovascular diseases, including high blood pressure, coronary heart disease, congestive heart failure, stroke, and congenital heart defects, are the leading cause of death in the Western world, claiming more lives than any of other major illness. It is well accepted that cardiovascular diseases leading to heart failure involve not only contractile dysfunction but also death of cardiomyocytes. Death of cardiomyocytes is the ultimate result of convergence of multiple signaling pathways during cardiovascular diseases, triggered by events such as nutrient and oxygen deprivation, ion imbalance, and excessive production of reactive oxygen species.

In the heart, cell death can occur by necrosis, apoptosis, or autophagy [1–3]. Necrosis is characterized by organelle swelling and disruption of cellular membranes, releasing their cytoplasmic and nuclear contents in the surrounding tissue where they elicit an inflammatory response. The term ''necrosis'' is believed by some to be inappropriate and has been proposed to be replaced by the term ''oncosis'' to denote ischemic cell death accompanied by swelling (derived from ''onkos,'' meaning swelling) [4]. Accordingly, the term ''necrosis'' should be reserved for the changes that occur after cell death regardless of the pathway by which the cells died [5].

In contrast, apoptosis is characterized by preserved cell boundaries with minimal inflammation. The characteristic morphological changes include shrinkage of the cytoplasm, membrane blebbing, condensation of the nuclear chromatin, chromosomal DNA fragmentation, as well as fragmentation and packaging of cellular contents into membrane-enclosed vesicles (apoptotic bodies), which are phagocytosed by macrophages or neighboring cells [6].

These distinct morphological changes of cells undergoing apoptosis are accompanied by a number of biochemical changes: degradation of the chromosomal DNA into high molecular weight [7, 8] and oligonucleosomal fragments [9], cleavage of a specific subset of cellular polypeptides [10–12],

as well as externalization of phosphatidylserine at the cell surface [13] and other changes which enable recognition by phagocytes [14]. Cleavage of specific cellular polypeptides is accomplished by a family of enzymes termed caspases.

In autophagy, proteins and organelles are degraded in the lysosomal pathway so that their constituents can be used as energy substrates by the cell. As with apoptosis, autophagic cell death is regulated and associated with DNA fragmentation, but unlike apoptosis it is caspase independent and morphologically resembles necrosis [15].

3. Apoptosis and the Heart

Apoptosis or programed cell death is an essential physiological process which plays a critical role in controlling the number of cells in development and throughout life by removal of cells at the appropriate time. However, apoptosis is also involved in a wide range of pathological conditions, including immunological disorders, neurodegenerative diseases, acquired immunodeficiency syndrome, and cancer [16]. The notion that apoptosis might be an important mechanism in the development of the heart and in the pathogenesis of cardiovascular diseases emerged relatively late compared to other fields of medicine, possibly because characteristic morphological features are less frequently observed in the heart than in some other tissues, for example, epithelial tissues. However, there is mounting evidence that apoptosis contributes to the normal embryonal development of the heart similar to other organs. After birth, apoptosis is assumed to be involved in the morphogenesis of the conduction system, including the sinus node, AV node, and His bundle, as well as in different heart diseases such as ischemic heart disease, heart failure, idiopathic dilated cardiomyopathy, and myocarditis [17, 18].

Despite the extensive research efforts during the last 15 years, the relative contribution of apoptosis and necrosis to cell loss in heart disease remains controversial. The type of cell death in the heart often depends on the time in the natural history when it is studied: necrosis is a feature of early heart failure, especially if caused by ischemia, but the cause of cell death in chronic heart failure is mainly apoptotic or autophagic [19–21]. Moreover, it is possible that apoptotic and necrotic cell deaths represent two ends of continuum of response to an injury [22]: cells may initially begin to die from apoptotic mechanism, but as cellular energy declines, the cells continue to die of necrosis [23].

4. Caspases

At the molecular level, apoptosis is tightly regulated and is mainly orchestrated by the activation of caspases [24]. Caspases, or cystein-dependent aspartate-specific proteases, are a family of cysteine proteases which have

specificity for tetrapeptide motifs-containing aspartate and cleave their substrates after the aspartate residue. In humans, caspases are ubiquitously expressed cytosolic proteases synthesized as zymogens (inactive enzyme precursors, procaspases, which require a biochemical change to become an active enzyme). Procaspase consists of an N-terminal prodomain, followed by a large subunit of about 20 kDa, p20, and a small subunit of about 10 kDa, p10. It becomes activated by proteolytic cleavage at Asp residues between the different subdomains, which leads to the formation of an active caspase, a tetramer consisting of two large and two small subunits [25, 26], and causes the release of the prodomain.

Fourteen caspases have been identified in mammals, referred to as caspase-1 to -14, and at least seven of them are involved in apoptosis. Depending on the structure of the prodomain and its function, caspases are divided into three groups. Caspases with a large prodomain are referred to as inflammatory caspases belonging to group I (caspase-1, -4, -5, -11, -12, and -14) and initiator caspases belonging to group II (caspase-2, -8, -9, and -10). Caspases with a short prodomain are named effector caspases and belong to the group III (caspase-3, -6, and, -7) [27, 28].

The large prodomains of procaspases contain structural motifs that belong to the death domain (DD) superfamily involved in the transduction of the apoptotic signals. This superfamily consists of the DD, the death effector domain (DED), and the caspase recruitment domain (CARD). Each of these motifs interacts with other proteins by homotypic interactions. DED is found in procaspase-8 and -10, and CARD is found in procaspase-1, -2, -4, -5, -9, -11, and -12. DED and CARD are responsible for the recruitment of initiator caspases into death- or inflammation-inducing signaling complexes, resulting in proteolytic autoactivation of caspases that subsequently initiates inflammation and apoptosis [26, 29, 30].

The short prodomains found in the three effector caspases have no clearly defined function, and their structure is currently unknown. As they are removed during caspase activation, further investigation on this domain would probably reveal interesting findings [25].

5. Activation of Caspases

Signaling for apoptosis can be initiated from outside the cell (extrinsic or death receptor pathway) or from inside the cell (intrinsic or mitochondrial pathway) [31, 32]. In both pathways, signaling results in the activation of initiator caspases. Active initiator caspases then sequentially activate downstream effector caspases such as caspase -3, -6, and -7. Once activated, caspases cleave a variety of intracellular polypeptides, including major structural elements of the cytoplasm and nucleus, components of the DNA repair

machinery, and a number of protein kinases disassembling important architectural components of the cell, contributing to the morphological and biochemical changes characteristic of apoptotic cell death.

5.1. Extrinsic (Death Receptor) Pathway of Caspase Activation

Extrinsic (death receptor) pathway of caspase activation during apoptosis involves the binding of death ligands to cell surface receptors (e.g., Fas/CD95/Apo-1 or TNF receptor), recruitment of adaptor molecules Fas-associated death domain (FADD) or TNF receptor-associated death domain (TRADD) to the cytosolic end of the receptor, and formation of the death-inducing signaling complex (DISC) at the plasma membrane. DISC recruits and activates the initiator caspases, caspase-8 or -10.

There are two different cell types concerning further CD95-induced caspase activation pathway. In so-called type I cells, high amount of active caspase-8 is formed at DISC, which directly cleaves and activates downstream procaspase-3. In type II cells, including cardiomyocytes, the DISC formation is reduced, but enough active caspase-8 is generated to cleave and activate the cytosolic protein BID (BH3 interaction domain death agonist), a proapoptotic Bcl-2 protein, which in turn causes mitochondrial damage leading to the activation of caspase-9. Caspase-9 activates procaspase-3 and links the extrinsic and intrinsic pathways [26].

5.2. Intrinsic (Mitochondrial) Pathway of Caspase Activation

Intrinsic (mitochondrial) pathway of caspase activation is initiated by the permeabilization of the mitochondrial outer membrane by proapoptotic members of the Bcl-2 family, resulting in a release of cytochrome *c* and other proteins from the intermembrane space of mitochondria into the cytosol. Cytochrome *c* translocation to the cytosol may follow a number of possible mechanisms. However, once in the cytosol, cytochrome *c* binds to apoptosis protease activating factor (Apaf-1) and in the presence of dATP or ATP facilitates Apaf-1 oligomerization and the recruitment of procaspase-9. The formation of this caspase-activating complex, termed the apoptosome, results in the activation of procaspase-9, and this in turn cleaves and activates the effector caspase-3 and -7. Activated effector caspases cleave key substrates in the cell and produce the cellular and biochemical events characteristic for apoptosis [33–35].

6. Activation of Caspases in the Heart

There is evidence to suggest that both extrinsic and intrinsic pathways of caspase activation occur in the heart [36]. Some other signaling pathways have been suggested to mediate apoptosis in cardiomyocytes [37–39].

However, it appears that in cardiomyocytes, being typical type II cells, apoptosis predominantly proceeds via the intrinsic (mitochondrial) pathway [3, 40].

Mitochondrial pathway of caspase activation has been demonstrated in various experimental models and humans to contribute significantly to cardiomyocyte apoptosis in the heart [41–44]. Studies suggest that mitochondrial-mediated apoptosis contributes to cardiomyocyte loss through intermembrane space proteins release, Bcl-2 protein involvement, and procaspase activation [41, 45, 46]. Due to the energy required by heart muscle, mitochondria are particularly abundant in cardiomyocytes. Under physiological conditions, prosurvival mechanisms exist to protect the myocardium from inappropriately triggered apoptosis [3].

In contrast to the mitochondrial pathway, the role of the death receptor pathway in ischemia/reperfusion injury is controversial. It appears that cardiomyocytes are relatively resistant to Fas-mediated apoptosis. Nevertheless, recent studies suggest a potential role of Fas in the heart disease. Soluble Fas ligand was found to be elevated in patients with congestive heart failure [47], and Fas mRNA was expressed in the failing human myocardium [48]. Moreover, there is increasing evidence that Fas can induce cardiomyocyte apoptosis during ischemia/reperfusion *in vivo* [49, 50]. Gomez *et al.* [51], on the contrary, reported that Fas pathway plays a minor role in a prolonged ischemia/reperfusion injury in mice.

It is controversial whether ischemia/reperfusion injury to the heart is mediated by different activation pathways. In a rat model of ischemia/reperfusion injury, caspase-9 was demonstrated to be activated during ischemia and remained activated throughout reperfusion. In contrast, activation of caspase-8 was only triggered during reperfusion. Therefore, different initiator caspases might be activated during different phases of ischemia/reperfusion injury. These results indicate that ischemia produces apoptosis through the intrinsic mitochondrial pathway, whereas reperfusion-induced cell death is additionally mediated through activation of an extrinsic death receptors pathway [52–54].

7. Functional Significance of Caspases

7.1. Role of Caspases During Embryonal Development

Much knowledge concerning the function of caspases was gained from targeted gene disruption studies in animals, especially in mice [55, 56]. Deletion of individual caspase-related genes from cell lines or animals (i.e., knockout mice) is necessary to explore the functional significance of individual caspases

in vivo. Many caspases-related genes have been deleted, yielding a variety of phenotypes, from embryonic or perinatal death to normal development. Targeted gene disruption has been described for caspase-1, -2, -3, -6, -7, -8, -9, -11, and -12. However, there are no published data about targeted gene disruption for the remaining caspases (caspase-4, -5, -10, -13, and -14). The data from some of these studies are summarized in Table 1.

The results of knockout mouse studies indicate that caspase function can be divided into two broad phenotypic classes, those that have primary crucial effect on animal development (proapoptotic caspases, i.e., caspase-3, -7, -8, and -9) and those that mediate immune system functions (proinflammatory caspases, i.e., caspase-1 and -11) [64, 73].

Mice that lack caspase-1, -11, or -12 do not have a significant decrease in viability and have no overt consequence on their development [57–59, 69, 72]. In contrast, caspase-3 and/or -9 ablation results in retarded developmental apoptosis and has a tremendous impact on animals' viability and phenotype [61, 67]. Similarly, caspase-8 knockout mice die *in utero* and are characterized by impaired formation of cardiac muscle and marked abdominal congestion with reduction of the number of hematopoietic precursors [65]. The size of the heart was reported to be almost normal, but the developing ventricular musculature was thin and sometimes similar to early mesenchyme. The trabeculae were thin and disorganized. Cultured fibroblasts from caspase-8 mice were resistant to death receptor-mediated apoptosis. These findings indicate that caspase-8 plays an important role in death induction by several receptors of Fas/TNF/NGF family. Similarly, Sakamaki *et al*. [66] described that protease-deficient caspase-8 mutant mice died *in utero* due to heart rupture which they believe was due to cardiomyocyte apoptosis.

7.2. Role of Effector Caspases During Apoptosis

The effector caspases cleave many vital cellular proteins, thus breaking up the nuclear scaffold and cytoskeleton. In addition, caspases activate DNases, which degrade nuclear DNA. These changes underlie the nuclear and cytoplasmic morphological alterations seen in apoptotic cells [74]. More than 280 cellular proteins have been identified to be targeted by activated caspases [75]. For most of them, the consequences of their cleavage are poorly understood. For some of them, on the contrary, cleavage can be linked to specific morphological changes. Among them are lamin and nuclear mitotic apparatus proteins, important components maintaining the structural shape of the nucleus, which are cleaved by activated caspase-3 and -6 [76]. DNA fragmentation factor and caspase-activated DNase are responsible for DNA fragmentation [77]. Structural proteins cleaved by activated caspases during apoptosis include actin, spectrin, gelsoin, fodrin, and focal adhesion proteins [74, 75].

TABLE 1
VIABILITY AND PHENOTYPE IN KNOCKOUT MICE WITH DELETION OF CASPASE GENES

Gene	Viability	Mutant phenotype	Cell death phenotype	Cardiac phenotype	References
Caspase-1	Yes	Normal development	Defects in death receptor-mediated apoptosis, resistant to lipopolysaccharide (LPS)-induced endotoxic shock	Normal	[57–59]
Caspase-2	Yes	Excess oocytes	Excess oocytes resistant to cytotoxic agents, defects in B-cell apoptosis by granzyme B and perforin, accelerated death of motor neurons	Normal	[60]
Caspase-3	Perinatal lethality	Excess brain tissue	Defective apoptosis in neuronal progenitor cells, forebrain malformation, reduced antigen-induced apoptosis of T cells	Normal	[61–63]
Caspase-6	Yes	Normal development	Not determined	Normal	[64]
Caspase-7	Embryonic lethality	Not determined	Not determined	Not determined	[64]
Caspase-8	Embryonic lethality	Impaired heart muscle development, decreased hematopoietic precursors	Defects in Fas- and TNF-initiated cell death	Myocardial thinning, reduced trabeculation	[65, 66]
Caspase-9	Perinatal lethality	Excess brain tissue	Defects in brain apoptosis, defects in cell death in response to UV or γ irradiation	Normal	[67, 68]
Caspase-11	Yes	Normal development	Resistant to LPS-induced endotoxic shock, defects in oligodendrocyte-mediated cell death	Normal	[69–71]
Caspase-12	Yes	Normal development	Defective endoplasmic reticulum-mediated apoptosis, reduced neuronal apoptosis by β-amyloid	Normal	[72]

Some caspase substrates, such as catenins, cadherins, plakoglobin, and desmoglein, maintain cell adhesion and cell–cell communications in adherens and gap junctions, or in desmosomes. Cleavage of these proteins causes loss of contact between cells [78, 79].

8. Caspases for Detection of Apoptosis

Despite extensive research, identification of apoptotic cells remains an important unresolved issue. Apoptosis can be recognized by characteristic morphological features, which are difficult to be found in the heart. Furthermore, morphology alone does not enable recognition of cells early in the apoptotic pathway. Detection of activated caspases appears to be a reasonable way to detect apoptotic cells, given the central role of caspases in the process of apoptosis. It must be kept in mind, however, that caspases may contribute to necrotic cell death [80, 81] and caspase-independent apoptosis does occur [80].

8.1. Techniques for Detection of Caspases

Several techniques for detection of activated caspases or their target proteins are available [82]. One of the most widely used techniques is *Western blotting*. Because the activation of caspases involves the transcatalytic cleavage of the zymogen procaspases, the cleavage products, differing in molecular weight from the zymogen, can be revealed electrophoretically and identified in Western blots using caspase-specific antibodies. Western blotting can be also used to detect target proteins which are cleaved by activated caspases during the apoptotic process. Many antibodies that can be used by this technique are commercially available, including antibodies against poly (ADP-ribose) polymerase (PARP), lamin B, and α-fodrin.

Another commonly used way to detect caspase activity is by *enzyme assay* that measures the cleavage of synthetic substrates on their incubation with lysates of apoptotic cells [83]. Commercially available substrates are conjugated either to the chromophore or to a fluorochrome. On cleavage of the substrate, the liberated chromophore/fluorochrome is spectrofotometrically/fluorometrically detected.

Both techniques can be applied to most experimental models of apoptosis, including cells in culture and biopsies. In addition, techniques based on the cleavage of synthetic caspase substrates can be applied to caspases, activated *in vitro* and *in vivo* [82, 83]. Whereas Western blotting is time consuming and mainly a qualitative assay, the detection of caspase activity by cleavage of synthetic substrates is a quantitative, relatively fast and sensitive method [82]. However, both techniques do not provide information about the type and distribution of the cells with activated caspases in the tissue examined.

Immunohistochemistry, on the other hand, enables identification of activated caspases or their cleaved products in fixed archival tissue sections. This technique allows identification of cell(s) undergoing caspase activation, as well as analysis of the distribution of cell(s) in the tissue. Specific antibodies to various caspases are now commercially available, the most frequently studied being caspase-3. Studies in various human tissues and cells have shown that immunohistochemical detection of activated caspase-3 is a useful tool for identifying apoptotic cells in archival material, even before all of the morphological features of apoptosis occur [84–86]. Several target proteins cleaved by caspases can also be detected by immunohistochemistry for example PARP [87], actin [88, 89], and lamin B [90].

8.2. Comparison of Techniques for Detection of Caspases to TUNEL Method

Biochemically, apoptosis is characterized by the internucleosomal degradation of chromosomal DNA to form a series of double-stranded fragments that are multiples of 180–200 base pairs in length. These fragments give a characteristic DNA ladder pattern on gel electrophoresis [91, 92] and can be detected by several cytochemical methods, the most extensively used being the terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated dUTP nick end labeling (TUNEL) [93–95]. The detection of ladder pattern and TUNEL positivity has been adopted as a marker of apoptosis.

Despite its wide use, the specificity of the TUNEL method for detection of apoptosis has been seriously questioned. There is mounting evidence that TUNEL is unreliable for the identification of apoptosis if used alone. DNA strand breaks are not a unique feature of apoptosis, since they have been found in various tissues, also in necrosis, during repair of reversibly damaged DNA, active gene transcription, and postmortem autolysis [85, 96–98].

Similar observations have been reported for the heart. Ohno *et al.* showed that so-called apoptotic myocytes in myocardial infarction (MI) presenting positive TUNEL and DNA ladders are ultrastructurally oncotic (necrotic) myocytes with DNA fragmentation [99]. In accordance, combining the techniques of TUNEL, a Taq polymerase-generated probe, and nuclear replication markers, Kanoh *et al.* showed that TUNEL positive cardiomyocytes in patients with dilated cardiomyopathy were viable cells in the process of DNA repair rather than apoptotic cells [100]. Furthermore, TUNEL-labeled cardiomyocytes from patients with either ischemic or dilated cardiomyopathy showing signs of active gene transcription lacked immunopositivity for activated caspase-3 [101]. In a recent study, Koda *et al.* suggested that DNA breaks represent an epiphenomenon of hypertrophy [102].

TUNEL labeling may also show significant variation with different histological fixatives, mode of fixation, length of fixation time, and enzyme pretreatment, resulting in more than 10-fold different TUNEL positive rates [86, 94, 103–106].

9. Apoptosis and Caspases in MI

Studies in various animal models and in human hearts suggest that apoptosis does occur in ischemia/reperfusion injury of the heart, though the relative contribution of apoptosis in comparison with necrosis to cell loss in ischemia/reperfusion injury is still controversial. Cardiomyocyte apoptosis was first reported by Gottlieb *et al.* [107], who studied the ischemia/reperfusion in rabbit hearts and found the hallmark of apoptosis in ischemic/reperfused hearts but not in the normal or ischemic-only rabbit hearts. Identification of apoptosis was based on the presence of fragmented DNA in electrophoretic gels, on *in situ* nick end-labeling assays, and on electron microscopy. They concluded that apoptosis may be a specific feature of reperfusion injury in cardiac myocytes. Subsequent studies have shown that apoptosis probably occurs both in ischemia and reperfusion [108]. It appears that apoptosis is more prominent after ischemia followed by reperfusion than after ischemia alone [109, 110].

Apoptosis has been demonstrated to occur also in human MI. Studies of heart tissue obtained at autopsies from patients, who died of MI, suggest that apoptotic cardiomyocytes were most prominent in the border zones of MI (Fig. 1A and B) [111–123], whereas few were present in the remote myocardium [113, 114, 118–121]. The most commonly used method for detection of apoptosis in these studies was TUNEL. Using this method, a highly variable apoptotic rate was found, ranging from 0.8% [114] to 43% [118] in the border zone of MI, and from 0.05% [114] to 38% [118] in the remote myocardium. Using immunohistochemistry for activated (cleaved) caspase-3, a much lower apoptotic rate was reported in human MI [123].

Little is known about the expression of other caspases in human MI. We have found positive immunohistochemical reaction for initiator caspase-8 and -9 in cardiomyocytes in the border zone of MI (Zidar, unpublished observation), consistent with the suggestion that both extrinsic and intrinsic pathways of caspase activation occur in the heart [36]. However, we have not observed a significant difference in caspase-8 and -9 expression in patients who received reperfusion treatment compared to those who did not. Thus, we were not able to confirm the hypothesis based on experimental studies that ischemia produces apoptosis through the intrinsic mitochondrial pathway,

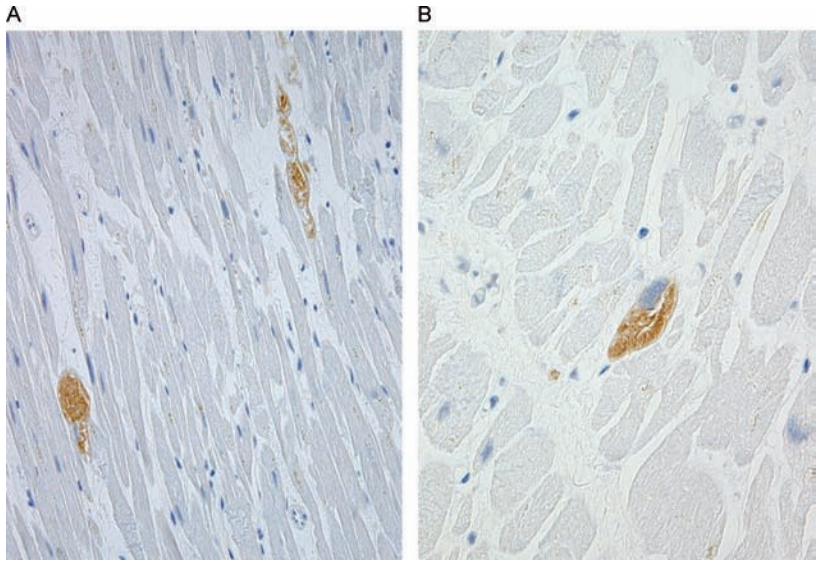

FIG. 1. Acute MI. (A, B) Immunohistochemical staining for activated caspase-3: positive reaction in cardiomyocytes in the border zone.

whereas reperfusion-induced cell death is additionally mediated through activation of an extrinsic death receptors pathway [53].

Positive reaction for caspase-9 has been also observed in cardiomyocytes bordering fibrous scar after MI, indicating that ongoing apoptosis might be important in ventricular remodeling after MI (Fig. 2B).

It is possible to monitor cardiac apoptosis *in vivo* in patients with acute MI. Hofstra *et al.* [124] used Tc-99m-labeled annexin V; annexin V has a high affinity for phosphatidylserine which is known to be externalized from the inner mitochondrial membrane to the outer mitochondrial membrane during the process of apoptosis [125]. An increased uptake of annexin V in the infarcted region was demonstrated in patients with MI after reperfusion treatment.

9.1. MORPHOLOGICAL FEATURES OF APOPTOTIC CARDIOMYOCYTES

It is controversial whether apoptotic cardiomyocytes in human MI exhibit characteristic morphological features such as cell shrinkage, condensation of chromatin, and fragmentation into apoptotic bodies [18]. It is also unknown how an apoptotic cardiomyocyte is released from the tight connection

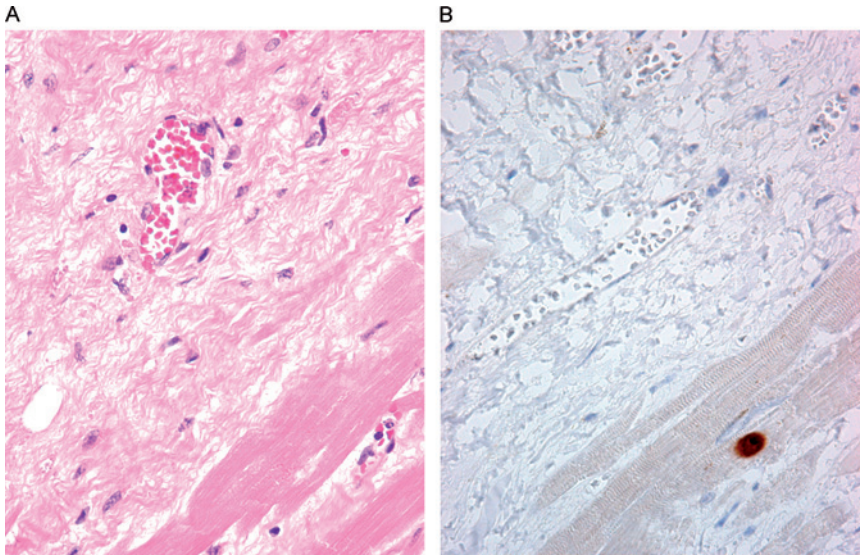

FIG. 2. Fibrous scar after MI. (A) Hematoxylin and eosin. (B) Immunohistochemical staining for caspase-9: positive reaction in a cardiomyocyte bordering the scar.

of the intercallated disk, and how it is removed from cardiac tissue: by macrophages, by neighboring cardiomyocytes, or both [126].

We have never found apoptotic bodies in autopsy samples of human MI. Furthermore, when we studied β-catenin, a component of adherens junctions at the intercallated disk (Fig. 3A), we have not observed any changes in β-catenin expression in acute MI, despite the fact that β-catenin has been shown to be cleaved by activated caspases, contributing to the disassembly of the cytoskeleton organization at cell–cell adhesion during apoptosis [127]. We have found, instead, changes in β-catenin expression in cardiomyocytes bordering the scar after MI (Fig. 3B). Some of these cardiomyocytes coexpressed caspase-3. This observation indicates that ongoing activation of caspases in cardiomyocytes results in cleavage of various structural proteins thus contributing to remodeling and development of heart failure after MI (Section 10).

It remains to be determined why apoptotic features are rarely, if ever, found in MI. It is possible that in acute MI, cardiomyocytes begin to die from apoptotic mechanism, but as cellular energy declines the cells continue to die of necrosis [22, 23]. Another possibility is that complex and large cells such as cardiomyocytes may not be able to show the classical morphological characteristics of apoptosis as originally described by Wyllie *et al*. [6], despite the activation of the cell death program [124].

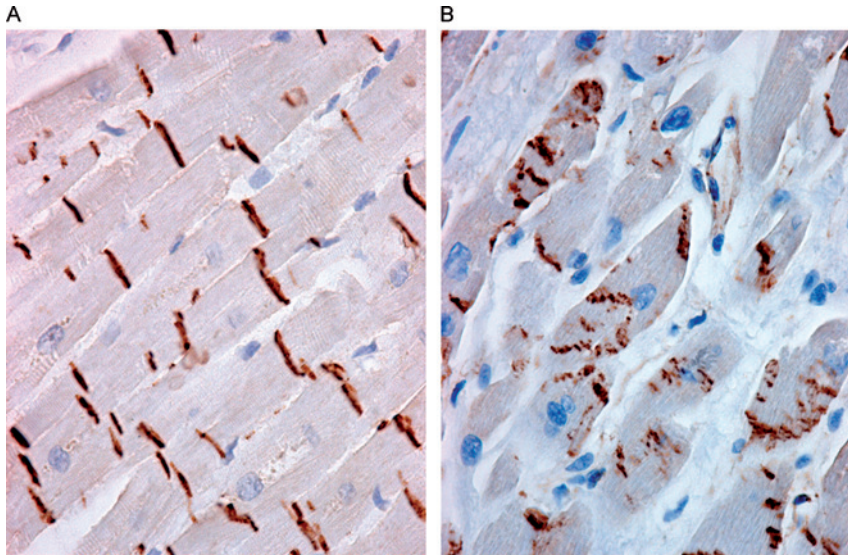

FIG. 3. Immunohistochemical reaction for β-catenin. (A) Positive reaction in intercallated disk in the normal human heart. (B) In cardiomyocytes bordering a scar after MI, β-catenin is disorganized, fragmented, and located in the cytoplasm.

Apoptotic features have been occasionally found in other cardiac disorders in humans [128–131] and experimental models [107, 132–135]. Takemura *et al.* [135] studied Fas-induced cardiomyocyte apoptosis by electron microscopy, and found extensive condensation of nuclear chromatin and shriveled cytoplasm, fragmented nuclei, and apoptotic bodies. Apoptotic features correlated with positivity for TUNEL and caspase-3. A distinct morphological feature was the abundance of lipid-like structures in the cytoplasm at the early phase and high incidence of plasma membrane rupture at the later phase. Apoptotic bodies were observed to be phagocytosed by neighboring cardiomyocytes.

10. Apoptosis of Cardiomyocytes in Ventricular Remodeling After MI

Cardiomyocyte apoptosis has been demonstrated to occur months after acute MI, both in humans [113, 120, 136–138] and experimental animals [139–141]. Apoptotic rate was higher in the periinfarct region especially if persistent or recurrent ischemia was present, and lower in the remote

myocardium, but still higher than in the control hearts. A gradual decrease of apoptotic rate over time has been reported, with end-stage ischemic heart failure having only a modest, but significant increase vs controls [120].

Ongoing apoptosis in the border zone and remote myocardium results in a reduction in cardiomyocyte number and might be therefore an important pathogenetic mechanism of ventricular remodeling after MI. Ventricular remodeling is characterized by progressive chamber dilatation, wall thinning, and systolic/diastolic dysfunction [142] beginning days after MI and persisting for weeks and months after the initial insult both at the site of MI and in the healthy myocardium [120, 143–146].

Apoptosis contributes to ventricular remodeling and heart failure not only through a reduction of the contractile cell mass, but probably also through another mechanism: activated caspases can cleave contractile proteins, such as actin, myosin, and troponin, leading to contractile dysfunction [1, 147]. It appears that all cardiomyocytes showing apoptotic changes do not die immediately; if the process of apoptosis is not complete in myocytes, it may differentially affect cytoplasmic proteins and nuclear substrates. Lack of nuclear fragmentation facilitates continuous loss of cytoplasmic proteins and may allow such cells to persist for prolonged periods in the myocardium, thus contributing to contractile failure in the surviving cells [42, 148, 149]. Consistently, caspase-3 activation is associated with a reduction in contractile performance of the left ventricle by destroying sarcomeric structure [150], and the degree of the myosin cleavage correlates with the contractile performance of the heart [151]. Moreover, caspase inhibition can reduce the occurrence of heart failure or impede its progression following MI by attenuating ventricular remodeling and protecting against myocardial protein cleavage [152–154].

11. Apoptosis of Other Cell Types in the Evolution of Fibrous Scar

Apoptosis is involved at multiple points in the evolution of MI, and comprises not only cardiomyocytes but also inflammatory cells, as well as cells of granulation tissue and fibrous tissue. In contrast to cardiomyocytes, little attention has been paid until recently to the significance of apoptosis of these cells in the evolution of MI [123, 152, 155].

Takemura *et al.* [155] described TUNEL positivity, and Hayakawa *et al.* [152] described caspase-3 positivity in infiltrated and proliferated interstitial cells in an experimental model of MI, suggesting that apoptosis might play an important role in the disappearance of these cells after MI. Consistent with these studies, we found positive reaction for cleaved caspase-3 in neutrophil granulocytes in the interstitium in acute MI in humans (Fig. 4B).

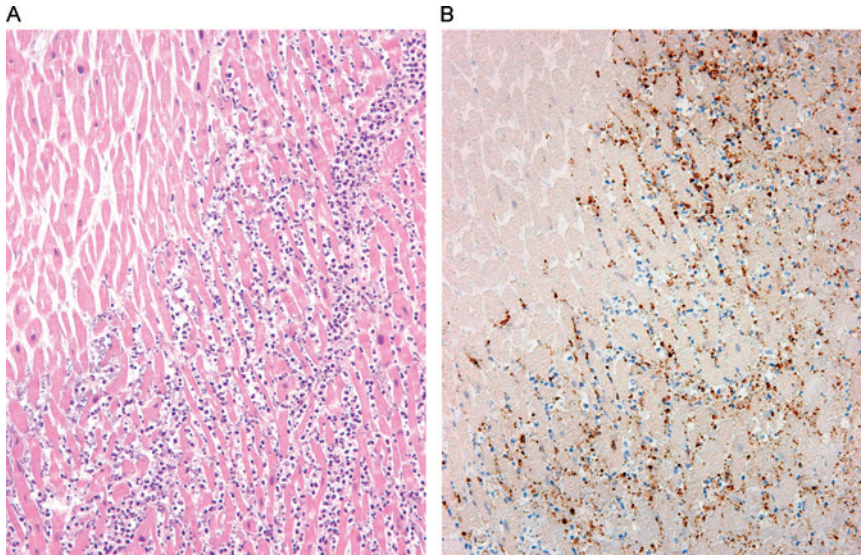

FIG. 4. Acute MI with interstitial infiltration of neutrophil granulocytes. (A) Hematoxylin and eosin. (B) Immunohistochemical staining for activated caspase-3: positive reaction in numerous neutrophil granulocytes. Apoptotic bodies are also positive.

In subacute MI, characterized histologically by the proliferation of granulation tissue and fibrous tissue, positive reaction for cleaved caspase-3 was observed in mononuclear inflammatory cells, myofibroblasts, and vascular endothelial cells (Fig. 5B). The number of positive cells decreased as granulation tissue was transforming into a fibrous scar [123].

The significance of elimination of noncardiomyocytes by apoptosis in MI has not been fully determined [156, 157]. Inflammatory cells accumulate in the infarcted region to scavenge the necrotic tissue. The first to arrive to the site are neutrophil granulocytes, which are an essential part of the acute inflammatory response to tissue injury and are thus a first-line defense of the organism. They probably play a key role in the early stages of repair after MI [158]. However, they might contribute to myocardial damage by release of oxygen-derived free radicals, proteases, leukotriens, and activation of the complement system [158], especially in situation in which inflammatory response is believed to be enhanced, for example, in reperfusion [159]. Furthermore, there is evidence to suggest that enhanced neutrophil infiltration might be important in the pathogenesis of the ventricular rupture after MI: a more intensive interstitial neutrophil infiltration of the infarcted myocardium was found in patients with heart rupture than in those without heart rupture [160].

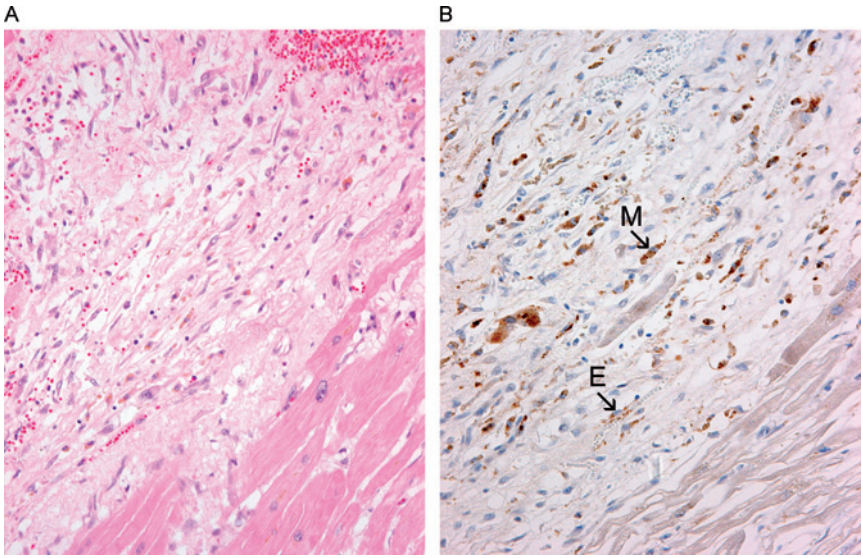

FIG. 5. Proliferation of granulation tissue in subacute MI. (A) Hematoxylin and eosin. (B) Immunohistochemical staining for activated caspase-3: positive reaction in granulation tissue, mononuclear inflammatory cells, myofibroblasts (M), and vascular endothelial cells (E).

Consistently, in experimental studies it has been shown that inhibition of neutrophil accumulation protects against myocardial ischemia/reperfusion injury [161] and reduces the incidence of the heart rupture after MI [162].

It is therefore of vital importance that after the scavenging process is completed, inflammatory cells are eliminated promptly so that surviving tissue is not further damaged. The elimination of inflammatory cells by apoptosis appears reasonable as toxic components are not released to the surrounding tissue [156] and has been confirmed in experimental models of acute inflammation [163]. It is believed that apoptosis of neutrophils and their subsequent clearance by macrophages is a critical event in the resolution of inflammation. Neutrophils die rapidly by a constitutive program of apoptosis both during aging *in vitro* and at inflamed sites *in vivo* [164, 165]. The key molecular controls of neutrophil apoptosis remain to be elucidated, but evidence is emerging that neutrophils depend on both intrinsic and extrinsic signals to determine their life span [166].

Necrotic tissue in MI is replaced by granulation tissue. Granulation tissue consists of numerous capillaries, inflammatory cells, fibroblasts, myofibroblasts, and extracellular matrix components. As repair proceeds, there is a

decrease in cellularity, ending with the formation of a fibrous scar. Experimental studies and studies in humans have shown that decrease in cellularity during transformation of granulation tissue into a scar is also mediated by apoptosis: morphological evidence of apoptosis, TUNEL positivity, and/or caspase-3 positivity have been described in inflammatory cells, vascular endothelial cells, and myofibroblasts [123, 155, 167].

Even though the significance of apoptosis in the healing process after MI is now well accepted, there is little information about the effect of inhibition of apoptosis on the healing process and scar formation after MI. Surprisingly, studies have suggested a beneficial effect of caspase inhibition. Hayakawa *et al.* [152] found that caspase inhibition during the subacute and early chronic stages of large MI improved the survival rate and ventricular remodeling at the chronic stage. These findings were attributed to the the effect of blocking the granulation tissue apoptosis in the rat hearts, in which cardiomyocyte apoptosis is rare. They also found phenotypic alteration of granulation tissue cells: there was an increase in the number of myofibroblasts, small blood vessels, and collagen fibers [152]. Similarly, Chandrashekhar *et al.* [154] reported that caspase inhibition, started soon after MI and continued for 4 weeks, preserves myocardial contractile proteins, reduces systolic dysfunction, and attenuates ventricular remodeling. This beneficial effect was attributed to reduced cardiomyocyte apoptosis in the noninfarcted myocardium and to reduced myocardial fibrosis which might be the result of reduced myofibroblast apoptosis.

12. Anti-Caspase Treatment

Apoptosis, in contrast to necrosis, is well characterized and its highly regulated nature makes it a potential target for therapeutic intervention. Caspases, being the key effector molecules in apoptosis, are a particularly attractive target for pharmacological modulation of cell death [26]. A number of specific caspase inhibitors have been developed based on the substrate cleavage sites of the caspases [168]. These peptides act as pseudosubstrates for active caspases and are therefore competitive inhibitors [169]. The chemical mechanism of action of the synthetic inhibitors is determined by the chemical groups to which peptides are linked. Linking the appropriate peptide to fluoro- or chloromethyl ketone (-cmk, -fmk) groups produces irreversible inhibitors. Peptides linked to aldehyde groups (–CHO) act as reversible inhibitors [169].

Specific and nonspecific (broad-spectrum) caspase inhibitors are now available, for example ZVAD-fmk is a broad-spectrum caspase inhibitor, while Z-DEVD- and YVAD-fmk are specific inhibitors of caspase-3 and -1 [170].

Broad-spectrum inhibitors may be preferred for acute injury where short-term administration is possible, whereas chronic diseases are best approached with selective inhibitors which target specific initiator caspases [171].

Although several potential therapeutic agents have been tested in animal models of ischemia/reperfusion heart injury with some success, nearly none of the specific antiapoptotic agents have reached the stage of clinical research [172]. The studies which have examined the effect of caspase inhibitors on ischemia/reperfusion models are summarized in Table 2. Broad-spectrum caspase inhibitors have been shown in many studies to reduce cardiomyocyte apoptosis, to reduce the size of MI, and to preserve heart function after MI [173–175, 178, 181]. The protective effect of caspase inhibitors can be seen when these agents are administered before or after the onset of ischemia but are most prominent when introduced before the onset of reperfusion [172, 175, 178]. Selective caspase inhibitors, on the other hand, have been reported to have varying effect: they have been found to reduce cardiomyocytes apoptosis, but the infarct size remained unchanged [177, 178, 181].

The majority of reported studies have focused attention on the short-term effect of caspase inhibition on ischemia/reperfusion injury to the heart. However, it has been well documented that apoptosis might be even more important later, contributing significantly to left ventricular remodeling and development of heart failure after MI [113, 136, 138–141]. It is therefore of utmost importance to understand the long-term effects of caspase inhibition. Few studies focusing attention on long-term caspase inhibition suggested that such treatment can reduce the occurrence of heart failure after MI by reducing apoptosis of cardiomyocytes in remote myocardium and by attenuating ventricular remodeling [152–154]. The possible effect of caspase inhibition on scar formation after MI was discussed in Section 11.

Although some experimental results look promising, important concerns have been raised regarding the use of caspase inhibitors in clinical practice. We need further information regarding the exact pathways that need targeting, the timing of anti-caspase therapy, and the mechanism by which the body reacts to such therapy.

Which of the caspases must be inhibited in order to achieve reduced tissue damage? One approach is to inhibit initiator caspases such as caspase-8 and -9. However, inhibiting the initiator caspase of the extrinsic or intrinsic pathways may not be sufficient since in many instances both pathways may be activated. Another approach is to inhibit the effector caspases since the both extrinsic and intrinsic pathways converge in these caspases. This approach has been successful in sepsis [184]. However, the most commonly used approach is combination of both and is targeting initiator and effector caspases, using broad-spectrum (nonselective) inhibitors [185].

TABLE 2
STUDIES SHOWING THE EFFECT OF TREATMENT WITH CASPASE INHIBITORS IN EXPERIMENTAL MODELS OF ISCHEMIA/REPERFUSION INJURY TO THE HEART[a]

Experimental model (method)	Inhibitor	Timing	Effect in the caspase inhibitor-treated group	References
I/R injury in rats (30-min ischemia, 24-hour reperfusion)	ZVAD-fmk (nonselective caspase inhibitor)	30 min before CA occlusion, repeated at 6-hour interval until 24 hour after reperfusion	Better LV contractility, lower LV filling pressure Reduced size of MI Reduced number of TUNEL positive myocytes	[173]
I/R injury in rabbits (30-min ischemia, 3-hour reperfusion)	YVAD-cmk (nonselective caspase inhibitor)	10 min before CA occlusion, 3.5-hour infusion; repeated before reperfusion	Reduced size of MI Reduced TUNEL positive myocytes in infarcted region	[174]
I/R injury in rats (45-min ischemia, 3-hour reperfusion)	ZVAD-fmk (nonselective caspase inhibitor)	Before ischemia	Reduced size of MI in pre- and postischemia administration	[175]
	BocD-fmk (nonselective caspase inhibitor)	Before reperfusion	Reduced size of MI only in preischemia administration	
I/R injury in isolated rat heart (35-min ischemia, 2-hour reperfusion)	ZVAD-fmk (nonselective caspase inhibitor) Z-IETD-fmk (caspase-8 inhibitor) Z-LEHD-fmk (caspase-9 inhibitor) Ac-DEVD-cmk (caspase-3 inhibitor)	5 min before reperfusion, for 15 min	Reduced size of MI with all caspase inhibitors	[176]
I/R injury in rats (30-min ischemia, 6-hour reperfusion)	YVAD-CHO (caspase-1 and subfamily inhibitor) DEVD-CHO (caspase-3 and subfamily inhibitor)	5 min before ischemia	No reduction of size of MI Inhibited myocyte DNA fragmentation Inhibited myocyte caspase activation	[177]

I/R injury in isolated rat heart (30-min ischemia, 2-hour reperfusion)	YVAD-cmk (nonselective caspase inhibitor)	At the start of reperfusion	Improved LV function Reduced size of MI Reduced number of TUNEL positive myocytes	[178]
	Ac-DEVD-cmk (caspase-3 inhibitor)		No effect on LV function	
	Z-LEHD-fmk (caspase-9 inhibitor)		No reduction of MI size No reduction of number of TUNEL positive myocytes	
Rabbit cardiomyocytes (metabolic inhibition and recovery)	IDN-1501 caspase inhibitor IDN-1529 caspase inhibitor IDN-1965 caspase inhibitor	Before, during, or after metabolic inhibition	Increased survival of cardiomyocytes in all settings, but correlating with earlier treatment	[179]
I/R injury on isolated rat hearts (30-min ischemia, 30-min reperfusion)	Ac-DEVD-CHO (caspase-3 inhibitor)	15 min before I/R or 5 min before reperfusion	Improved LV function Inhibition of caspase-3 activation No reduction of apoptosis No reduction of troponin-I cleavage	[180]
Isolated rabbit heart (30-min ischemia, 2-hour reperfusion)	MMPSI (caspase-3/7 inhibitor) ZVAD-fmk (nonselective caspase inhibitor) DEVD-fmk (caspase-3/7 inhibitor)	Heart: 15 min before ischemia	Reduced size of MI Reduced apoptosis (MMPSI)	[181]
Rabbit cardiomyocytes (16 hour of simulated ischemia, 2-hour simulated reperfusion)	YVAD-cmk (nonselective caspase inhibitor)	Cardiomyocytes: before ischemia	No reduction of MI size	

(*continues*)

TABLE 2 (*Continued*)

Experimental model (method)	Inhibitor	Timing	Effect in the caspase inhibitor-treated group	References
I/R injury in rats (1 hour of ischemia followed by reperfusion)	Boc-Asp-fmk (broad-spectrum caspase inhibitor)	Third day after MI, continued for 4 weeks	Greater survival, decreased left ventricular remodeling and better cardiac function at 12 weeks Reduced apoptosis and granulation at 2 weeks	[152]
I/R injury in pigs (1-hour ischemia, 7-day reperfusion)	IDN-6734 (broad-spectrum caspase inhibitor)	At reperfusion, continued for 24 hour	Improved LV function, attenuated remodeling post MI at 7 days No significant change of MI size and plasma troponin-I values	[153]
MI in rats (surgical LAD ligation)	Z-Asp-2, 6DCBMk (broad-spectrum caspase inhibitor)	Before LAD ligation, continued for 28 days	Reduced myocardial caspase-3 activation Reduced troponin-I cleavage Improved LV function Attenuated LV remodeling	[154]
MI in mice (surgical LAD ligation)	DEVD-CHO (caspase-3 and subfamily inhibitor)	Before LAD ligation, continued for 7 days	Improved survival (yet statistically not significant) Improved LV function Attenuated LV dilatation	[182]
MI in rats (surgical LAD ligation)	Caspase-3 inhibitor II Calpain inhibitor XII	Immediately after LAD ligation	Reduced size of MI Improved LV function Attenuated LV remodeling Reduced apoptosis	[183]

[a]I/R = ischemia/reperfusion; CA = coronary artery; LV = left ventricle; MI = myocardial infarction; and LAD = left anterior descending branch of left coronary artery.

Timing of anti-caspase therapy is an important issue with many unsolved questions. The different outcomes of reported studies may reflect the different times the inhibitors were administered. This underscores the importance of defining the optimum time for therapeutic intervention. It remains to be determined whether the anti-caspase therapy should target the early ischemic injury, reperfusion, or even chronic injury [185, 186].

How will the body react to treatment with caspase inhibitors? Caspases are responsible for several important physiological functions, besides apoptosis which removes damaged cells, they are also responsible for cytokine activation, cleaning up free radicals, and maintenance of the cytoskeleton. These functions may be adversely affected by caspase inhibitors. Long-term treatment with caspase inhibitors might therefore cause cancer, infections, and autoimmune disorders. Thus, it might be desirable to limit activation of prosurvival signaling to the heart. One possible solution would be to manipulate survival signaling pathways through local genetic or pharmacological interventions [187].

13. Conclusion

There is accumulating evidence that apoptosis plays an important role at multiple points in the evolution of MI not only in the acute phase but also in remodeling and development of heart failure after MI.

Despite extensive research, many aspects of cell death including the molecular regulatory mechanisms, as well as the relative contribution of apoptosis and necrosis, and possibly other types of cell death to cardiomyocyte loss remain to be elucidated. An important unresolved issue is also the identification of apoptotic cardiomyocytes.

Being a highly regulated process, apoptosis represents an attractive novel approach for the treatment of cardiovascular disease. Although some results of experimental studies look promising, we need more information regarding the timing and the long-term effect of inhibition of apoptosis not only on myocardium but also on other tissues. All of these issues will have to be resolved before antiapoptotic treatment can be used in clinical practice.

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DEAMIDATED GLIADIN PEPTIDES AS TARGETS FOR CELIAC DISEASE-SPECIFIC ANTIBODIES

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Abbreviations

CD	celiac disease
ELISA	enzyme-linked immunosorbent assay
IgA	immunoglobulin A
IgG	immunoglobulin G
MHC	major histocomapatibility complex
tTG	tissue transglutaminase

The standard IUB/IUPAC one-letter code for amino acids is used throughout.

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1. Abstract

Celiac disease (CD) is an (auto)immunologically mediated intestinal intolerance against proteins from wheat (gluten) and related cereal proteins. Tissue transglutaminase (tTG) has been identified as the autoantigen in CD. Although ultimate diagnosis is based on histological analysis of small intestinal mucosa obtained via tissue biopsy, assessment of autoantibodies can provide substantial help in the evaluation of CD. Gliadin antibodies are directed against the native disease-provoking cereal proteins. Despite their initial usefulness, these antibodies have lost diagnostic importance due to their poor specificity and sensitivity as CD markers. Recently, it was found, however, that gliadin antibodies from sera of patients with active CD preferentially recognized deamidated gliadin peptides. The use of deamidated gliadin peptides in immunoassays has significantly improved the usefulness of gliadin antibodies in diagnosis of CD to that observed with autoantibody assay methods (endomysium antibodies, antibodies against tTG). The antibody epitopes (B-cell epitopes) reflect substrate specificity of tTG and resemble peptide sequences known to be strongly T-cell stimulatory (T-cell epitopes) in CD. The assay applying deamidated gliadin peptides measures a new species of antibodies, which is different from conventional gliadin antibodies as well as from autoantibodies and will likely provide new information on pathophysiological mechanisms of CD.

2. Introduction

Celiac disease (CD, synonyms: celiac sprue or gluten-sensitive enteropathy) is an (auto)immunologically mediated intolerance against proteins from wheat (gluten) and related cereal proteins in the diet. The enzyme tissue transglutaminase (tTG) has been identified as autoantigen in CD [1]. Despite initial estimates, CD is now more common than previously assumed. Besides classical CD with fully expressed symptoms (diarrhea, vomiting, abdominal pain, weight loss/failure to thrive), there are mono- and oligosymptomatic findings that can occur with atypical milder silent (and latent) forms of the disease. These disease forms together account for a prevalence of up to 1%. Although CD is a common disorder in Europe and in populations of European ancestry (North and South America, Australia), it has also been found in regions where it was historically considered extremely rare including North Africa, the Middle East, and Southeast Asia. Because CD is spread worldwide as in a common "Global Village," greater levels of awareness and attention on gluten intolerance are needed in both the Old and New World [2]. This disorder has to be treated by a lifelong dietary modification to avoid offending cereal proteins, an approach also suggested for the milder forms of CD. Final diagnosis of CD is based on histological examination of

the duodenal mucosa. This invasive procedure is indicated both when disease is clinically suspected or when CD autoantibodies are present. It should be noted that autoantibodies have high diagnostic sensitivity and specificity for CD approaching and exceeding values of 95%. In contrast, detection of antibodies directed against the disease-provoking cereal proteins (gliadin antibodies) has lost diagnostic importance. These antibodies are regarded as much less specific and sensitive for CD. However, the validity of gliadin antibodies in the diagnosis of CD has increased considerably when deamidated variants are used as immunoassay targets. Interestingly, these deamidated peptides are produced *in vivo* by the action of tTG under the inflammatory conditions prevailing in the small intestinal mucosa of CD patients under a gluten-containing diet. In this regard, data on the epitope specificity of gliadin antibodies of CD patients are discussed. Further, the epitope specificity of gliadin antibodies of CD patients, which should reflect the enzyme activity of tTG, is compared with substrate specificity of this enzyme. Current knowledge of sequence requirements for binding of T-lymphocyte stimulatory gliadin-derived peptides to major histocompatibility complex (MHC) class II molecules associated with CD is also discussed. These data propose for a new role of gliadin antibodies in diagnosis of CD and may even suggest a possible function of gliadin antibodies in causation of CD.

3. Prolamins as Cereal Storage Proteins

CD represents a malabsorption disorder induced by ingestion with food of cereal storage proteins of wheat, barley, and rye. Although oats are well tolerated by most CD patients [3, 4], some concern remains [5, 6]. The cereal storage proteins toxic for CD patients are insoluble in aqueous salt solutions, but can be extracted from flour by aqueous alcohol. According to the classical definition [7, 8], cereal storage proteins soluble in aqueous alcohol without reduction of intermolecular disulfide bonds are termed prolamins and the proteins that are soluble in aqueous alcohol only after additional reduction treatment are called glutelins. This classification is also supported by the Working Group on Prolamin Analysis and Toxicity [9] and by the Codex Alimentarius Commission [10] and will be used throughout this chapter (Table 1). It should be noted that another classification does exist [11–14] but will not be used. In the latter, the term prolamins is applied to cereal storage proteins soluble in aqueous alcohol irrespective of reduction of disulfide bridges and thus comprises both mono- and polymeric storage proteins.

The wheat storage proteins *without* interchain disulfide bonds are monomeric and are termed gliadins, those *with* interchain disulfide bonds are polymeric and are called glutenins. Gliadins plus glutenins form gluten. Originally, it was assumed that the toxicity for CD patients was restricted to

TABLE 1
CLASSIFICATION OF STORAGE PROTEINS FROM TRITICEAE[a]

		Triticeae storage proteins		
		Wheat	Rye	Barley
HMW	Polymeric	HMW subunits of glutenins	HMW secalins	D-hordeins
MMW	Monomeric	ω-Type gliadins	ω-Secalins	C-hordeins
LMW	Monomeric	α-Type and γ-type gliadins	40 K γ-secalins	γ-Hordeins
	Polymeric	LMW subunits of glutenins	75 K γ-secalins	B-hordeins

[a]HMW = high molecular weight; MMW = medium molecular weight; LMW = low molecular weight. Gluten represents the sum of gliadins and glutenins from wheat. According to another classification, the term prolamin is used for both the monomeric and the polymeric storage proteins [11–14].

the gliadin fraction from wheat and the respective prolamins of related cereals; however, there are now clues that some wheat glutenins may be toxic [15–17].

The different prolamins represent groups of highly polymorphous proteins with molecular weights between 30,000 and 90,000 kDa. Search of the U.S. National Center for Biotechnology Information (NCBI) database for the phrase "prolamin AND (barley OR wheat OR rye OR triticeae OR gliadin OR secalin OR hordein)" (January 17, 2006) [18] revealed 335 different entries of peptide sequences. Prolamins contain a high proportion of glutamine (up to 50%) and proline (up to 30%) with an amino acid sequence consisting of repeated blocks of one or more short peptide motifs. The proline- and glutamine-rich sequences of prolamins form hydrophilic β-turns [19–21], which are usually located at the surface of proteins. The QQPFP sequence motif (standard IUB/IUPAC one-letter code for amino acids [22]) is contained up to 15 times within a prolamin molecule. This motif is a component of a supersecondary structure (β-structures and poly-L-proline II helix) and seems to represent an immunodominant B-cell epitope [23]. A strong polyproline II helical propensity was also reported for the PQPQLPY peptide (from α-type gliadins) and tTG may have a preference for polyproline II helical substrates [24].

4. Pathogenesis of CD

In genetically susceptible individuals, ingestion of cereal prolamins from wheat, barley, rye, and possibly oats initiates an inflammatory disorder during which the small intestinal mucosa is damaged. This process is accompanied by malabsorption, activation of the intestinal immune system, and

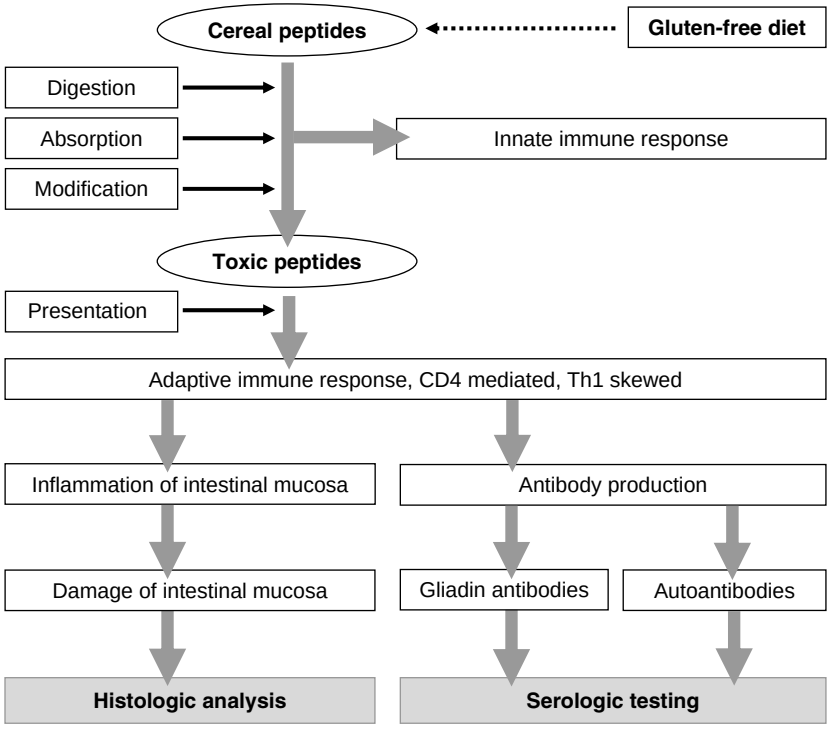

FIG. 1. Brief survey of pathogenesis and diagnosis of CD. Food peptides from wheat and related cereals are partially digested and absorbed. Remaining peptides can be modified by tTG. Toxic peptides bind to MHC class II molecules (antigen presentation). For binding, specific HLA alleles (DQ2 or DQ8) are required. Peptide presentation to CD4 T cells initiates mechanisms of adaptive immune response. As a result, the intestinal mucosa is damaged, which can be analyzed in intestinal biopsy material (gold standard for final CD diagnosis). Further, antibodies against gliadin and against tTG as autoantigen are produced, from which autoantibodies are regarded as the main help in diagnosis. Due to their lower diagnostic validity, gliadin antibodies have lost importance. However, according to new findings, this species of antibodies may gain new weight if for detection deamidated gliadin peptides are applied. The therapy of CD consists in elimination of the offending cereal proteins from food (so-called gluten-free diet). Furthermore, cereal peptides are also able to activate innate immune responses.

production of antibodies against the corresponding cereal food proteins and of autoantibodies against tTG (Fig. 1). The intolerance against prolamins is generally assumed to be lifelong [25, 26]; however, some observations question this claim [27, 28]. The main therapy consists in exclusion of the disease-triggering cereal proteins from the diet (so-called gluten-free diet).

Before gluten proteins from food are able to interact with cells of the intestinal immune system, they have to pass through the gastrointestinal tract.

Most food macromolecules are nearly completely digested to monomers and short oligomers by the high hydrolytic potential of the gastrointestinal luminal fluid and plethora of enzymes residing in the microvillus membranes of intestinal enterocytes. Due to their high proline content, gliadins are poorly digested and larger peptides remain. In the past, it was believed that a peptidase could be missing in CD patients resulting in the accumulation of toxic peptides in the intestinal lumen [29]. Several results suggested a defective mucosal digestion of gliadin peptides [30–33]; however, a peptidase specifically missing in CD patients was never identified. It was shown that a peptide comprising 33 amino acids from the N-terminal part of α2-gliadin, a representative of α-gliadin, remains remarkably stable after digestion with peptic and pancreatic enzymes and even after incubation with rat or human brush border membrane preparations [34]. Shorter peptides from this 33-mer also resisted intestinal digestion. Dipeptidyl peptidase IV and dipeptidyl carboxypeptidase I were identified as rate-limiting enzymes in digestive breakdown [35]. Another peptide (26-mer) from γ-gliadin (rich in proline and glutamine) also resisted proteolysis [36]. Supplementation of digestion with a bacterial enzyme, prolyl endopeptidase, led to a more efficient hydrolysis of gliadin peptides [37]. Prolyl endopeptidase decreased the amount of intact gliadin peptides crossing the intestinal biopsy specimens of CD patients, but could not completely prevent the intestinal passage of toxic or immunostimulatory metabolites [38]. However, pretreatment of food gluten with prolyl endopeptidase reduced gluten-induced malabsorption in CD [39] and destroyed epitopes important for subsequent activation of T cells [40]. Enzyme therapy based on animal digestive extracts also was successful in reducing symptoms in CD patients [41]. Collectively, these data demonstrate that even if there is no specific peptidase deficiency demonstrable in CD patients, large gluten peptides likely reach and interact with the intestinal epithelium.

The mechanism by which gluten-derived peptides are absorbed through the intestinal epithelium in order to interact with immune cells is still largely unknown. Binding of gliadin peptides was demonstrated to rat microvillus membrane proteins [42–46], to isolated glycoproteins of rat brush border membranes [47], to cells of the intestinal epithelial cell line HT-29 [48,49], to isolated rat enterocytes and human enterocytes from CD patients in remission as well as from controls [50], and to crypt cells of human biopsies from patients with active CD but not from disease controls or patients in remission [51]. In spite of these intensive investigations, the mechanism of binding still remains unknown.

After binding, gliadin is endocytosed [52] and translocated to HLA-DR antigen-containing endocytic vesicles [53] and Golgi complexes [54] of small intestinal enterocytes in active CD. Alternatively, gliadin may be transported paracellularly across the epithelium. It was found that zonulin, a protein

involved in regulation of permeability of tight junctions is increased in intestinal tissues during acute CD [55]. *In vitro*, gliadin-induced zonulin release in intestinal epithelial cells was found to open tight junctions and increase intestinal permeability [56], thus favoring passage of environmental antigens possibly involved in the pathogenesis of CD.

Following absorption from the intestinal mucosa, gluten peptides stimulate intestinal T lymphocytes to promote antibody production and release of inflammatory mediators. Stimulation of lymphocytes by gluten is seen as main step in the chain of pathogenetic events in CD and is, therefore, of central interest. Other effects of gluten peptides on intestinal epithelial cells [57, 58] and other cells [59–62] which do not seem to be directly related with activity intestinal immune system (''direct toxicity'' of gluten peptides) and induction of innate immune responses (for review see [63, 64]) will not be discussed here.

The intestinal mucosa harbors distinct populations of T lymphocytes. For example, CD4+ T lymphocytes are predominant within the lamina propria. Other T-lymphocyte populations reside in the epithelium (intraepithelial lymphocytes). The majority of these are CD8+ with a minority CD4− and CD8−. In CD, the number of T cells in the lamina propria and epithelium is increased. Although intraepithelial T cells have potent cytolytic and immunoregulatory capacity, their role in the pathogenesis of CD is not well understood. It has been assumed that intraepithelial lymphocytes, by upregulation of natural killer receptors, kill enterocytes. Gluten can induce natural killer receptors as well as expression of their ligands on enterocytes by stimulating the expression of interleukin-15 [65]. However, CD4+ T cells are considered the key component of the anti-gluten response [66]. The CD4+ T cells of the lamina propria are restricted by MHC class II molecules. In CD patients, these lamina propria CD4+ lymphocytes can be stimulated by gluten peptides bound to MHC class II molecules on the surface of antigen-presenting cells. This interaction results in release of proinflammatory cytokines such as interferon-γ [67, 68] and the ultimate destruction of the intestinal mucosa. Thus, the CD4-mediated proinflammatory immune response in CD is Th1 skewed. Another effect of stimulation of CD4 T cells of lamina propria is the induction antibodies to gliadin and the autoantigen tTG.

The central event in stimulation of lamina propria T cells of CD patients is presentation of gluten-derived peptides. There are two prerequisites for stimulation: (1) presence of the DQ2 or DQ8 haplotype of MHC molecules and (2) modification of gluten peptides for optimal fit into the binding pocket of DQ molecules.

In a large population-based twin study [69], it was shown that in Italy 75% of monozygotic twin pairs were concordant for CD compared with only 11% of dizygotic twin pairs. From these results, it was concluded that environmental

factors apart from gluten have only little or even no effect on the pathogenesis of CD. From 15 concordant monocygotic twin pairs, 12 had the DQ2 haplotype, 1 had the DQ8 haplotype, and 2 had a DRB1*7 allele. The DRB1*7 allele was shown to be linked with the DRB4*01 allele so that these two individuals were positive for Dw53, which is also strongly implicated in the pathogenesis of CD [70]. However, only a small percentage of the 30% of Europeans who inherit HLA-DQ2 and/or DQ8 actually develop CD. Thus, other genes than HLA should be implicated in CD. It was calculated that HLA genes confer only up to 40% of the genetic risk for CD and that the rest is attributable to non-HLA genes [71]. However, large genome-screening studies failed until now to identify other genes that exert a major effect. Rather, a wealth of genetic characteristics that individually exerts little effect might collectively contribute to gluten intolerance together with HLA.

The role of HLA molecules in the pathogenesis of CD is seen in their function of antigen presentation. After binding gluten peptides, MHC molecules associate with T-cell receptors of intestinal CD4+ T cells, resulting in a stimulation of T cells. Peptides derived from native gliadin, however, showed low affinity for DQ2 [72]. Studies of peptide-binding motifs of DQ2 and DQ8 revealed the importance of negatively charged residues in several positions [73–76] not typical for native gliadin peptides.

In 1997, tTG was identified as autoantigen of CD and gliadin was described to be a preferred substrate for this enzyme [1]. Subsequently, gliadin was found to be cross-linked to tTG in tissue sections of monkey esophagus [77]. Hypotheses were then proposed to explain how gliadin cross-linked to tTG could cause the synthesis of autoantibodies directed against tTG (formation of a neoantigen, epitope spreading, intramolecular help) [77, 78]. However, tTG was not only shown to be able to cross-link gliadin to amine donors but also to deamidate glutamine residues within gliadin, thus giving rise to glutamic acid residues. Selective deamidation of gliadin by tTG [79–83] was shown to strongly enhance gliadin-specific T-cell reactivity. However, deamidation of gluten peptides by tTG is not always a prerequisite for binding to MHC molecules and initiation of immune responses [16, 84, 85].

5. Serological Diagnosis of CD

The criteria reported by the Working Group of the European Society of Pediatric Gastroenterology and Nutrition in 1990 [86] still represent the basis for diagnosis of CD. Clinical symptoms and serology together with histological examination of small intestinal biopsy material are collectively used to diagnose disease. Final confirmation still depends on demonstration

of typical histological changes in the mucosa of intestinal biopsy material which is regarded as gold standard [87–89]. According to Marsh [90], there are consecutive stages of progressive abnormalities of the small intestinal mucosa. These include: type 1 (infiltrative, increased numbers of intraepithelial lymphocytes), type 2 (hyperplastic, crypthyperplasia), and type 3 (destructive, villous atrophy, flat lesion). Marsh also recognized a type 4 lesion (irreversible hypoplastic/atrophic lesion) in which malignant transformation can develop. However, interpretation of histological findings is subjective and requires experience. Further, some patients are reluctant to undergo intestinal biopsy. Therefore, a less invasive method of diagnosis is desirable [87].

Serological diagnosis is based on estimation of antibodies against gliadin and of autoantibodies directed against tTG. Gliadin antibodies have been known for more than 40 years [91]. However, their diagnostic sensitivity and specificity for CD is low. The immunoglobulin A (IgA) class has a somewhat higher specificity than the immunoglobulin G (IgG) class [88]. Increased concentrations of gliadin antibodies can also be found in a variety of other conditions not related to CD, for example inflammatory bowel disease [92], IgA nephropathy [93], HIV infection [94], rheumatoid arthritis [95], as well as in some apparently normal individuals. Furthermore, gliadin antibodies were also described in neurological disorders [96, 97]. The most frequently published neurological syndromes associated with gliadin antibodies are cerebellar ataxia (gluten ataxia) and peripheral neuropathy (celiac neuropathy). However, the association between gliadin antibodies and neurological diseases is, for the most part, weak [98]. Consequently, the role of gliadin antibodies in pathogenesis of neurological disorders is highly debatable [99].

Autoantibodies in patients with CD were identified a few years following discovery of gliadin antibodies [100, 101]. These autoantibodies were directed against components of the extracellular matrix. Because they were assayed by indirect immune fluorescent microscopy, they were named according to their tissue substrate, that is reticulin antibodies [101–103], endomysium antibodies [104, 105], jejunum antibodies [106], and human umbilical cord antibodies [107]. After the discovery of tTG as autoantigen of CD [1], enzyme-linked immunosorbent assays (ELISAs) were generated from enzyme preparations from guinea pig [108–110]. Subsequent ELISAs used human recombinant tTG [111–116].

The diagnostic efficiency of autoantibody determination was found to exceed that of gliadin antibodies [117, 118]. Autoantibody tests were based mainly on the determination of IgA immunoglobulins against endomysium, human umbilical cord, and tTG. These autoantibodies were highly sensitive and specific with values for both parameters exceeding 95% in most studies [118–121].

Improved quality of autoantigen preparation (human recombinant antigen vs guinea pig liver enzyme) in ELISAs led to a diagnostic accuracy comparable to indirect immunofluorescent detection of endomysium antibodies [115, 117, 118, 120, 122–127]. These advances in ELISA technology have led to a progressive replacement of the more labor-intensive and -subjective immunofluorescent techniques.

The high diagnostic accuracy of autoantibody tests for CD has questioned the need for intestinal biopsy [128], especially in pediatric populations [129]. Because histological and serological examination has limitations (and also HLA typing, an expensive procedure not suitable for routine diagnostic use), it is still advised to apply a combination to clarify the full breadth of the gluten-sensitive spectrum [130].

Estimation of autoantibodies in CD is based mainly on detection of IgA class immunoglobulins. However, CD is associated with selective IgA deficiency [131]. About 2% of CD patients are IgA deficient in Italy [132, 133] and Ireland [134]. In cases of IgA deficiency, the corresponding IgG antibodies should be evaluated. Indirect immunofluorescent detection of IgG anti-endomysium antibodies has drawbacks due to secondary antibodies against human IgG often nonspecifically binding to connective tissue fibers [135]. However, IgG class antibodies against tTG have shown high specificity for CD in cases of IgA deficiency [136–139].

Caution should be exercised when patients with liver diseases are tested for antibodies against tTG. Increased levels of tTG antibodies in liver disease (especially primary biliary cirrhosis) often lead to false positive values for antibodies against tTG in ELISA. This phenomenon has been attributed to hepatic (over)expression of tTG [140]. Furthermore, the presence of high levels of immunoglobulins in these patients can additionally interfere with ELISA quantitation [141,142]. Increased small bowel permeability in cirrhotic patients [143, 144] may also contribute to alteration in immune response to endogenous tTG [141]. In these patients, the diagnosis of CD may not be confirmed by intestinal biopsy [140, 145, 146]. Interestingly, these patients are usually negative if autoantibodies are determined by immunofluorescent assay [140, 141, 145, 146]. False positive results in cirrhotic patients cannot be overcome by use of the human recombinant tTG. It should be noted that purified tTG antigen may be distorted during attachment to the ELISA microtiter plate wells, resulting in binding of nonspecific antibodies [147].

In CD, autoantibody assays have generally replaced gliadin antibody assays. Measurement of gliadin antibodies is, however, advantageous in small children. Autoantibodies are often absent in CD children under 2 years of age [148, 149]. In another study, antibodies against tTG were found to develop not before an age of 1.3 years [150]. Increased concentration of gliadin antibodies may be helpful in the investigation of other

conditions not related to CD (see above). Furthermore, in psoriasis [151, 152], increased concentrations of gliadin antibodies were found in the absence of anti-endomysium antibodies. In one study, expression of tTG in the dermis responded to a gluten-free diet [152].

There were several attempts to increase the diagnostic accuracy of gliadin antibodies. As early as 1989, it was known that gliadin antibodies of CD patients differed in their reactivity toward different gluten-derived peptides but no correlation between gliadin antibodies and disease state was found [153]. Later, the IgG and IgA antibody reactivity against an overlapping set of synthetic peptides (ELISA) that covered the entire sequence of α-gliadin was measured from CD patients, controls with increased gliadin antibodies, and healthy children. Antibodies mainly recognized peptides derived from the N-terminal region of α-gliadin, containing the motif QPFXXQXPY. However, gliadin antibodies in CD patients and controls with increased gliadin antibodies recognized the same linear epitopes. From these results it was concluded that serological investigation of the specificity of these antibodies using a peptide ELISA does not allow appropriate discrimination between patients and controls [154].

6. Gliadin Antibodies of CD Patients Recognize Deamidated Gliadin

Although the gliadin-deamidating activity of tTG toward gliadin was known since 1998 [79–82], attention was focused on the deamidation effect on binding of modified gliadin peptides to HLA molecules. This was due to the fact that the typical mucosal lesion in CD is basically regarded as a result of T-cell activation after presentation of gliadin peptides. Antibodies in CD were considered of relatively minor importance in disease pathogenesis and were generally viewed as side products of the immune response of some usefulness in diagnosis. Several attempts were made to screen different gliadin amino acid sequences for epitopes of antibodies of CD patients [154–157]. Unfortunately, these studies used sequences from native unmodified gliadin.

To identify the gliadin epitope, we screened libraries of phage-displayed peptides for binding to antibodies from CD patients [157]. The technique of screening of phage-displayed peptides (phage display) is based on application of a large pool of filamentous coli phage that express a vast number of different short peptides fused to phage coat proteins (combinatorial library of random peptides) on their surface [158–160]. Each single phage expresses only peptides of one common sequence. Due to the size of the phage pool, the number of different expressed peptides is tremendous and can be tested for

binding ligands of interest. If antibodies are applied as ligands, those phage with peptides on their surface mimicking the antibody epitopes will be bound and can be amplified in further rounds of selection. Finally, the phage DNA can be sequenced and the peptide sequence responsible for binding can be deduced from the DNA sequence. We screened a phage-displayed dodecapeptide library with human IgA from sera positive for anti-endomysium antibodies and with high concentration of gliadin antibodies [157]. As a result, 200 different antibody-binding dodecapeptide sequences were identified. These were synthesized onto cellulose membranes and subjected to further binding studies with human sera. Only four of the dodecapeptides showed a significantly stronger binding of IgA of sera with high gliadin antibody concentrations compared to sera low in gliadin antibodies. All of them contained the tripeptide motif PEQ. Contrary to the phage display experiments, the PEQ sequence could not be found when short peptides (synthesized onto cellulose membranes) covering in overlapping manner the complete native sequence of α- and γ-type gliadins were investigated for binding to gliadin antibodies (pepscan). But if the third amino acid residue in one of the main peptides detected in the pepscan investigations, QPQQPF, was substituted by glutamic acid yielding a PEQ motif, binding of IgA antibodies from sera of CD patients increased considerably. From these findings, together with knowledge about the deamidating activity of tTG, we concluded that gliadin antibodies of CD patients selectively recognized deamidated gliadin.

Later, other investigators [161] reported that gliadin peptide sequences QQLPQPQQPQQSFPQQQRPF, amino acid positions 134–153 of a γ-type gliadin [80] and QLQPFPQPQLPYPQPQS, amino acid positions 56–75 of a α-type gliadin [162] were specifically deamidated by tTG. This study tested binding of IgA and IgG from sera of CD patients to the native peptide and the glutamine–glutamic acid substituted peptides. These authors found that selective deamidation specifically increased recognition by gliadin antibodies from CD patients.

These results demonstrated that the epitope repertoire of gliadin antibodies of CD patients was restricted to a small number of similar amino acid sequences. In this regard it is worth mentioning that human antibodies of the IgE class, which can be found in wheat allergic patients, are also commonly directed against the (nondeamidated) QQPFP and PQQPF motifs [163, 164]. Furthermore, the native gliadin peptide QQPFP is also the main epitope of monoclonal antibodies raised against gliadin or secalin in mice [23]. The restricted number of epitope motifs in gliadin was promising for the development of antibody assays based on a small number of defined gliadin-analogous peptides.

The analysis of the epitope repertoire of antibodies against tTG in CD patients was not, however, conclusive enough to allow the construction of peptide-based tests for estimation of autoantibodies [165–168].

7. Enhanced Diagnostic Potential of Glutamine–Glutamic Acid Substituted Gliadin-Analogous Peptides

Screening of phage-displayed peptides revealed PEQ as important motif recognized by gliadin antibodies of CD patients [157]. Therefore, we investigated all possible nonapeptides bearing a central PQQ from representative sequences of α-, γ-, and ω-type gliadins (for sequences see [169–171], respectively) and their glutamine–glutamic acid substituted peptide counterparts. Altogether we studied 51 different PQQ-containing sequences in a luminescence assay [127]. Binding of IgA from sera of patients with CD was increased when PQQ was substituted by PEQ in nearly all peptides (Fig. 2).

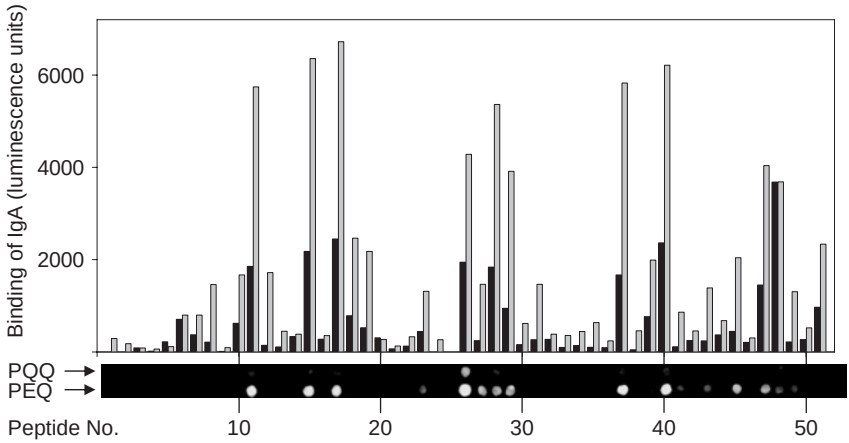

FIG. 2. Binding of IgA from sera of CD patients (positive for endomysium antibodies) to native and glutamine–glutamic acid substituted gliadin peptides. All 51 nonapeptides bearing a central PQQ motif from α-, γ-, and ω-type gliadin and the corresponding 51 substituted peptides with central PEQ were synthesized onto cellulose membranes. Peptides numbered consecutively from N- to C-terminus first of α-, then of γ-, and finally of ω-type gliadin. Binding of IgA detected by means of a luminescence assay applying antihuman IgA conjugated with peroxidase and luminol as substrate [127]. Top: recognition of the peptides by 41 human sera (means). Black columns: peptides with central PQQ. Gray columns: peptides with central PEQ. Bottom: reactivity of a representative serum (upper line peptides with central PQQ, lower line corresponding peptides with central PEQ).

A common feature of the PEQ-containing peptides, which are strongly bound by CD patients' antibodies, was a C-terminal PFP. Of the 51 glutamine–glutamic acid substituted nonapeptides studied, the sequences FSQPEQPFP, SQQPEQPFP, PQQPEQPFP, PLQPEQPFP, PIQPEQPFP, and TQQPEQPFP were recognized by the highest number of CD sera. Interestingly, each contained the common heptapeptide QPEQPFP. Because 10% CD patients were nonreactive with the above peptides, we investigated other peptides that may be used to complement the assay. Although the peptide PEQLPQFEE was recognized only by about one-half of CD patients, it detected several of the patients not detected when the QPEQPFP-containing peptides were used. Thus, for diagnostic purpose, PEQLPQFEE should be used in combination with a QPEQPFP-containing nonapeptide. If PLQPEQPFP and PEQLPQFEE were used within one assay a diagnostic accuracy of 93.8% was obtained. This accuracy was substantially higher than that of gliadin antibodies. It should be stressed that the control group in our study included 14 patients with high gliadin antibody concentration. Only three of these controls were also positive for peptide antibodies, a finding that underscores the specificity of the test [127]. Of course, further variation of the amino acid sequence of peptides could even improve sensitivity and specificity of the peptide antibody test. For routine use, however, the technique has to be simplified and transferred from a luminescent-based assay performed on cellulose membranes to a microtiter plate-based format.

Later, a commercial assay was developed using deamidated gliadin peptides for antibody detection in CD patients (INOVA Diagnostics, San Diego, CA) [172]. Although peptide length was reported to be less than 30 amino acids, the exact amino acid sequence was not disclosed. The authors concluded that the new ELISA was superior in sensitivity and specificity to the standard gliadin antibody format and agreed much better with assays that determined antibodies against tTG and the endomysium [173]. Due to lack of clinical information, the new assay was only correlated to autoantibody assay data. It could be shown that the results of the peptide test showed 97% concordance with results for anti-endomysium and anti-tTG antibody assays. More importantly, of the 56 sera negative for autoantibodies but positive for conventional gliadin antibodies, 54 (96%) were also negative for antibodies against deamidated gliadin peptides. Furthermore, the IgG-based version of the assay was found to detect all cases of IgA-deficient CD patients [172]. The diagnostic value of the test was further substantiated by investigating sera of biopsy proven adult CD patients vs sera of non-CD controls [173]. These data indicated that the new deamidated gliadin peptide assay was an effective and reliable tool for the accurate diagnosis CD in clinical practice [174]. In addition, we were able to develop an ELISA version of the test applying our glutamine–glutamic acid substituted peptides described above [127] with high diagnostic accuracy [175].

However, the question remains, why a new ELISA test when the assay for tTG antibodies already approaches a diagnostic accuracy of ~100%? It should be stressed that the new peptide ELISA neither is a substitute for already existing autoantibody tests nor is it measuring the same antibody species by another method (as is the case for assays for antibodies against endomysium and tTG). The new peptide ELISA measures a new species of antibodies different from conventional gliadin antibodies and autoantibodies against tTG. Therefore, the new peptide ELISA might provide new and additionally useful information on pathogenesis of CD. However, further investigations will need to determine if the new peptide ELISA is better suited for diagnosis in small children in whom autoantibodies are still absent [148, 149]. In addition, the peptide assay may prove to be more stable and yield better reproducibility vs tests using large (recombinant) proteins, for example tTG, in which a native conformation appears crucial for proper antigenicity [147, 176]. The peptide assay may be additionally beneficial if it avoids nonspecific binding to antibodies against tTG (human recombinant), commonly observed in liver disease [140, 141, 145, 146].

8. Does Epitope Specificity of Gliadin Antibodies of CD Patients Mirror Substrate Specificity of tTG?

Several studies have investigated substrate specificity of tTG. After incubation with tTG, gluten peptides were obtained with a strong stimulatory effect on T cells of CD patients. Analysis by mass spectrometry revealed a highly restricted pattern of deamidation with defined glutamine residues identified as tTG targets [79, 80]. Substituting amino acids in the vicinity of glutamine residues in gliadin demonstrated that the amino acid proline in position +2 favored deamidation, whereas proline in positions +1 and +3 (N- to C-terminus) exerted a negative effect [177, 178]. Screening the complete sequence of α-gliadin after tTG treatment confirmed the deamidation of 19 of 94 glutamine residues and the critical role of nearby proline amino acid residues [179]. Analysis of peptides from a peptic–tryptic digest of gliadin resulted in 10 peptides in which tTG was able to attack glutamine residues with proline in position +2 [180]. Using a phage display system, the QXP sequence within larger peptides was also shown to be a good substrate for tTG [181]. We screened a library of gliadin peptides spotted onto cellulose membranes (pepspot) and demonstrated that tTG exclusively attacks the first glutamine residue in the PQQPFP motif [23]. We found, however, that substitution of the amino acid phenylalanine in position +3 by tyrosine tremendously decreased tTG activity. This effect was, however, partly counterbalanced if a leucine residue resided in position +1 [182].

These features of substrate specificity of tTG are mirrored by epitope specificity of gliadin antibodies of CD patients. The common core of the nonapeptides specifically recognized by antibodies of CD patients is QPEQPFP with the amino acid proline residing in position +2 [127]. However, in accordance with results from studies of substrate specificity of tTG [182], exchange of phenylalanine residue for tyrosine completely abolished binding of IgA of CD patients [127].

To comprehensively address this phenomenon, we modified the sequence of the nonapeptide FSQPEQPFP (derived from our experiments as a good epitope for IgA from CD patients) by phenylalanine–tyrosine and glutamine–leucine substitutions (Table 2). Binding of antibodies to the peptide was very low after glutamine–leucine substitution and was completely lost after phenylalanine–tyrosine exchange. We also tested peptides from the N-terminal region of α-type gliadins identified (after deamidation) as potent T-cell epitopes [34, 82, 162, 183]. All of these sequences contained the PELPYP motif instead of the PEQPFP motif. We demonstrated that PEQPYP and PELPYP (as part of the nonapeptides) were not recognized by IgA from CD patients (Table 2).

Our results agree with investigations on binding of CD antibodies to a deamidated fragment of a longer peptide comprising positions 56–73 of A-gliadin, an α-type gliadin. This peptide, QLQPFPQPQLPYPQPQS, after a glutamine–glutamic acid substitution at position 65 (underlined) also contained the immunodominant CD-specific epitope [162]. However, IgA from sera of CD patients did not bind better to the deamidated version of the peptide than to the native sequence [161, 184]. Admittedly, in the first study [161], the deamidated version was detected by IgG better than the native

TABLE 2
REACTIVITY OF IGA FROM SERA OF PATIENTS WITH BIOPTICALLY PROVEN CD WITH DIFFERENT SYNTHETIC NONAPEPTIDES[a]

Peptide	Diagnostic sensitivity (%)	Source of sequence
FSQPEQPFP	90	[127]
FSQPEQPYP	0	Modified according to [127]
FSQPELPYP	3	Modified according to [127]
FSQPELPFP	26	Modified according to [127]
PFPQPELPY	0	Fragment 60–68 of peptide 57–73 [162]
FPQPELPYP	0	Fragment 61–69 of peptide 57–73 [162]
YPQPELPYP	0	Fragment 74–83 of 33-mer peptide [34]
PYPQPELPY	3	Fragment 73–82 of 33-mer peptide [34]

[a]Effect of glutamine–leucine and phenylalanine–tyrosine substitutions on antibody binding. Peptides spotted onto cellulose membranes. Antibody binding measured in a luminescence assay [127]. At least 39 sera of CD patients positive for endomysium antibodies tested.

peptide (84% vs 47% of sera reactive). However, in the same study, a peptide from γ-gliadin containing the QPQQPQQSFPQQQRPF sequence (positions of glutamine–glutamic acid substitutions underlined) was much more effective in discriminating CD and control sera [161].

The QPQQPFP sequence and the shorter hexapeptide PQQPFP are only contained in γ- and ω-type gliadins, not α-type gliadins (BLAST database, U.S. National Center for Biotechnology Information for exact matches of the PQQPFP motif for gliadin sequences [185]). Therefore, the CD-specific IgA antibodies against deamidated gliadin peptides [127] are directed predominantly against γ- and ω-type prolamins but should react only weakly with α-type in which the PQLPYP sequence is predominant together with PQQPYP, QQLPYP, and QQQPFP.

Studies of epitope specificity of gliadin antibodies and substrate specificity of tTG point to a role of proline and phenylalanine in amino acid positions +2 and +3 in glutamine deamidation. In contrast, investigations of T-cell stimulatory peptides reveal several potent T-cell stimulatory peptides from the N-terminal part of α-type gliadin containing the PELPY motif. In this connection, it was shown that substitution of phenylalanine in the +3 position or other amino acids in the glutenin peptide Glt-156 inhibited the T-cell proliferative response [186].

Interestingly, the epitope of human gliadin antibodies of CD patients is similar to that of mouse monoclonal antibodies that recognized the native amidated form. We compared the epitope specificity of nine different monoclonal antibodies raised against gliadin or secalin. The epitope specificity differed only slightly according to the length of the sequence (five to eight amino acids). Remarkably, the amino acid phenylalanine constituted the main common requirement for antibody binding and in most cases substitution of this aromatic amino acid by tyrosine was not tolerated [23]. This finding suggested that the presence of phenylalanine represented an important structural condition for antibody binding.

9. Relation Between B- and T-Cell Epitopes of Gliadin

The gluten-triggered pathogenic reaction in CD, that is destruction of the intestinal mucosa, is generally seen as CD4 T-cell-mediated immune response in the lamina propria together with innate immune responses through interleukin-15 induction (recent reviews [187, 188]). Although these antibodies appear to be specific indicators of disease, their pathogenic role remains unclear [189]. Autoantibodies are not regarded as disease initiators and are unlikely to cause disease symptoms [190]. Gliadin antibodies are considered nonspecific and secondary to enhanced permeability of the intestinal mucosa due to tissue destruction. However, we hypothesized earlier [157] that gliadin

antibodies may not only represent an epiphenomenon of CD but may play a decisive role in the delivery of gliadin peptides into antigen processing cells of the gut mucosa. After binding of gliadin to membrane-bound B-cell receptors or to soluble gliadin antibodies which in turn can associate with Fc receptors, gliadin could be taken up and delivered for intracellular processing. The subsequent presentation by DQ2 molecules would initiate an efficient T-cell response. The repetitive nature of the epitopes may represent an important prerequisite for cross-linking of Fc and B-cell receptors and thus for activation of B and accessory cells. We stressed that B-cell epitopes can be found as identical or homologous sequences in peptides reported as ligands of HLA-DQ2 molecules and in T-cell epitopes, pointing to a role of B-cell epitopes as targets for immunoglobulins for delivery into MHC class II-expressing cells [157].

Recognition of a dominant T-cell epitope of A-gliadin by IgA antibodies of patients with CD was also described in another study [184].

The rules of gliadin T-cell epitope binding to the disease-associated DQ2 molecules in CD were redefined [191]. By comparing nine gluten epitopes, a (deamidated) nonapeptide consensus sequence P-Q-P-Q-Q-P/Q-F/Y-P-Q was obtained as peptide-binding register with proline residues in positions 1, 3, 6, and 8 but never in positions 2, 4, 7, and 9 and with the main deamidations crucial for T-cell recognition in positions 4 and 6 (underlined). This sequence agrees well with the epitope of gliadin antibodies in CD patients. The striking similarity between B- and T-cell epitopes might support a still unrecognized or underestimated role of gliadin antibodies in the pathogenesis of CD.

10. Conclusions

Gliadin antibodies in sera of patients with active CD preferentially recognize several deamidated gliadin peptides. The amino acid sequence of the epitopes of gliadin antibodies is in agreement with data on substrate specificity of tTG showing that this enzyme is responsible for generation of the deamidated epitope motifs. Furthermore, the gliadin antibody epitope (B-cell epitope) is very similar to sequences described as strongly T-cell stimulatory (T-cell epitopes). Determination of antibodies against deamidated gliadin peptides has high diagnostic accuracy approaching that of autoantibody assays (endomysium antibodies, antibodies against tTG). Further improvement and refinement of the gliadin-analogous peptide sequences in this unique assay system will likely increase the validity of the test. The assay applying deamidated gliadin peptides measures a new species of antibodies, which is different from conventional gliadin antibodies as well as from autoantibodies directed against tTG. Therefore, the new peptide ELISA provides new information on pathophysiological mechanisms of CD.

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UROKINASE RECEPTOR VARIANTS IN TISSUE AND BODY FLUIDS

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Abbreviations

AML	acute myeloid leukemia
ATF	amino-terminal fragment of uPA
BPH	benign prostatic hyperplasia
DFP	diisopropyl fluorophosphate
ELISA	enzyme-linked immunosorbent assay
FPR	formyl peptide receptor
FPRL1/LXA4R	FPR-like receptor-1/lipoxin A4 receptor
G-CSF	granulocyte colony-stimulating factor

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GPI	glycosylphosphatidylinositol
GPI-PLD	GPI-specific phospholipase D
HEK	human embryonic kidney
HIV-1	human immunodeficiency virus-1
HSC	hemapoetic stem cells
LMW-uPA	low molecular weight uPA
m	murine
mAb	monoclonal antibody
MMP	matrix metalloproteinase
NSCLC	non-small cell lung cancer
pAb	polyclonal antibody
PAI-1	plasminogen activator inhibitor-1
s	soluble
SCLC	small cell lung cancer
tPA	tissue-type plasminogen activator
TR-FIA	time-resolved fluorescence immunoassay
uPA	urokinase-type plasminogen activator
uPAR	uPA receptor
uPAR(I)	uPAR domain I
uPAR(I–III)	intact uPAR
uPAR(II–III)	uPAR domain II + III

1. Abstract

The cellular receptor for urokinase, urokinase-type plasminogen activator receptor (uPAR) plays a central role in localizing its ligand, urokinase-type plasminogen activator (uPA) and thereby the plasminogen activation to the cell surface. uPA converts the proenzyme plasminogen to plasmin, which is involved in degradation of the extracellular matrix. In addition, uPA also cleaves uPAR, liberating the ligand-binding domain I, uPAR(I) and leaving the cleaved form, uPAR(II–III) on the cell surface. This cleavage inactivates the binding potential of uPAR toward uPA and vitronectin. uPAR can be shed from the cell surface and both intact and cleaved uPAR variants have been identified in tissue and body fluids. Identification and characterization of cleaved uPAR variants are dependent on monoclonal antibodies with known epitope specificity. Some of these have also been useful for the immunohistochemical localization of uPAR in tumor tissue. A number of immunoassays have been designed to measure uPAR and the collective amounts of all uPAR forms measured by enzyme-linked immunosorbent assay (ELISA) in tumor lysates or blood correlate to prognosis in several forms of cancer. However, the amounts of uPAR(I) and uPAR(II–III) may be

directly related to the uPA activity and therefore be even stronger prognostic markers. Immunoassays measuring the individual uPAR forms have recently been designed and can be used to investigate this. This chapter is focused on the mechanism of uPAR cleavage and characterization and identification of the different uPAR forms in biological tissues and body fluids using immunologic methods. Monoclonal antibodies against uPAR are reviewed as well as different immunoassays used to investigate the prognostic potential of uPAR. Finally, an overview of the localization and prognostic significance of uPAR in different cancers and other malignancies is included.

2. Introduction

The urokinase-type plasminogen activator receptor (uPAR/CD87) is essential for cell surface-associated plasminogen activation, mediated by its ligand, urokinase-type plasminogen activator (uPA) [1–4]. uPA binds with high affinity to the intact, three domain receptor protein, uPAR(I–III), which is attached to the cell surface by a glycolipid anchor. The proenzyme plasminogen is converted into plasmin by uPA. Plasmin is a key enzyme for degradation of extracellular matrix proteins in a variety of biological processes, where tissue destruction and cell migration are involved [5–8]. Even though uPAR plays a crucial role in cell surface plasminogen activation, no obvious phenotype was observed, when unchallenged uPAR-deficient mice were born and closely examined [9, 10]. However, macrophages isolated from $uPAR^{-/-}$ mice were not effective in potentiation of uPA-mediated cell surface plasminogen activation, unlike macrophages from wild-type mice. When uPAR-deficient mice were made double deficient by crossing with mice deficient in tissue-type plasminogen activator (tPA), fibrin deposits were found in the liver of the adult double-deficient animals. These deposits were not observed in mice with either uPAR or tPA deficiency alone, demonstrating that uPAR possesses a functional role in fibrinolysis [11]. When the $uPAR^{-/-}$ mice were challenged with *Pseudomonas aeruginosa* infection, the induced neutrophil recruitment was reduced compared to that in wild-type mice [12]. Furthermore, leukocyte recruitment to sites of acute inflammation was dramatically decreased in uPAR-deficient mice [13–15].

In addition to binding to uPAR(I–III), uPA can cleave uPAR(I–III) in the linker region between domains I and II, liberating domain I and leaving the cleaved form, uPAR(II–III) on the cell surface [16, 17]. uPAR(I–III) is shed from the cell surface by cleavage at or near the glycolipid anchor, and the soluble form, suPAR(I–III) as well as the cleaved soluble form, suPAR(II–III) have been identified in biological fluids [8, 18–22]. Two crystal structures of a soluble form of the human uPAR(I–III) have been reported [23, 24].

This knowledge will provide new insights to be used in the design of inhibitors targeting the interaction between uPA and uPAR(I–III). Inhibition of uPA binding to uPAR(I–III) using monoclonal antibodies (mAbs), derivatives of uPA with no proteolytic function, or synthetic peptides has been demonstrated to have pronounced effects on plasminogen activation, primary tumor growth, and dissemination of cancer cells in model systems [25–32]. In tumor tissue from cancer patients, uPAR is often expressed at the invasive front [33, 34] and high levels of uPAR in tumor tissue as well as in blood are correlated with poor prognosis for patients with different types of cancer [35–41]. Elevated levels of uPAR in blood from patients with infectious diseases like Human immunodeficiency virus-1 (HIV-1) are also correlated with adverse prognosis [42–45]. In most of these studies, uPAR has been measured using assays, which detect the total amounts of the different uPAR forms. However, knowledge of the levels of the individual uPAR(I–III), uPAR(II–III), and uPAR(I) forms might provide improved prognostic information.

This chapter will concentrate on the different uPAR forms, how they are generated, in what tissues and body fluids they have been detected, and how they may be measured in patient samples by immunoassays. The mAbs used for assay design and immunohistochemistry will be characterized with respect to domain specificity. Finally, an overview of the prognostic findings that has been made in relation to several types of cancers is given.

2.1. Full-Length, Glycolipid-Anchored uPAR, uPAR(I–III)

Twenty years ago, a high-affinity-binding site for uPA was demonstrated on the surface of peripheral blood monocytes and cultured cells of the human histiocytic lymphoma cell line, U937 [46]. The expression of uPAR on the cell surface of many cell types has since then been demonstrated, including a variety of neoplastic cell lines as well as nonneoplastic cells such as neutrophils, macrophages, keratinocytes, placental trophoblasts, endothelial, and smooth muscle cells [7, 33, 47–51]. The human uPAR gene has been mapped to chromosome 19q1.3 [52].

The uPAR protein was initially purified from lysates of phorbol ester-stimulated U937 cells by affinity chromatography using diisopropyl fluorophosphates (DFP)-inactivated uPA [53, 54]. uPAR is anchored in the plasma membrane by a glycosylphosphatidylinositol (GPI) moiety and it consists of 283 amino acids in its processed form [55, 56]. The protein is composed of three domains and each domain contains ~90 amino acids. The domains are connected by linker regions with a length of 15–20 amino acids [57, 58]. The disulfide bonds in the N-terminal domain I have been experimentally determined and the pattern of cysteine residues in the sequence has revealed

structural homology between the three domains of uPAR(I–III) [59]. uPAR(I–III) is a heavily glycosylated protein with five potential glycosylation sites for N-linked carbohydrates, N^{52} on domain I, N^{162} and N^{172} on domain II, and N^{200} and N^{233} on domain III [60]. There are experimental evidence that all but the N^{233} glycosylation site are utilized [61]. The glycosylation of domain I influences uPA binding, as the affinity for uPA was shown to be decreased around fivefold by mutating $N^{52}\rightarrow E$ and thus preventing glycosylation of domain I [62]. The glycosylation of uPAR(I–III) is markedly heterogeneous varying widely among different cell types and even within one cell type [53, 63].

uPAR(I–III) has an important function in extracellular proteolysis as binding of the zymogen pro-uPA to uPAR(I–III) results in a strong enhancement of cell surface plasminogen activation [2]. This effect is dependent on the concomitant binding of pro-uPA to uPAR(I–III) and of plasminogen to its cell surface-binding sites [64]. Abrogating the binding between pro-uPA and uPAR(I–III) using the domain I-specific mAb R3 inhibits this potentiation of plasmin generation [1]. Domain I of uPAR(I–III) contains the functional binding site for uPA [65], but the liberated uPAR(I) binds uPA with very low affinity [6]. The intact uPAR molecule is required for the high-affinity binding [66, 67].

The requirement of membrane localization of uPA has also been shown in a different system. In the anthrax toxin-protective antigen, the furin cleavage site was replaced by a sequence specifically cleaved by uPA. This uPA sensitive protective antigen is activated by uPAR(I–III)-bound uPA leading to internalization of the added recombinant cytotoxin, consisting of anthrax toxin lethal factor fused to the ADP-ribosylation domain of *Pseudomonas* exotoxin A. This treatment leads to cell death; however, the cells can be protected by incubation with mAb R3, thus showing that the membrane localization of uPA is also important for cleavage of the protective antigen [3].

In the elegant studies by Zhou and coworkers, simultaneous overexpression of catalytically active uPA and uPAR in keratinocytes in the presence of plasminogen resulted in a pathological phenotype of the skin characterized by extensive alopecia, induced by involution of the hair follicles, epidermal thickening, and subepidermal blisters [4, 68]. No skin phenotype was evident in mice with targeted overexpression of either uPA or uPAR alone, of catalytically inactive uPA together with uPAR, or in the absence of plasminogen.

In addition to its role in pericellular proteolysis, uPAR(I–III) plays a role in cell adhesion by binding to the extracellular matrix protein vitronectin [69]. There has been some controversy as to where the binding site for vitronectin is located in the uPAR structure. However, the domain I-specific mAbs, R3 and R5, prevented binding of vitronectin to cell surface-expressed uPAR(I–III) as well as to soluble uPAR(I–III), whereas the mAbs, R2 and

R4, with their epitopes located on uPAR(III)had no effect. Thus, the main binding site for vitronectin is located in domain I even though, as is the case for uPA binding, the entire uPAR(I–III) is required for high-affinity binding [70–72]. The precise functional epitope for vitronectin binding has yet to be identified. However, uPA and vitronectin have distinct binding sites since concomitant binding of uPA and vitronectin to uPAR(I–III) is possible and binding of uPA to uPAR(I–III) augments vitronectin binding [69, 70, 72]. This enhanced vitronectin binding is independent of the catalytical activity of uPA since pro-uPA and the catalytic inactive amino-terminal fragment (ATF) of uPA have the same effect [69]. uPAR(I–III) binds vitronectin through the somatomedin B domain of vitronectin [73, 74] and it competes with the uPA inhibitor, plasminogen activator inhibitor-1 (PAI-1) for binding to vitronectin, demonstrating overlapping binding sites [73, 74]. Thus, both uPA and PAI-1 modulate the physical binding of vitronectin to uPAR(I–III) independent of their enzymatic and inhibitory activities.

Besides the uPA and vitronectin binding, the direct association of uPAR(I–III) with different integrins has been demonstrated using coimmunoprecipitation. In a study where the human embryonic kidney cell line 293 (HEK293), being devoid of uPAR, was transfected with either uPAR(I–III) or uPAR(II–III), it was shown that antibodies to the integrin subunits α_v, α_3, α_5, and β_1 coimmunoprecipitated with the intact receptor but not with the cleaved form [75]. In both HEK293 cells transfected with uPAR(I–III) and MDA-MB-231 breast cancer cells, uPAR was found in complex with $\alpha_3\beta_1$ [76]. In the human epidermoid carcinoma cell line HEp3, uPAR was associated with $\alpha_5\beta_1$ and the binding was augmented by uPA. In this study, the fibronectin-dependent Ras extracellular signal-regulated kinase activation was inhibited by an anti-β_1 integrin function-blocking antibody as well as by the anti-uPAR mAb R2 binding to domain III, and to a lesser extent by anti-uPAR mAbs recognizing domain I [77]. The association of uPAR with the β_2 integrin subunit has also been described [78, 79]. Although most studies on interactions between uPAR and integrins point to the requirement of intact uPAR, binding of suPAR(II–III) to the $\alpha_4\beta_1$, $\alpha_6\beta_1$, $\alpha_9\beta_1$, and $\alpha_v\beta_3$ integrins has been reported [80].

2.2. Cleavage of uPAR(I–III)

By use of mAbs with known domain specificity, a molecular variant of uPAR-lacking domain I, uPAR(II–III), was first identified on U937 cells [16]. Subsequently, uPAR(II–III) was identified on several neoplastic cell lines, in xenotransplanted human mammary and ovarian tumors as well as in tumor tissue lysates from ovarian cancer patients [20, 81–85]. In solution, purified uPAR(I–III) can be cleaved in the linker region between domains I and II by

moderate concentrations of uPA (i.e. 10 nM), though it is a slow reaction. Low molecular weight (LMW)-uPA, lacking the epidermal growth factor like receptor-binding domain, can also cleave uPAR(I–III) in solution [16]. Thus, binding of uPA to uPAR(I–III) through this domain is not required for the cleavage to occur. Binding of an inactive form of uPA, DFP-uPA to domain I of uPAR(I–III), does not prevent added active uPA from cleaving uPAR in solution [17]. In contrast to this slow cleavage in solution, uPA-mediated cleavage on the cell surface is much faster and this acceleration is dependent on binding of uPA to uPAR(I–III), since the cleavage is inhibited by preincubation of the cells with DFP-uPA [16, 17]. Furthermore, LMW-uPA added to cells results in a similar time course for uPAR(I–III) cleavage, as uPA added to purified uPAR(I–III) in solution [16] (Fig. 1). uPA bound to uPAR(I–III) on the cell surface, therefore, cleaves a neighboring uPAR(I–III) molecule [17].

The two N-terminal sequences of uPAR(II–III) obtained by cleavage of purified uPAR(I–III) with uPA were identical to those of uPAR(II–III) purified from U937 cells. The two cleavage sites are between R^{83} and A^{84} (^{81}SGRAV^{85}) and between R^{89} and S^{90} (^{87}YSRSR^{91}) [17]. The cleavage of

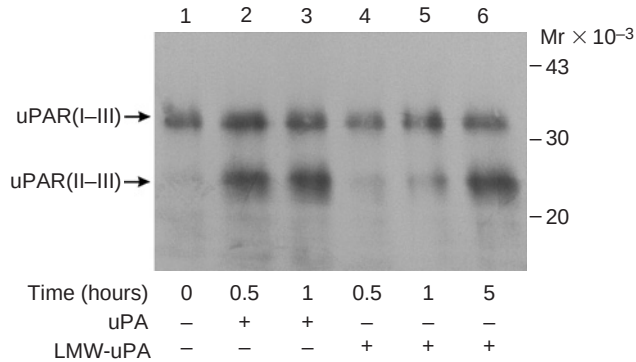

FIG. 1. Time course of uPA and LMW-uPA-catalyzed cleavage of uPAR on the cell surface. U937 cells were grown for 48 hours in the presence of fetal calf serum, phorbol ester, and 10 μg/ml of the anticatalytic anti-uPA mAb clone 5. The cells were washed with phosphate-buffered saline and subsequently with glycine buffer pH 5 to remove receptor-bound uPA. After neutralization, cells were either harvested immediately (lane 1) or transferred to 20 ml of serum-free culture medium, either with 10 nM uPA (lanes 2 and 3) or with 10 nM LMW-uPA (lanes 4–6). The cells were then incubated for 0.5 hour (lanes 2 and 4), 1 hour (lanes 3 and 5), and 5 hours (lane 6) at 37°C. After incubation the cells were harvested, lysed, and subjected to temperature-dependent phase separation [53]. Detergent-phase samples from 10^6 cells were denatured under reducing conditions and deglycosylated by incubation with 0.5 U *N*-glycosidase F for 20 hours. Samples were analyzed by SDS-PAGE and Western blotting using 2 μg/ml of mAb S1, recognizing the reduced forms of uPAR(I–III) and uPAR(II–III) [17].

uPAR(I–III) on U937 cells and cells from the human breast cancer cell line MDA-MB-231 can be inhibited by culturing the cells in the presence of the antihuman uPA mAb clone 5, which we have previously demonstrated to inhibit the catalytic activity of human uPA [16, 17, 83]. In addition, murine uPAR(I–III), muPAR(I–III), cleavage on Lewis lung carcinoma cells is inhibited by culturing the cells in the presence of an anti-murine uPA (muPA) polyclonal antibody (pAb) [83]. Interestingly, whereas there is a high degree of species specificity in the ligand binding between uPA and uPAR(I–III) [8, 63], this is not the case for the cleavage since both muPA and human uPA can readily cleave human uPAR(I–III) [16]. Furthermore, Chinese hamster ovary cells transfected with human uPAR(I–III) [86] liberated uPAR(I) into the media (Fig. 2A, lane 4), demonstrating that hamster uPA also cleaves human uPAR(I–III). This liberated domain I was purified from the cell culture media by immunoaffinity chromatography using the uPAR(I)-specific mAb R9 [65, 87]. The purified protein comigrates with domain I obtained by uPA cleavage (Fig. 2A, lanes 3 and 4). Plasmin is able to cleave purified

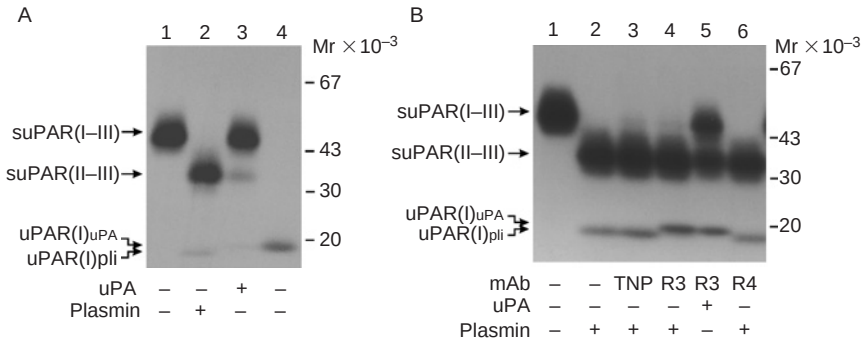

FIG. 2. Plasmin and uPA-catalyzed cleavage of suPAR(I–III). (A) ^{35}S-labeled recombinant suPAR(I–III) was purified on an immunoaffinity column with the domain I-specific mAb R3 immobilized [86]. Lane 1 is 10 nM suPAR(I–III), lane 2 is 10 nM suPAR(I–III) incubated for 2 hours at 37°C with 50 nM plasmin (pli), lane 3 is 10-nM suPAR(I–III) incubated for 20 hours at 37°C with 500-nM uPA, and lane 4 is ^{35}S-labeled domain I purified from cell culture media on an immunoaffinity column with the domain I-specific mAb R9 immobilized, after removing suPAR (I–III) from the media by immunoaffinity chromatography, employing the domain III-specific mAb R2. (B) Ten nM suPAR(I–III) was preincubated with 67 nM of the indicated mAbs for 2 hours at 37°C prior to addition of either 50 nM plasmin (lanes 2, 3, 4, and 6) or 500 nM uPA (lane 5) and continued incubation for 2 or 20 hours at 37°C. Lane 1 is suPAR(I–III) alone, lane 2 is suPAR(I–III) incubated with plasmin only, lane 3 shows preincubation with a subtype control mAb, lanes 4 and 5 show preincubation with mAb R3, lane 6 shows preincubation with the domain III-specific mAb R4. The radioactive samples have been separated by SDS-PAGE prior to autoradiography of the dried gel.

uPAR(I–III) and suPAR(I–III) with the same efficiency and the cleavage sites include those found for uPA (Fig. 3) [16, 86, 88]. However, uPAR(I) obtained by plasmin cleavage of purified suPAR(I–III) does not comigrate with the liberated domain I purified from cell culture media (Fig. 2A, lanes 2 and 4). uPAR(I) produced by plasmin cleavage has a higher electrophoretic mobility suggesting cleavage sites inside of domain I. This intradomain cleavage is inhibited by preincubation of suPAR(I–III) with mAb R3 (Fig. 2B, lane 4). Preincubation with mAb R3 does not prevent cleavage by plasmin in the linker region and consequently does not influence uPA-mediated cleavage (Fig. 2B, lane 5). Since the functional epitope of mAb R3 is located to E^{33}, L^{61}, and K^{62} (Section 3) and the substrate specificity of plasmin predicts hydrolysis of the peptide bond on the carboxyl side of K and R, the cleavage site inside domain I is most likely between K^{62} and I^{63} (Fig. 3).

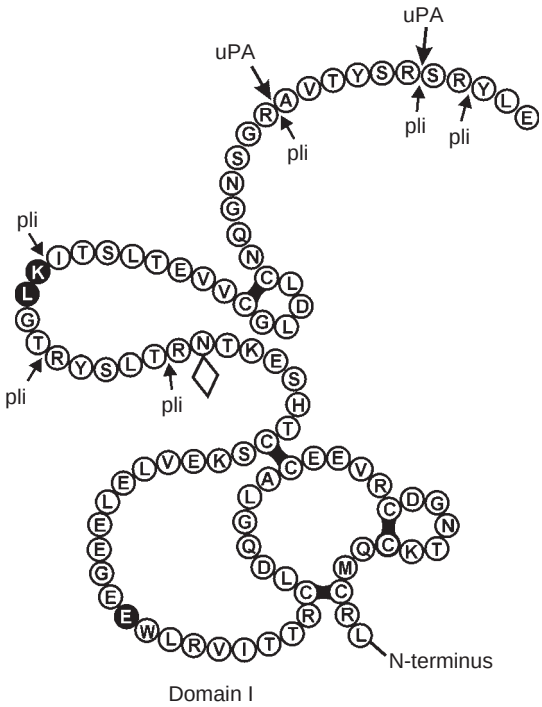

FIG. 3. uPA and plasmin cleavage sites in the linker region and in domain I of uPAR. The uPA cleavage sites in uPAR have been experimentally determined [17]. The plasmin (pli) cleavage sites have been deduced from its substrate specificity hydrolyzing peptide bonds with R or K residues on their carboxyl side. The amino acids of the functional epitope of mAb R3 are indicated [8].

In vivo it was demonstrated that active muPA is required for cleavage of muPAR(I–III). Cleaved muPAR was only observed in skin extracts from bitransgenic mice with keratinocyte-targeted overexpression of muPAR(I–III) and catalytically active muPA, but not catalytically inactive muPA [4]. It was excluded that muPA-activated plasmin was responsible for the cleavage of muPAR(I–III) by crossing the mice in order to obtain bitransgenic mice overexpressing muPAR and active muPA but in a plasminogen-deficient background. When analyzing skin extracts for the level of functional, muPA-binding muPAR(I–III), this was found to be the same whether the bitransgenic mice were in a wild-type, heterozygote, or plasminogen-deficient background [68]. This demonstrates that in these keratinocytes where muPA and muPAR(I–III) were overexpressed, muPA and not plasmin was responsible for the cleavage of muPAR(I–III). However, it cannot be ruled out that in different cell types other proteases can cleave uPAR(I–III). Incubation of U937 cells with a relatively high concentration of neutrophil elastase (100 nM) also liberated domain I from the cell-bound uPAR(I–III) [89]. Furthermore, in human foreskin microvascular cells, it was found that matrix metalloproteinase-12 (MMP-12) released domain I from cell-anchored uPAR(I–III) [90].

The identification of uPAR(II–III) in U937 cell lysates using Western blotting is hampered by the identical electrophoretic mobility of uPAR(II–III) and an intracellular, differently glycosylated form of uPAR(I–III) [53]. However, removing the carbohydrates clearly separates the variants, since uPAR(I–III) daltons and uPAR(II–III) have apparent molecular masses of 35,000 and 27,000 after complete deglycosylation, respectively [16]. Glycosylation has been suggested to regulate uPAR(I–III) cleavage. In the cell line ARO, derived from an anaplastic thyroid cancer, uPAR(I–III) was found to be heavily glycosylated and resistant to cleavage by added uPA. uPAR(I–III) from another thyroid cell line, TAD-2, was not as heavily glycosylated and was efficiently cleaved by uPA. After deglycosylation, the apparent molecular mass of uPAR(I–III) from both cell lines was 35,000 daltons, indicating that the only difference between uPAR(I–III) on ARO cells and TAD-2 cells was the extent of glycosylation [91]. However, it cannot be excluded that a mutation in the linker region between domains I and II in uPAR(I–III) from ARO cells or a difference in the GPI anchor (Section 2.3) was responsible for the observed resistance to proteolysis by uPA. The cleavage of uPAR(I–III) prevents uPA binding and cell surface plasminogen activation.

uPAR cleavage could also affect internalization, since it has been demonstrated that intact cell surface uPAR(I–III) is required for internalization of the uPA–PAI-1 complex [92]. After internalization, uPAR(I–III) was found to be recycled to the cell surface [93]. Internalization also requires the presence of another receptor, a member of the low-density lipoprotein

receptor family, and it has been shown that there is a direct binding between domain III of uPAR and the α_2-macroglobulin receptor/low-density lipoprotein receptor-related protein [94, 95]. No studies have been carried out to investigate if and how the cell surface cleavage product uPAR(II–III) is internalized.

2.3. Shedding of uPAR Forms

The soluble form of uPAR(I–III), suPAR(I–III), was initially identified in plasma from patients with paroxysmal nocturnal hemoglobinuria. This form of the protein was not shed from the cell surface but secreted by leukocytes from these patients due to a deficiency in their ability to synthesize or attach a functional glycolipid anchor [96]. In plasma and serum from healthy individuals, only shed, intact uPAR has been identified and purified [18, 20, 82, 86, 97, 98], whereas both intact and cleaved suPAR is found in plasma from acute myeloid leukemia (AML) patients [18]. In ascites and cystic fluid from ovarian cancer patients, suPAR(I–III) is present in high concentrations and suPAR(II–III) has also been identified [21, 82, 99]. The mechanism of shedding and the enzymes responsible have not been fully elucidated. A study using six ovarian cancer cell lines revealed that the majority of the suPAR released from the cell surface was generated by GPI anchor cleavage, catalyzed by endogenous cellular GPI-specific phospholipase D (GPI-PLD), whereas the GPI-PLD from serum was not involved [22]. Downregulation of the endogenous GPI-PLD by antisense mRNA greatly affected the amounts of suPAR detected in the media. uPAR release was also inhibited by the metalloproteinase inhibitor 1,10-phenanthroline, which also inhibits GPI-PLD, but serine proteinase inhibitors had no effect [22]. In contrast, addition of only 10 nM of the serine proteinase plasmin to monolayers of human SV40-transfected bronchial epithelial cells resulted in liberation of uPAR(I–III) into the medium. The plasmin cleavage site was located to R^{281} by MALDI-MS analysis of the proteolytic fragmentation of synthetic peptides covering the 12 C-terminal amino acids [88]. The same investigators found that addition of the neutrophil serine proteinase cathepsin G released uPAR(I–III) from the cell surface of U937 cells but the concentrations used were not physiologically relevant (between 250 nM and 1 μM). Furthermore, no experiments have been conducted to investigate if inhibition of endogenous plasmin or cathepsin G activities prevented the release of uPAR from the cell surface. Both these enzymes are capable of cleaving uPAR after R^{281} close to the C-terminus but whether these proteolytic cleavages are physiologically important remains to be investigated.

Since both intact and cleaved suPAR forms have been identified in biological fluids and as the only observed difference between uPAR(I–III)

and suPAR(I–III) is the lack of the GPI anchor, it was expected that they would be susceptible to cleavage by the same proteases in the linker region between domains I and II. Surprisingly, purified plasma suPAR(I–III) from healthy individuals could not be cleaved by overnight incubation with 100 nM uPA, in contrast to GPI-anchored uPAR(I–III). Both forms were, however, efficiently cleaved by 50-nM plasmin [86]. Recombinant $suPAR_{1–277}$ behaves in this respect like plasma suPAR(I–III) [86]. *In vitro* cleavage of $suPAR_{1–277}$ by MMP-3, MMP-12, MMP-19, and MMP-26 revealed one major cleavage site positioned after T^{86} in the linker region [100]. So far no studies have been conducted to clarify whether suPAR(II–III) is generated *in vivo* by cleavage of suPAR(I–III) or if the cleavage takes place on the cell surface and both forms are subsequently shed. Using keratinocytes with targeted overexpression of muPAR and either an active or inactive form of muPA, cleaved smuPAR was only detected in the media from cells expressing active muPA [4]. As discussed above (Section 2.2), muPA cleaves muPAR(I–III) on the cell surface of these keratinocytes [68]. Since uPA cleaves uPAR(I–III) but not suPAR(I–III) [86], these experiments taken together indicate that in this case muPAR(I–III) is cleaved on the cell surface, leading to shedding of both the intact and cleaved forms. The different uPAR variants thus far identified in tissue and body fluids are illustrated in Fig. 4.

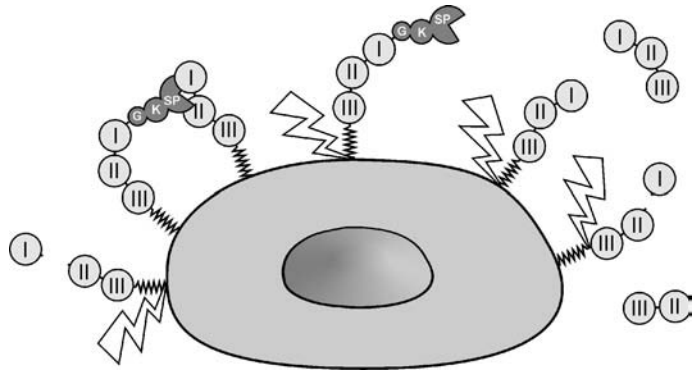

FIG. 4. uPAR variants present on the cell surface and in solution. The illustration shows uPAR(I–III)-bound uPA-mediated cleavage of a neighboring uPAR(I–III) on the cell surface, liberating uPAR(I) into the medium and leaving uPAR(II–III) on the cell surface [17]. The GPI-linked uPAR forms are shed from the cell surface either by GPI-anchored cleavage catalyzed by endogenous cellular GPI-PLD [22] or possibly by proteolytic cleavage after R^{281} [88, 89]. The uPA domains depicted are the growth factor domain (G), the kringle domain (K), and the serine protease domain (SP).

The obvious molecular difference between uPAR(I–III) and suPAR(I–III) is the lack of at least the lipid moiety of the GPI anchor. This does not influence their ligand-binding properties. However, the glycolipid-anchored attachment to the cell membrane is required for internalization of the uPA–PAI-1 complex [92]. Furthermore, the GPI anchor governs the conformation or flexibility of the linker region between domains I and II, resulting in the different susceptibility to uPA cleavage. suPAR(I–III) cannot be cleaved by physiologically relevant concentrations of uPA [86]. Furthermore, an antibody raised to a peptide covering A^{84}–E^{94} of the linker region reacts with GPI-anchored uPAR(I–III) but the reactivity is lost after removal of the lipid moiety by Pi-PLC, and suPAR(I–III) is not recognized by this antibody [86]. Conformational alterations as a consequence of removal of the GPI anchor have been reported for several proteins, including Thy-1 glycoprotein [101], Ly-6A.2 [102], carcinoembryonic antigen [103], and CD59 [104]. In the recently reported crystal structures of a soluble form of uPAR(I–III), the region comprising amino acids 84–90, which is a part of the linker region, was poorly defined in the electron density map and is therefore not included in the structures [23, 24].

Cleavage of uPAR(I–III) in the linker region between domains I and II prevents uPA, vitronectin, and integrin binding. Cleavage of suPAR(I–III) in the linker region was, however, found to be required for its chemotactic function [105]. This chemotactic property of cleaved suPAR could be mimicked by a peptide constituting a sequence, that is, $S^{88}RSRY^{92}$, present in the linker region [106]. suPAR(II–III) containing this chemotactic epitope was shown to be a ligand for the seven-transmembrane receptor, formyl peptide receptor (FPR)-like receptor-1/lipoxin A4 receptor (FPRL1/LXA4R) and to be capable of activating this receptor [107]. HEK293 cells transfected with the FPRL1/LXA4R bound $suPAR(II–III)_{88–274}$ with an apparent K_d of 83 nM. Furthermore, an antiserum against FPRL1/LXA4R specifically blocked the chemotactic effect of $suPAR(II–III)_{88–274}$ on THP-1 cells and peripheral blood monocytes. The ligand-binding site of $suPAR(II–III)_{88–274}$ is likely to reside in the seven-transmembrane domain of FPRL1/LXA4R, as it competes with other ligands binding to this area [107].

3. Monoclonal Antibodies to uPAR

mAbs are important tools for functional, structural, and localization studies as well as for quantification of the antigen in question. mAbs were initially raised against uPAR(I–III) purified from U937 cell lysates by DFP-uPA affinity chromatography [1]. One of these mAbs, R3 was used to show that uPA binding to uPAR is required for cell-induced potentiation of

plasminogen activation [1]. These mAbs react in Western blotting with uPAR under nonreducing conditions, but not after chemical reduction, indicating that the mAbs recognize conformational but not linear epitopes [16]. The domain reactivity of these mAbs was initially determined using immunoprecipitation of I^{125}-labeled individual domains of uPAR(I–III) [1]. Among the mAbs obtained from this fusion, 3 are specific for domain I whereas the 14 others bind mainly to domain III. For two of the domain I-specific mAbs, R3 and R5, the functional epitopes have been determined using alanine-scanning mutagenesis and analysis of the biomolecular interaction by surface plasmon resonance [8, 65]. The functional epitope of mAb R5 comprises the uPAR residues R^2, E^{16}, A^{18}, L^{19}, and G^{20} and R5 functions as a noncompetitive inhibitor of uPA binding, displacing uPA from uPAR(I–III). The mAb R3 is a competitive inhibitor of uPA binding to uPAR(I–III) with the functional epitope localized to uPAR residues E^{33}, L^{61}, and K^{62} [8, 26]. Neither R3 nor R5 have epitopes overlapping with the uPA-binding site consisting of amino acids R^{53}, L^{55}, Y^{57}, and L^{66}, even though the amino acids important for R3 binding are in proximity to the uPA-binding site [65]. Another inhibitory mAb, R23, requiring both domains II and III for binding, was obtained using recombinant $suPAR_{1–277}$ as the antigen [108]. It has not been clarified whether the epitope recognized by R23 is located in the linker region between domains II and III or if the epitope is composed of amino acids in both domains II and III. It will be of great interest to map the R23 epitope and locate it on the three-dimensional structure in order to elucidate the inhibitory mechanism of this mAb.

mAbs reacting with uPAR have also been obtained by immunizing mice with human peripheral blood mononuclear cells. These mAbs were directed against differentiation markers called Mo antigens, for which the expression was induced during the myeloid-monocyte-macrophage sequence of differentiation [109–111]. Comparing the cDNAs for Mo3 and uPAR(I–III) revealed the identity of these two proteins, and two of the Mo3-specific mAbs were subsequently found to prevent uPA binding to uPAR(I–III) [112]. mAbs were also raised against cells of the human monocytic cell line THP-1 [113, 114]. One of these mAbs, VIM-5 binds to domain I and prevents uPA binding to uPAR(I–III) [115].

For studies on uPAR cleavage and glycosylation, it has been important to visualize the reduced and alkylated forms of uPAR. mAbs recognizing these forms of the receptor were generated by immunization of mice with reduced and alkylated $suPAR_{1–277}$ [17]. These mAbs do not react with native uPAR forms but have been useful for detection of the enzymatically deglycosylated forms of uPAR. Another collection of 12 mAbs specific for the reduced forms of uPAR was obtained by immunizing with recombinant, nonglycosylated uPAR(I–III) consisting of amino acids 1–284 and produced

in *Escherichia coli* [28]. All of these mAbs reacted with uPAR under reducing conditions, whereas seven also reacted with uPAR under nonreducing conditions. Three of the mAbs were directed against domain I, and their epitope locations were mapped using overlapping peptides representing linear sequences in uPAR. mAb IIIF10 is a competitive inhibitor of uPA binding and the epitope was localized to amino acids 52–60 of uPAR(I–III). This sequence includes three of the four amino acids present in the functional epitope for uPA as determined by alanine scanning. Although no domain II-specific but several domain III-specific mAbs were obtained from the fusion, where uPAR(I–III) from U937 cells was used as immunogen [1, 116], four mAbs reacting with amino acids 125–132 of domain II were generated when the immunogen was recombinant uPAR, expressed by *E. coli* [28]. The mAbs described above are those that have been most frequently used in uPAR studies, but the list is not exhaustive.

4. Immunoassays for the Quantification of uPAR

The cell surface plasminogen activation system has an active role in cancer invasion. Thus, both uPA and PAI-1 have been shown to be prognostic markers mainly in breast cancer [117–119]. Therefore, it was early proposed that measurement of uPAR might also be of value in identifying patients with aggressive tumors and hence poor prognosis. This provided impetus for the establishment of immunoassays to be used for sensitive and specific measurements of uPAR in tumor tissue lysates and blood from cancer patients once anti-uPAR antibodies were available. In the different enzyme-linked immunosorbent assays (ELISAs) described in this chapter, uPAR(I–III) is used as the standard but in many cases the biological samples contain a mixture of uPAR forms. Therefore, the concentrations measured should be expressed as molar concentrations, not as ng/ml or ng/mg. In order to compare the amounts of uPAR measured in donor plasma samples by the different immunoassays, the amounts are expressed in moles. For simplicity, the ELISA formats that have been used in the prognostic studies described in Section 4 will be referred to as E1, E2, and so on. The immunoassays, the antibodies employed, and the uPAR forms they measure are summarized in Table 1.

The first ELISA designed for uPAR measurements with an analytical sensitivity of 19.5 pmol uPAR/liter was developed using two mAbs, R8 for catching and biotinylated R2 for detection [116]. These mAbs recognize nonoverlapping epitopes on domain III of uPAR and therefore, this ELISA measured all known forms of uPAR except uPAR(I). A 20% decrease in the recovery was reported when uPA/uPAR complexes were measured. This assay was never used on patient material because of the

TABLE 1
uPAR Forms Measured by Different Immunoassays

Immunoassay	uPAR variants measured	References
E1 ELISA [a]C-Ab: anti-rsuPAR pAb [b]D-Ab: R3, R5; uPAR(I) R2; uPAR(III)	uPAR(I–III), uPAR(II–III), uPAR(I) uPAR(I–III)/uPA complex	[87]
E2 ELISA C-Ab: anti-rsuPAR pAb D-Ab: R3, R5; uPAR(I) R2; uPAR(III)	uPAR(I–III), uPAR(II–III), uPAR(I) uPAR(I–III)/uPA complex	[120]
E3 ELISA C-Ab: anti-suPAR pAb D-Ab: anti-suPAR pAb	uPAR(I–III), uPAR(II–III), uPAR(I) uPAR(I–III)/uPA complex	[97]
E4 ELISA C-Ab: R2; uPAR(III) D-Ab: anti-rsuPAR pAb	uPAR(I–III), uPAR(II–III), uPAR(I–III)/uPA complex	[41]
E5 ELISA C-Ab: anti-rsuPAR pAb D-Ab: R2; uPAR(III)	uPAR(I–III), uPAR(II–III), uPAR(I–III)/uPA complex	[122]
E6 ELISA C-Ab: anti-rsuPAR pAb D-Ab:IIIF10; uPAR(I)	uPAR(I–III), uPAR(I)	[38]
E7 ELISA C-Ab: anti-rsuPAR pAb D-Ab: HD13.1	Not known	[38]
TR-FIA 1 C-Ab: R2; uPAR(III) D-Ab: R3; uPAR(I)	uPAR(I–III)	[108]
TR-FIA 2 C-Ab: R2; uPAR(III) D-Ab: R23; uPAR(II–III)	uPAR(I–III), uPAR(II–III)	[108]
TR-FIA 3 C-Ab: R5; uPAR(I) D-Ab: R3; uPAR(I)	uPAR(I)	[108]

[a] C-Ab = coating antibody.
[b] D-Ab = detecting antibody.

low sensitivity [116]. In order to increase the sensitivity and be able to measure all uPAR forms collectively, we designed an ELISA using a rabbit anti-suPAR pAb for catching and a combination of three biotinylated mAbs for detection. The antigen used for raising the pAb was recombinant $suPAR_{1–277}$. The mAbs used for detection were R2 and the two domain I-specific antibodies, R3 and R5. This assay thus detects all forms of uPAR, including uPAR(I), and is

referred to as E1 herein. The analytical sensitivity was increased more than 10 times to 1.6 pmol uPAR/liter [87]. E1 was first validated for measuring uPAR in breast cancer tissue extracts and lysates and later for use with blood plasma samples [98]. The median level of suPAR in a plasma pool from 39 healthy individuals was 28 pmol/liter as measured with E1.

The E1 ELISA was further optimized for use with plasma samples by replacing the biotinylated mAbs and end-point peroxidase measurements with nonbiotinylated mAbs and a rabbit anti-mouse pAb coupled to alkaline phosphatase. This kinetic ELISA enabled accurate calculation of the uPAR concentration from multiple time-point measurements of the linear color development obtained with a phosphatase substrate system [120]. The use of nonbiotinylated mAbs was prompted by the finding that biotinylation of mAbs could change not only their affinity but also their specificity. These problems were found to be most pronounced when the ratio of biotin to mAb was high and plasma samples were analyzed [121]. When this kinetic ELISA, called E2, was used for the measurement of uPAR in plasma samples from 93 healthy individuals, the median concentration was 39 pmol suPAR/liter [120]. Mizukami *et al.* developed another ELISA, E3, employing an anti-suPAR pAb raised against Pi-PLC-released uPAR from U937 cells. Using this pAb for catching and detection, the E3 ELISA determined a median concentration of suPAR of 65 pmol/liter in a plasma pool from 20 healthy volunteers [97]. The E3 ELISA should hypothetically have measured all forms of uPAR but it used biotinylated antibodies for detection without reporting the biotin/antibody ratio. It is therefore unknown how much influence the biotinylation had on antibody affinity and especially specificity.

The assay E2 was later inverted, using the high-affinity mAb R2 as catching antibody while the anti-suPAR pAb was employed for detection. This was also a kinetic ELISA. This ELISA, E4, quantifies uPAR(I–III), uPAR (II–III), and uPA/uPAR complexes and gives a lower background signal in EDTA plasma samples compared to the E2 assay [41]. However, serum collections are more commonly used in clinical studies than plasma, and when serum samples were analyzed with E4, rather high-non-specific signals were occasionally observed. This problem was solved by adding heparin to the assay buffer, which prevented nonspecific signals. The exact mechanism is unknown but the highly charged heparin polyanion probably binds to the solid phase not allowing the molecules responsible for the unspecific signal to bind [122]. This kinetic assay, E5, uses the anti-suPAR pAb for catching and the mAb R2 for detection [40, 122] and like the E4 assay, it detects all uPAR forms except uPAR(I). The analytical sensitivity of E5 is 0.13 pmol/liter and the median suPAR concentrations in citrate plasma and serum pools from healthy individuals were 75 and 85 pmol/liter, respectively [122].

Kotzsch *et al.* have developed two uPAR ELISAs measuring different forms of uPAR both using a pAb, raised in chicken against $suPAR_{1–277}$

as the catching antibody. One of the ELISA formats, E6, uses the domain I-specific mAb, IIIF10, as the detecting mAb. E6 measures nonoccupied uPAR(I–III) and liberated domain I. The other ELISA, called E7, employs a mAb HD13.1 raised against $suPAR_{1–277}$ for detection. Since the domain specificity of this mAb is not identified, it is unknown which uPAR variants the assay measures [38].

Two GPI-anchored and three soluble forms of uPAR have been identified in human tissue and body fluids (Fig. 4). The amounts of these in tissues and circulation will reflect not only the level of cellular expression but also the activity of the cleavage enzymes, including those of the plasminogen activation system. Thus, the levels of the cleaved uPAR forms may have a stronger prognostic significance than merely the total uPAR content. To enable studies of the prognostic potential of the individual uPAR forms, we designed specific time-resolved fluorescence immunoassays (TR-FIAs) using different combinations of mAbs. The detection system using time-resolved fluorescence was crucial, since biotinylation of detecting mAbs would cause problems with both sensitivity and specificity, when measuring plasma and possibly also serum samples [121]. TR-FIA 1 quantifies only nonoccupied uPAR(I–III), while TR-FIA 2 measures nonoccupied uPAR(I–III) and uPAR(II–III). The molar concentration of uPAR(II–III) can thus be calculated by subtracting the molar quantities measured by TR-FIA 1 from those measured by TR-FIA 2. TR-FIA 3 determines the amount of uPAR(I) in a sample. The specific detection of liberated uPAR(I) in the assay was obtained by adding an inhibitor, AE120, that prevents mAb R3 from binding to uPAR(I–III), but allows R3 binding to the liberated domain I [108]. The detection limits for both TR-FIA 1 and 2 were 0.3 pmol/liter and for TR-FIA 3, it was 1.9 pmol/liter. In a citrate plasma pool from healthy volunteers, 42 pmol/liter of uPAR(I–III) and 26 pmol/liter of uPAR(I) were measured and the calculated uPAR(II–III) content was 39 pmol/liter. This corresponds to the ELISA-measured amount using E5, which determined the total amount of suPAR in citrate plasma from healthy individuals to 75 pmol/liter compared to 81 pmol/liter as measured with TR-FIA 2, since the E5 ELISA does not measure uPAR(I).

5. uPAR in Cancer

5.1. Breast Cancer

Thus far, no domain I-specific mAbs that detect uPAR in paraffin sections are available. The mAbs that have proven useful for immunohistochemistry have epitopes residing in domains II and III and none of these recognize uPAR(II–III) selectively [28, 33]. Therefore, the signals obtained after

immunohistochemical staining of a tissue sample will originate from both uPAR(I–III) and uPAR(II–III) and will be referred to as uPAR in the following. In breast cancer tissue, the localization of uPAR was studied by three independent research groups using different antibodies, though obtaining consistent results [28, 33, 123]. In the first study, mAb R2, reacting with an epitope in the C-terminal part of domain III, stained uPAR in sections of formalin-fixed paraffin-embedded tissue from diagnosed invasive ductal breast carcinomas obtained from 40 women [33]. We found uPAR immunoreactivity in 34 of the 40 cases. In 32 of the cases, uPAR was detected on macrophages immediately surrounding the malignant epithelium. In 5 of the 34 positive specimens, uPAR was found on cancer cells. There was a weak positive signal in most cases from tissue neutrophils, but only in 2 of the 34 positive cases a weak staining of endothelial cells was obtained. Normal appearing epithelium and normal female breast tissue were negative [33]. The localization of uPAR mainly to macrophages, some cancer cells, and a few endothelial cells was confirmed by the two other studies, in which paraffin-embedded tissue sections from invasive breast carcinomas (59 and 20 cases) were stained using an anti-uPAR pAb in one study and an mAb specific for domain II in the other [28, 123]. The immunohistochemical localization of uPAR in different cancer forms is summarized in Table 2.

In an attempt to optimize the conditions for measurements of uPAR in breast tumor tissue, the amounts extracted using three different buffers were compared [87]. A Triton X-114-containing buffer routinely used for extraction of GPI-linked uPAR from cells [53] solubilized the highest amounts of uPAR from the breast cancer tissue. The second most efficient was a Triton X-100-containing acetate buffer, pH 4.2, which was optimized for the extraction of uPA [124]. The least efficient buffer contained no detergent and is generally used for preparations of cytosols [125]. Whereas the first two buffers will extract both GPI-anchored and soluble forms of uPAR, the cytosol buffer is only capable of extracting soluble uPAR forms. Surprisingly, ELISA measurements, using E1, of cytosols and acidic Triton X-100 buffer extracts from 505 primary breast tumors revealed that uPAR levels above

TABLE 2
IMMUNOHISTOCHEMICAL LOCALIZATION OF uPAR IN CANCER

Cancer	uPAR positive cell types	References
Breast	Macrophages, cancer cells, endothelial cells	[28, 33, 123]
Colorectal	Macrophages, neutrophils, cancer cells	[34]
Liver	Macrophages, myofibroblasts, cytokeratin 7 positive hepatocytes	[128]
Prostate	Macrophages, neutrophils	[140]

the median in cytosolic extracts were significantly associated with a shorter overall survival. The amounts of uPAR extracted with the acidic Triton X-100 buffer had less prognostic impact. In a group of node positive postmenopausal women, cytosolic uPAR was found to be a very strong predictor of overall as well as relapse-free survival [37]. In a different study measuring cytosolic extracts from 878 primary breast tumors, the prognostic significance of these forms of uPAR was confirmed. The E1 ELISA was slightly modified in this study using nonbiotinylated mAbs and an HRP-conjugated goat anti-mouse pAb for detection [126]. Higher levels of uPAR were measured in steroid hormone receptor negative tumors in this study, but the uPAR levels were found to be unrelated to menopausal status or grade of differentiation. The performances of E6, E7, and a commercial uPAR ELISA have been compared in another study. Only the combined levels of uPAR(I–III) and uPAR(I) measured with E6 in tumor tissue extracts correlated with disease-free survival in the 199 patients tested [38]. It would be interesting to compare the amounts measured by E6 with those measured by TR-FIA 1 and 3 described above and to investigate if the prognostic impact of either uPAR(I–III) or uPAR(I) alone would be more significant.

Measuring prognostic parameters such as uPAR in tumor tissue extracts or lysates requires that fresh tumor tissue is available from surgery or a biopsy. The tissue has to be frozen without delay and the proteins extracted prior to analysis. This requires considerable cooperation between surgeons, pathologists, and the immunoassay laboratory. Since the pathologist must have priority in selection of material for diagnosis and also because a tumor is rather heterogeneous, the piece that is available for extraction and immunoassay might not be completely representative of the whole tumor. Peripheral blood on the contrary is considerably more homogeneous and easier to collect. In the studies mentioned above, the level of soluble uPAR forms extracted from breast cancer tissue with the cytosol buffer was shown to have prognostic significance [37]. It was therefore reasonable to propose that the soluble forms found in blood might also be prognostic markers. A collection of sera made from preoperatively collected blood from 274 breast cancer patients without evident distant metastases was analyzed using E5, measuring all uPAR forms except domain I [40]. From 188 of these patients, cytosols were prepared from the primary tumor. Measurements of sera from 174 female blood donors revealed that there was an association between the age of the donor and the suPAR level with an increase of the suPAR level with age. The suPAR measurements of the patient sera were accordingly adjusted with respect to the age of the patient. Even though the mean suPAR level in sera from the breast cancer patients (124 pmol/liter) was not much higher than that in sera from the healthy volunteers (98 pmol/liter), the age-adjusted serum suPAR levels in the patients were significantly higher compared with

those of the donors. A significant correlation was found between high serum suPAR levels in breast cancer patients and poor outcome and this was independent of lymph node status, tumor size, and estrogen receptor status. There was no correlation between the cytosolic and serum suPAR levels in samples from 188 of the patients [40].

5.2. Colorectal Cancer

In colon cancer, uPAR is localized mainly to tumor-infiltrating macrophages and neutrophils at the invasive front of the primary tumor [34]. Of the 30 cases of colonic adenocarcinoma investigated, a strong signal of immunoreactivity was seen in all cases at the invasive foci. Some cancer cells were stained in 19 of the cases. uPAR was detected with mAb R2, employing mAb R4 as a control. The uPAR-expressing cell types were verified and the subcellular localization of uPAR was determined by an immunoelectron microscopical analysis of 12 cases of colon cancer [127]. In this study, mAbs R2 and R4 and an anti-uPAR pAb were used with identical results on frozen sections. The localization of uPAR to the plasma membranes of macrophages indicates that they were engaged in cell surface plasminogen activation. The uPAR positive cancer cells displayed uPAR on the plasma membrane and also in their rough endoplasmic reticulum, indicating intracellular synthesis. In contrast to the light microscopy study, some reactivity was detected on the plasma membrane of some endothelial cells. In several fibroblasts, immunoreactivity was found in the lumen of the endoplasmic reticulum with little expression along the plasma membrane, which is a typical staining pattern for cells that actively secrete protein [127].

In hepatocellular carcinoma, uPAR was also found on macrophages, on myofibroblasts, and in a few cases on cytokeratin 7 positive hepatocytes. Identical results were obtained whether the specimens were stained with mAb R2, R4, or the pAb against $suPAR_{1-277}$ [128]. Interestingly, uPAR and uPA were often colocalized.

In a study including tumor tissue resected from 161 colorectal cancer patients, proteins were extracted from the tissue using a Tween 80-containing buffer. The uPAR content was measured using an ELISA, where the domain specificity of the catching mAb was not revealed and thus it is unknown which uPAR forms were measured. The study, however, identified the uPAR concentration as an independent and significant prognostic factor for 5-year overall survival [129]. In a more recent study where tumor tissue extracts from 71 colorectal cancer patients were analyzed using another ELISA, high levels of uPAR (>91 fmol uPAR/ng protein) were significantly associated with shorter overall survival ($p = 0.0248$). The levels of uPAR were also found to correlate with the presence of liver metastases as patients

with tumors and liver metastases had higher levels of uPAR than those without [130].

suPAR was detected in plasma from Duke's stage D colorectal cancer patients and the suPAR levels were elevated as measured with the E2 ELISA [120]. This study was expanded and the prognostic significance explored by measuring preoperatively obtained EDTA plasma from 591 patients with colorectal cancer using the E4 ELISA. Overall survival was significantly related to plasma suPAR levels. An arbitrary cut point equal to the median of all patients (45 pmol uPAR/liter) divided the patients with Duke's stage B, C, and D tumors [131, 132] into statistically different survival groups. Since the survival of these patients was recorded as death of all causes, the survival of an age- and gender-matched population was used for comparison. Interestingly, for patients with Duke's stage B cancer and with suPAR levels below the cut point, the survival did not differ from the age- and gender-matched normal population [41]. Thus, in the most clinically difficult patient group for decision making, measurements of plasma suPAR were able to identify patients with the highest risk. In a multivariate analysis that included stage and other known prognostic indicators of colon cancer survival, an elevated preoperative suPAR level was an independent prognostic indicator for shorter overall survival [41].

High expression of uPAR has been reported in gastric cancer [133]. Heiss *et al*. demonstrated a prognostic value of uPAR in bone marrow aspiration biopsies from gastric cancer patients with cytokeratin positive tumor cells in the bone marrow at the time of primary surgery. Staining with a uPAR mAb showed that the presence of uPAR positive tumor cells in the bone marrow predicted early relapse and was a strong independent prognostic marker [134, 135].

5.3. Lung Cancer

The prognostic significance of uPAR has been studied in tumor tissue extracts from patients with non-small cell lung cancer (NSCLC). When the 228 patients were subgrouped into squamous cell carcinomas ($n = 84$), adenocarcinomas ($n = 106$), and large cell carcinomas ($n = 38$), the median level of uPAR was 83.3 fmol/mg protein in the adenocarcinomas and 45.3 and 42.0 fmol/mg protein in the squamous and large cell carcinomas, respectively [136]. In this study, tumor extracts were made using the acidic Triton X-100-containing buffer [124] and uPAR measured with the E1 ELISA. For uPAR measured in extracts from the patients with squamous cell carcinoma, there was a significant correlation between high uPAR level and short overall survival ($p = 0.038$) [39]. The highest uPAR levels were measured in extracts of the 106 adenocarcinomas. These levels did not correlate with survival

[136]. This was also the case for uPAR in tumor extracts from large cell carcinomas, possibly due to the low number of patients [39]. When 63 of 77 extracts from the squamous cell carcinoma patients were reanalyzed using TR-FIA 3, the measured amounts of domain I were found to be associated with survival. The prognostic impact of uPAR(I) was stronger compared to that of total uPAR measured previously [35]. Intact uPAR was not measured in this study since the recovery was very poor when the assay was validated in the tumor tissue extracts. The reason for this could be the presence of uPA in these extracts, which will form complexes with uPAR(I–III) and therefore cannot be measured by TR-FIA 1 [35, 108]. The prognostic significance of the combined amounts of domain I and uPAR(I–III) in tumor extracts from NSCLC patients was demonstrated by Werle *et al.* using the E6 ELISA [137].

The median suPAR level, as measured with the E1 ELISA in citrate plasma from 17 patients with NSCLC, was significantly increased compared to that in citrate plasma from 30 healthy donors, while there was no increase in the uPAR level in citrate plasma from 14 patients with small cell lung cancer (SCLC) [138]. In another study using a different ELISA, elevated levels of circulating suPAR were found both in serum samples from patients with NSCLC and SCLC [139].

5.4. Prostate Cancer

In prostate cancer, uPAR is located on neutrophils and macrophages as demonstrated in tissue from 16 patients with histologically confirmed prostate adenocarcinoma [140]. uPAR protein was detected in specimens from all patients. The antibody used for immunohistochemical staining was an anti-$suPAR_{1–277}$ pAb [87] and the staining pattern of the control mAb R2 was identical. In 8 of the 16 cases of prostate cancer, uPAR was identified in a subpopulation of macrophages mainly found in the interstitial tissue. In all 16 cases, the anti-uPAR pAb stained intravascular neutrophils, while none of the cases demonstrated staining in cancer cells. When benign prostate hyperplasia (BPH) from nine patients were analyzed, eight patient samples had uPAR positive macrophages, which were located in the lumen of glands, intraluminal, and in one case in the interstitial tissue. In seven cases, intravascular neutrophils stained positive for uPAR [140].

A possible correlation between serum suPAR levels and prostate cancer disease progression was investigated in serum from 72 treatment naive patients with confirmed prostate cancer [141]. suPAR was elevated in serum from prostate cancer patients compared to that from healthy individuals, and the mean concentrations were 127 ± 39 pmol/liter in healthy controls ($n = 70$) and 179 ± 91 pmol/liter in the patient sera ($n = 72$). No age dependence of the suPAR level in sera from male donors was found.

When patients were divided according to their serum suPAR content, the overall survival of patients with elevated suPAR levels was significantly shorter than that of patients with serum suPAR levels indistinguishable from those of healthy controls [141].

The presence of the different suPAR variants in EDTA plasma from a prostate cancer patient was analyzed by size exclusion chromatography and measurement of the fractions obtained with the three different TR-FIAs. This EDTA plasma contained detectable amounts of uPAR(I) in contrast to an EDTA plasma pool from healthy donors. The levels of suPAR measured with TR-FIA 2 were considerably higher and eluted at a later position in the patient sample than in the donor plasma, thus indicating the presence of suPAR(II–III) in the patient sample [108]. In a recent study, the concentrations of uPAR(I) as well as the calculated suPAR(II–III) were found to be significantly elevated in serum samples from patients with prostate cancer compared to the concentrations in serum from men with benign prostatic conditions. Specific measurements of uPAR(I) were found to improve specificity of prostate cancer detection [142].

5.5. Gynecologic Cancer

High uPAR levels in breast, colorectal, and lung cancers predict short overall survival. However, the opposite was found for uPAR in tumor tissue from ovarian cancer patients [143]. Proteins in this study were extracted from the tumor tissue using the Triton X-100-containing acidic buffer [124]. The levels of uPAR, measured with the E4 ELISA, were lower in benign as compared to invasive or borderline tumors. However, among the malignant tumors, the more advanced and poorly differentiated tumors contained lower levels of uPAR than the well-differentiated, less advanced tumors.

The uPAR forms present in blood, ascites, and cystic fluids from ovarian cancer patients have been extensively characterized and their levels measured [21, 82, 99, 144]. Using the E2 ELISA, preoperatively sera taken from 87 patients with ovarian cancer were measured and compared to the serum levels in 40 age-matched healthy women. The levels of suPAR were increased in sera from the patients [144]. However, when the patients were ranked according to their FIGO classification [131], the highest levels were found in stage 2 patients (111 ± 20 pmol/liter) and then the levels decreased with increasing FIGO stage (98 ± 10 pmol/liter in stage 3 and 72 ± 7 pmol/liter in stage 4). Nevertheless, high levels of suPAR in preoperatively collected sera were found to correlate with poor survival. This is in contrast to the results obtained using tumor tissue extracts from ovarian cancer patients discussed above [143]. The amount measured in sera from healthy controls ($n = 40$)

was 49 ± 3 pmol/liter and the suPAR levels were independent of the age of the donor [144]. Similar results were obtained in another study where citrate plasma samples from 53 ovarian cancer patients were analyzed [145]. However, no relation to prognosis was observed when EDTA plasma from 47 women with FIGO stage 3 ovarian cancer was analyzed using the E5 ELISA, possibly due to the smaller number of samples studied [146].

Ovarian cancer patients with progressed disease often present with ascites/peritoneal fluid. In some women, ovarian cysts are detected containing cystic fluid. The concentrations of suPAR in these body fluids were compared with those in serum made from peripheral blood and blood aspirated from the surface veins on the tumor in 77 patients admitted for surgery of ovarian tumors [21]. In this study, elevated levels of suPAR were measured in serum from peripheral blood and tumor blood in the patients with more advanced disease. However, the concentrations of suPAR in the body fluids were quite different, in serum the measured concentrations were between 46 and 98 pmol/liter, in ascites/peritoneal fluid concentrations were between 293 and 586 pmol/liter, and in cystic fluids the concentrations were even higher, that is 651–8468 pmol/liter. The concentrations of suPAR in cystic fluids clearly separated benign and malignant cysts with predictive values above 90%. The levels of suPAR in cystic fluids could therefore be used in the early diagnosis of ovarian cancer patients. The suPAR in the cystic fluids was present both in intact and cleaved forms and at least some of the suPAR(I–III) was not occupied by uPA [21]. In another study, tumor tissue, serum, ascites, and urine from ovarian cancer patients were analyzed for their content of the different uPAR forms. Whereas all of tumor lysates, ascites, and urine contained uPAR(I–III) and uPAR(II–III), domain I was only present in urine samples. In serum, only intact suPAR was detected [82]. The antibodies used for identification were mAb R3 (domain I) and mAb R2 (domain III).

5.6. Leukemia

The uPAR expression on leukemic cells was compared to that on peripheral blood and bone marrow cells from healthy individuals in two studies using flow cytometric analysis. Both studies used the domain I-specific anti-Mo3f (also termed 3B10) and VIM-5 mAbs [147, 148]. The uPAR expression on neutrophils and monocytes in peripheral blood from healthy donors was verified in both studies and found uPAR expressed neither on resting B nor on resting T lymphocytes. uPAR expressed on monocytes and neutrophils could bind the added ATF of uPA [147], showing that at least some of the uPAR molecules present on these cells were unoccupied. When blasts from AML patients were analyzed, they were found to express uPAR and the

amount of uPAR correlated with the French-American-British (FAB) myeloid leukemia classification [149]. Most uPAR was found on cells of the M5 subgroup. Interestingly, intracellular pools of uPAR were detected in many blast cells of lower FAB classification, even though no uPAR was detected on the surface of these cells. Only 12% of AML patients had uPAR positive blast cells, suggesting that uPAR could be used for leukemia typing. In bone marrow, CD34 negative myelomonocytic precursor cells expressed uPAR, whereas all CD34+ cells from bone marrow and mobilized peripheral blood CD34+ cells were uPAR negative [148].

In addition to the cellular expression on malignant blast cells in AML, elevated levels of suPAR were found in plasma from leukemia patients [18]. In a longitudinal study, in which patients receiving chemotherapy were monitored, it was demonstrated that the suPAR level in plasma from patients with AML correlated with the number of circulating tumor cells and that these were reduced after chemotherapy. In plasma from AML patients, suPAR(II–III) was detected in addition to intact suPAR. This is in contrast to findings in plasma from healthy individuals and from the ovarian cancer patients described above [144]. suPAR(II–III) was also present in plasma made from bone marrow aspirates. The other cleaved form, uPAR(I), was only identified in urine. Lysates of the leukemic cells contained both intact uPAR and uPAR(II–III). The amounts of suPAR(II–III) in plasma and uPAR(I) in urine were decreased following chemotherapy. In healthy controls, intact uPAR was detected in lysates from mononuclear cells in blood and suPAR(I–III) in plasma and bone marrow aspirates, while suPAR(II–III) was detected in urine [18].

The granulocyte colony-stimulating factor (G-CSF) induces hematopoietic stem cell (HSC) mobilization into peripheral blood and this stimulation also induces uPAR expression on some cell types but not on others [19]. It was confirmed, using mAbs R2 and R4, that CD34+ cells with or without G-CSF stimulation and B and T lymphocytes do not express uPAR [147, 148]. However, uPAR expression was increased on myeloid precursors and CD14+ monocytes in donors after G-CSF administration. G-CSF induced an increase in the amount of uPAR(I–III) but not of uPAR(II–III) on the surface of peripheral blood mononuclear cells. The content of suPAR in serum collected from the donors was also increased after G-CSF stimulation and both intact and cleaved forms of suPAR were present. suPAR(II–III) containing amino acids 88–92 is able to induce monocyte chemotaxis by activating the low-affinity receptor for fMLP, as discussed in Section 2.3 [107]. This implies that the upregulation of uPAR by G-CSF results in production of the chemokine suPAR(II–III), resulting in increased mobilization of the CD34+ HSCs into the peripheral blood [19].

6. Other Diseases

The level of uPAR is elevated in tumor tissue and blood from cancer patients and uPAR is a prognostic marker when measured both in tumor tissue and in blood, as described in Section 5. When monocytes and T cells were infected with HIV-1, uPAR was found to be upregulated [150]. The levels of suPAR in serum from 314 HIV-1-infected patients were therefore measured using a slightly modified E4 ELISA [44]. All patient sera contained measurable amounts of suPAR and the median value was 120 pmol/liter. If the patients were divided in two groups according to the median, the patients with the higher suPAR levels had shorter follow-up time because of acquired immunodeficiency syndrome-related deaths. When the patients were grouped according to their clinical stage, the suPAR levels were found to be higher in patients with more advanced HIV infection. The results from this study were confirmed and extended in a study where heparin plasma samples from 99 HIV-infected patients and 59 control subjects were analyzed both with the E5 ELISA and TR-FIA 1–3. The HIV-infected patients had higher levels of both the ELISA-measured suPAR as well as all the measured individual forms of suPAR. Additionally, all levels were higher in patients with advanced disease in accordance with the first study. Furthermore, the prognostic value of the ELISA-measured suPAR was confirmed and the levels of both suPAR(I–III) and suPAR(II–III) but not uPAR(I) were, in addition, found to be independent predictors of short survival in these patients [42]. Notably, the E5 ELISA does not measure uPAR(I). In a longitudinal study where 29 treatment naive HIV patients were followed during a 5-year treatment with highly active antiretroviral therapy, the levels of suPAR decreased on initiation of treatment. The decrease was most pronounced during the first year and in patients with high suPAR levels prior to therapy [151].

Elevated suPAR levels in blood from patients with other infectious diseases have been reported and their prognostic value assessed. Although interesting results have been obtained, more studies are needed to confirm these findings, but the present evidence suggests that the blood levels of suPAR could be a marker of severe inflammation and immune activation [43, 45, 97, 152–154].

7. Conclusions and Perspectives

uPAR is a multifunctional molecule, present in intact and cleaved forms and involved in extracellular proteolysis, cell adhesion, mobility, and cell signaling events [5–8, 155–157]. It is located mainly on stromal cells at the

invasive front in breast and colorectal cancer and present in elevated levels in tumor tissue and blood from cancer patients as well as patients with infectious diseases. High levels of uPAR correlate with short survival in breast, colorectal, and lung cancer as well as in patients with HIV. Cleavage of uPAR could be an indication of an active plasminogen activation system. With the design of immunoassays that can quantify the individual uPAR variants, domain I was found to be a stronger prognostic marker than the total uPAR amount in tumor extracts of NSCLC patients [35]. It is possible that domain I or one of the other uPAR forms would have a stronger prognostic potential than the combined uPAR forms both in tumor tissue and blood from cancer patients. The use of either the total amount or the individual forms of uPAR for monitoring therapy response should be investigated. In AML, it has already been shown that the levels of suPAR(II–III) in plasma decrease following chemotherapy and this correlates with a decrease in the number of circulating tumor cells [18].

In a mouse cancer model for breast cancer, the volume of the lung metastases was demonstrated to be significantly reduced in mice deficient in uPA [158]. The therapeutic potential of blocking the interaction between uPAR (I–III) and uPA has been proven in several model systems and reviewed [157]. *In vivo* studies in mice using xenotransplanted human tumors and syngeneic tumors have demonstrated reduction of primary tumor growth, metastasis, and angiogenesis, by blockage of cellular binding of uPA by various antagonists including pAbs [25, 29, 159, 160]. mAbs raised against uPAR, blocking uPA binding, might thus be potential therapeutic agents [1, 26]. The presently available mAbs against uPAR, which prevent uPA binding, are directed against the human form of uPAR (Section 4). Due to species specificity, these will have no effect in mouse on the muPA–muPAR interaction. For therapy experiments in genetically induced mouse cancer models, murine mAbs specific for mouse uPAR are required. Mice deficient in uPAR have no defects in their humoral immune response [161]. Anti-muPAR mouse mAbs that inhibit the muPA–muPAR interaction have recently been obtained by immunizing $uPAR^{-/-}$ mice [162]. These mAbs have *in vivo* efficacy and will be important tools in therapeutic studies in murine cancer models. The results of such experiments could serve as proof of principle for further development of anti-uPAR mAbs for clinical use.

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PROTEOMICS IN CANCER

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1. Abstract

Proteomic studies have generated numerous datasets of potential diagnostic, prognostic, and therapeutic significance in human cancer. Two key technologies underpinning these studies in cancer tissue are two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS). Although surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF)-MS is the mainstay for serum or plasma analysis, other methods including isotope-coded affinity tag technology, reverse-phase protein arrays, and antibody microarrays are emerging as alternative proteomic technologies. Because there is little overlap between studies conducted with these approaches, confirmation of these advanced technologies remains an elusive goal. This problem is further exacerbated by lack of uniform patient inclusion and exclusion criteria, low patient numbers, poor supporting clinical data, absence of standardized sample preparation, and limited analytical reproducibility (in particular of 2D-PAGE). Despite these problems, there is little doubt that the proteomic approach has the potential to identify novel diagnostic biomarkers in cancer. In therapeutic proteomics, the challenge is significant due to the complexity systems under investigation (i.e., cells generate over 10^5 different polypeptides). However, the most significant contribution of therapeutic proteomics research is expected to derive not from single experiments, but from the synthesis and comparison of large datasets obtained under different conditions (e.g., normal, inflammation, cancer) and in different tissues and organs. Thus, standardized processes for storing and retrieving data obtained with different technologies by different research groups will have to be developed. Shifting the emphasis of cancer proteomics from technology development and data generation to careful study design, data organization, formatting, and mining is crucial to answer clinical questions in cancer research.

2. Introduction

In Europe, with an estimated 2.9 million new cases and 1.7 million deaths in 2004 only [1], cancer remains a major public health problem. It is expected that the aging population in industrialized countries will cause these numbers to increase even further. Because advances in traditional therapies such as surgery and radiotherapy have been widely exhausted, progress in cancer treatment is now expected from early diagnosis through screening programs and from tumor-specific drugs. In recent years, novel drugs like trastuzumab in breast cancer (reviewed in [2]), avastin or erbitux in colon cancer (reviewed in [3]), and imatinib in gastrointestinal stromal tumors (reviewed in [4]) have

delivered proof of principle in the efficacy of targeted cancer therapy. As such, there is general optimism that continued research in molecular biology, cellular biology, and biochemistry combined with advanced validation tools will provide drug developers with novel therapeutic alternatives.

Over the last 15 years, powerful high-throughput technologies, such as DNA microarrays, cDNA subtraction, and serial analysis of gene expression (SAGE), have been widely applied for identifying novel cancer-related genes and for classifying cancers at the molecular level [5]. Modern proteomic tools have also generated large datasets of potential diagnostic, prognostic, and therapeutic targets in a wide range of human cancers. Some of these are detailed below. Input from proteomics is important because significant differences in the abundance ratio of the mRNA transcript and the corresponding protein product have been observed for many genes and particularly for nonsecretory proteins in epithelial tissue [6].

2.1. Clinical Background for Proteomic Studies in Cancer

Most current prognostic and follow-up tests in clinical oncology are based on the detection and quantification of proteins in serum. For example, serum carcinoembryonic antigen (CEA) is an established prognostic biomarker routinely monitored during follow-up in colorectal cancer (CRC). This finding meets theoretical expectations for diagnostic tools because proteins are functional molecules that can be targeted to a specific cell type, that is they characterize tumor behavior and thereby the clinical phenotype. As such, it is the proteome not necessarily the genome that determines prognosis.

Many human diseases are diagnosed by protein-based testing in serum, for example troponin (myocardial infarction), SGOT/SGPT (liver cytolysis), and C-reactive protein (CRP) (inflammation). Protein tests are not, however, widely accepted for diagnostic purposes in cancer due to poor specificity, for example CEA in CRC, prostate-specific antigen (PSA) in prostate cancer, and α-fetoprotein (AFP) in liver cancer. In fact, serum protein levels appear affected by many physiological and biochemical conditions, not only by mutations in the genome or by cellular mRNA level.

These limitations have underscored the importance of developing novel proteomic technologies to improve the potential of classical cancer tests for use as a screening tool in the general population. For example, patterns of protein expression have been shown to yield more biologically relevant and clinically useful information than assays of single proteins. Thus, global discovery systems that effectively integrate large and sometimes disparate data [7] have gained much interest in cancer research. Because of the strength of this tactic, diagnostic companies are now integrating limited panels of additional cancer biomarkers into their established technology platforms.

3. Sampling for Proteomic Analysis

Many factors play a contributory role in the generation of reliable and accurate results in proteomic research. These include appropriate patient selection, the availability of adequate clinical data, precise disease definition according to worldwide pathology standards, and sample preparation. Even the best technology platform will deliver poor results if improperly collected samples are subjected to analysis. Unfortunately, the problem of adequate sampling appears to have been largely neglected in translational research in general and in proteomics specifically. These limitations make comparison of research results from different groups difficult, if not impossible.

In contrast to nucleic acids where polymerase chain reaction (PCR) allows the investigation of single cells, no amplification technology is available at the protein level. As such, proteomic studies require relatively large sample volume. Because of this limitation, it is not a surprising that most proteomic studies have been performed on whole tissue samples. This approach in cancer research is, however, unfortunate because cellular heterogeneity within a biopsy or tissue sample will likely impair the quality and reproducibility of information generated.

3.1. Cell Lines

Limited availability of human samples has prompted the use of cell lines in proteomic cancer research. In contrast to tissue samples, cell lines are well-defined and standardized biological systems particularly suited for methodological assays, that is, for validating novel proteomic research technology. Moreover, cell lines appear to be optimal systems for functional proteomics because interventional studies can be conveniently performed—an approach not possible in human biopsy specimens. Despite their apparent benefit, cell lines also have several limitations. Results obtained in expression proteomic studies in cell lines do not accurately reflect natural tissue physiology that contains a more heterogeneous cellular composition. Furthermore, cell lines are likely to have undergone selective pressures that result from their *in vitro* maintenance in culture systems. For example, in glioblastoma multiforme, a highly malignant brain tumor, numerous differences were identified in protein expression patterns between glioblastoma tumor biopsy samples and glioblastoma cell lines [8]. Finally, no normal cell lines are available for comparison purposes. Thus, cell lines appear valuable for functional proteomic studies and to a lesser degree in expression studies. However, their use for diagnostic studies is questionable.

3.2. Whole Tissue Biopsies

Traditional endoscopic and surgical procedures provide whole tumor samples well suited for microscopic examination and analysis in the pathology laboratory. The use of whole tissue tumor biopsies for proteomic studies has, however, raised several important issues that have been well demonstrated in CRC [9]. These include cellular heterogeneity in the different bowel parietal layers (mucosa, submucosa, muscularis mucosa, serosa) that may or may not be infiltrated, epithelial cell diversity in the mucosa itself, tissue infiltration by inflammatory cells such as lymphocytes, contamination with other body fluids, and protein degradation following tumor necrosis. In fact, epithelial cell content was found to vary between 9 and 67% in whole biopsies of normal mucosa and between 7 and 95% in tumor biopsies [10]. This study clearly demonstrates the likelihood of large cellular variation between tissue samples.

Tissue heterogeneity has also been demonstrated in prostate tissue. The prostate has three anatomical zones that include the peripheral and the transition zones (endodermal origin) and the central zone (mesodermal origin). When zonal proteomic patterns were characterized, 10 proteins with significant differential expression were identified thus suggesting functional differences [11]. As can be expected, tissue heterogeneity can unexpectedly increase result variability especially if this factor was not considered in the original study design. As such, proteomic differences may simply reflect sampling artifact or contamination rather than cancer-specific differences.

3.3. Purified Tumor Cells

The importance for proteomic studies of being performed on standardized samples cannot be emphasized enough. Several approaches have been developed to address the problem of tissue heterogeneity. Beads-based sample enrichment (BBSE) and laser capture microdissection (LCM) procedures are emerging as the methods of choice. The aim of these procedures is to eliminate, as much as possible, all confounding and contaminating factors in order to obtain a sample quality that can be compared with cell lines without the disadvantages of *in vitro* cultures.

3.3.1. *Laser Capture Microdissection (LCM)*

LCM permits the isolation of specific cell types for subsequent molecular analysis. This approach provides an opportunity for independent analysis of the tumor as well as its local microenvironment. The scope and limitations

of applications of LCM in cancer proteomics have been reviewed [12]. It is important to note that LCM can be applied in all cancers, whereas BBSE techniques have been developed only for epithelial cancers thus far. Despite its universal application, LCM remains a cumbersome and time-consuming technique. These drawbacks explain why LCM applications are still relatively rare in cancer research. However, LCM combined with two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been successfully employed using parallel analysis on selected breast tumors with or without overexpression of the HER-2 or neu oncogene [13]. Differentially expressed proteins included cytokeratin 19, an intermediate filament protein of the cytoskeleton. This finding suggested that cytokeratin 19 was involved in molecular events driving more aggressive tumor behavior. LCM was also applied for the isolation of specific cell types for subsequent proteomic analysis in CRC [14]. When LCM was used to enrich for both normal and malignant pancreatic ductal epithelial cells, the limited amounts of protein in the samples necessitated the detection of 2D-PAGE-resolved proteins by silver staining. However, loading equivalent amounts of protein for electrophoretic separation was found to be difficult because conventional means of measuring total protein in the samples were not sufficiently accurate [15].

3.3.2. *Beads-Based Sample Enrichment*

Ten years ago, our group developed an effective BBSE method for purification of large quantities (up to 10^7 cells) of epithelial cells from normal colonic mucosa, primary colorectal tumors, and liver metastases [16]. After preparation, samples contained more than 95% epithelial cells that was a decisive improvement when compared to whole tissue biopsy samples. Subsequently, we applied BBSE to more than 4000 patients suffering most epithelial cancers, including colonic, gastric [17], pancreatic [18], and other cancers. BBSE is demanding from the logistic point of view because it requires close collaboration between surgeons in the operating room, pathologists in the laboratory, and technicians performing the enrichment procedure. Despite this limitation, BBSE sample preparation time is faster than LCM and provides samples of superior cell amount and comparable quality. Furthermore, the quality of RNA and proteins can be assessed before expensive procedures are initiated and samples can be selected to meet industrial standards.

3.4. Body Fluids

Proteomic analysis of human body fluids is expected to provide screening tests, prognostic assays, and therapeutic monitoring tools. Easy clinical access to body fluids allows repeat analysis, an important feature in oncology,

where multiple measurements during follow-up are required to determine disease-free survival or tumor recurrence.

3.4.1. *Serum*

For many years, diagnosing and monitoring of many diseases has typically been conducted by use of enzyme-linked immunosorbent assays (ELISAs) that utilize a specific antibody to quantify a single protein of interest in a serum sample. Serum, the most abundant source of proteins in the human body, is also one of the easiest to collect. These properties have led to its broad use in diagnostic assessment for clinical chemistry laboratories worldwide. Thus, serum analysis combined with quantitative proteomic technology with pattern recognition algorithms appears to be a straightforward approach to identify new diagnostic cancer biomarkers. Despite its relative simplicity, this task is challenging. For example, the serum proteome is difficult to investigate because most proteins are obscured by the presence of ubiquitous proteins such as albumin. In fact, the 10 most abundant proteins in plasma account for about 90% of the total protein concentration in this compartment. Another difficulty relates to the very large dynamic concentration range of serum proteins (at least 9 orders of magnitude [19]). Finally, several preanalytic factors complicate serum analysis such as the time between sampling and analysis and differences related to sample type (plasma vs serum). The type of serum collection tube can also affect the proteomic profiles because of differences in clotting time. For example, *in vitro* manipulation of platelets has revealed that specific serum proteins were increased during the clotting process including some of those exhibiting time-dependent changes [20]. Because of these limitations, researchers have resorted to immunosubtraction techniques (combined with ion exchange and size exclusion chromatography) in order to detect serum polypeptides at levels below 10 ng/ml. This highly sensitive approach has clearly opened the door to discovery of specific, low-abundance cancer biomarkers [21].

3.4.2. *Plasma*

The Human Proteome Project (HUPO, www.hupo.org) initiated the Plasma Proteome Project (PPP) in 2002, and numerous laboratories have contributed to this ambitious project of deciphering all proteins contained in the human plasma. Plasma, the soluble component of the human blood, is believed to harbor thousands of distinct proteins that originate from a variety of cells and tissues through either active secretion or leakage from blood cells or tissues. For the reasons described above, HUPO recommends use of plasma instead of serum, with EDTA (or citrate) for anticoagulation and standardized sample preparation. HUPO proposes combinations of serum depletion, fractionation procedures, and MS/MS technologies, with explicit

criteria for evaluation of spectra, use of search algorithms, and integration of homologous protein matches. HUPO has created a publicly available knowledge base (www.ebi.ac.uk/pride or www.bioinformatics.med.umich.edu/hupo/ppp) that allows researchers to match results obtained in cancer tissue with 1274–9504 plasma proteins based on level of confidence.

3.4.3. *Urine*

Many components in urine are useful in clinical diagnosis and urinary proteins are known as important components to define many diseases such as proteinuria, kidney, bladder, and urinary tract diseases. Although sequential preparation of urinary proteins by gel filtration and ultrafiltration retains most urinary proteins, this approach results in low amounts of proteins for resolution by 2D-PAGE. High molecular weight proteins, however, can easily be detected and interestingly have been successfully used as urinary biomarkers related to lung cancer [22]. Thus, urine proteomic studies have a diagnostic potential not only for urinary tract cancers but also for other cancers. Moreover, it appears that the secretion by the kidney of hydrophilic proteins in urine and the smaller dynamic range of protein concentration, compared to serum, might be advantageous for proteomic studies.

3.4.4. *Bronchioalveloar Lavage Fluid*

Bronchoalveolar lavage fluid (BALF) is presently the most common way of sampling components of the epithelial lining fluid and provides the most accurate reflection of pulmonary airway protein composition. Proteomic studies of BALF have already been published and have contributed to a better knowledge of the lung structure at the molecular level and the study of lung disorders at the clinical level [23].

3.4.5. *Other Body Fluids*

Proteomic analysis of breast nipple aspirate fluid (NAF) [24], pancreatic juice [25], and bile [26] has also been performed and might be of relevance for specific cancer studies.

4. Proteomic Technologies Used in Cancer Proteomics

Proteomics is the study of the proteome—defined as the total set of proteins expressed in a given cell type at a given time [27]. Tremendous progress has been made in the past few years in generating large-scale cellular protein profiles, organelle composition, protein activity patterns, and datasets for protein–protein interactions.

4.1. 2D-PAGE

The key technologies underpinning cancer proteomic studies have been and remain 2D-PAGE for protein separation and mass spectrometry (MS) for protein identification. 2D-PAGE has been used for decades to separate complex mixtures of proteins, but recent technological improvements (in particular, immobilized pH-gradients and gels meeting industrial standards) have greatly enhanced resolution, sensitivity, and reproducibility of this separation method. 2D-PAGE is capable of displaying 10^3–10^4 polypeptide chains, characterized by a single isoelectric point (pI) and $M(r)$ value as coordinates. This technology provides separation of proteins according to their physicochemical characteristics independent of immunochemical methods typically used to separate proteins. Data output from 2D-PAGE is typically slow and analysis is limited to low-throughput means. These limitations prevent this technology from being used to rapidly screen large sample numbers, an important concept in biomarker discovery. Despite these considerations, 2D-PAGE has been effectively used as a discovery tool in numerous human cancers, both for expression and functional purposes.

4.2. Differential In-Gel Electrophoresis

Differential in-gel electrophoresis (DIGE) facilitates protein expression by labeling different populations of proteins with fluorescent dyes. Typically, paired samples from the normal and tumor region are labeled with Cy3 and Cy5. After analysis by differential analysis image software, protein spots that exhibit a significant difference in intensity are excised for in-gel tryptic digestion and MS analysis.

4.3. Isotope-Coded Affinity Tagging and Amino Acid-Coded Mass Tagging

The isotope-coded affinity tag (ICAT) technology enables the concurrent identification and comparative quantitative analysis of proteins present in biological fluids by coupling microcapillary chromatography with electrospray ionization tandem MS. A two-step approach was developed to identify proteins whose abundance changes between samples. In the first step, a software program for the automated quantification of ICAT reagent labeled peptides analyzed by microcapillary electrospray ionization TOF-MS determines those peptides that differ in abundance. In the second step, peptides are identified by tandem MS using an electrospray quadrupole TOF-MS and sequence database searching [28]. Amino acid-coded mass tagging (AACT)-assisted MS is an ICAT-derived method. This technology utilizes a number

of different heavy amino acids as internal markers that significantly increase the peptide sequence coverage for both quantitation and identification. Despite their promise, ICAT and AACT are still emerging technologies with limited application in cancer research.

4.4. Gel-Free Technologies

Over the recent years, proteomic technologies evolved rapidly and now offer new perspectives in discovery of new cancer biomarkers based on the detection of low molecular weight proteins or peptides by MS. Two main peptidomic approaches are currently under investigation. These include pattern recognition and single/oligo biomarker detection.

4.4.1. *Pattern Recognition*

Surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF)-MS provides a mean to rapidly assess complex protein mixtures in body fluids and, to a lesser degree, in tissues. Combined with pattern recognition algorithms, SELDI technology generates highly accurate diagnostic information. This system appeared to have potential in biomarker discovery and, to a lesser degree, as a clinical diagnostic assay platform [29], especially in human cancer. Despite its initial enthusiastic appeal, substantial skepticism developed for SELDI due to questionable reproducibility and lack of definitive protein characterization. In the meantime, it was possible to identify the peaks of interest by various techniques, that is by gel electrophoresis, in-gel trypsin digestion, and tandem MS. Identities of proteins could then be confirmed by conventional technologies like ELISA and immunodepletion assays.

4.4.2. *Single/Oligo Biomarker Detection*

The MALDI time-of-flight mass spectrometer (MALDI-TOF-MS) is a powerful analytical tool for comprehensive profiling of peptides. This sensitive instrument provides the ability to simultaneously detect minor peptide changes in complex biological mixtures. Usually, a few micrograms of sample are sufficient to create a detailed mass spectrum. The resolution and specificity of this analysis is further increased by coupling MS with liquid chromatography (LC) or other high-resolution technologies. For example, biological samples separated by means of high-performance LC (HPLC) are individually analyzed with MALDI-MS. The result of this peptide profiling process is the generation of a two-dimensional (2D) dataset with fraction number and mass-to-charge ratio (m/z) as coordinates. With proper methodological care, peptide abundance is represented by the mass spectra signal intensity. This can be visualized in a 2D intensity map, using a color intensity

code. Specialized software tools are often used to data mine via statistical analysis in order to obtain the most significant experimental changes [30].

4.5. Targeted Glycoproteomics

In human cancer, surface gylcoproteins have been shown to play a key role in immune response generation and metastasis (reviewed, for example, in [31]). Thus, there is an opportunity for direct targeting of glycoproteins in comparative proteomics analysis. This approach will provide analysis of oligosaccharides released from glycoproteins as well as the recovery and identification of proteins with aberrant glycosylation. Technological advances, in particular application of capillary electrophoresis in combination with MS and tandem MS, is expected to facilitate biomedical glycoscreening projects [32].

4.6. Reverse-Phase Protein Arrays-Based Studies

Reverse-phase protein arrays offer a robust new method of quantitatively assessing expression levels and the activation status of a panel of proteins. For this purpose, the lysate of protein(s) of interest is arrayed without selection via a capture molecule. This array can then be queried with an antibody or ligand probe, or an unknown biological component. Since an individual test sample is immobilized in each array spot, this array can be composed of a variety of different patient samples. Each array is incubated with one detection protein or antibody, and a single end point is measured across the arrayed cohort and can be directly compared across multiple samples. Replicates can be reproducibly printed at a given sitting, increasing quality control over a series of queried arrays (reviewed in [33]).

4.7. Antibody-Based Proteomic Studies

The significance of antibody microarrays in cancer research has been reviewed [34, 35]. Several applications of this technology have been reported, including protein analysis in serum, resected frozen tumors, cell lines, and on membranes of blood cells. Antibody microarray experimental formats can be categorized into two categories: direct labeling and dual antibody sandwich assays. Each format has its distinct advantages and disadvantages. In the direct labeling method, the covalent labeling of all proteins in a complex mixture provides a means for detecting bound proteins after incubation on an antibody microarray. If proteins are labeled with a tag, such as biotin, the signal from bound proteins can be amplified. In the sandwich assay, proteins captured on an antibody microarray are detected by a cocktail of specific detection antibodies.

4.8. Combinatorial Beads

Another technique that could facilitate biomarker discovery in the serum of cancer patients consists in the use of combinatorial beads to reduce the dynamic range of proteins. This technique consists in a library of combinatorial ligands coupled to small beads. This library comprises hexameric ligands composed of amino acids, resulting in millions different structures. When these beads are impregnated with complex proteomes (e.g., human body fluids) of widely differing protein compositions, they are able to significantly reduce the concentration differences and thus greatly enhance the possibility of identifying species of low abundance [36].

5. Proteomic Applications in Cancer

Over recent years, oncology has been a key focus of proteomic technologies as applied in both expression and functional studies. Expression proteomic studies are screening for differences in protein patterns between tumor and control tissues. Functional proteomic studies are defined as the combination of readouts with proteomics data from the same sample at different points of time (under different conditions), with the aim of detecting and prioritizing certain proteins or polypeptides of functional relevance.

5.1. Expression Proteomic Studies

As of today, expression proteomic studies are available for all common human cancers and some rare tumors.

5.1.1. *Lung Cancer*

Proteomic studies in lung cancer, the most common cancer worldwide, have been reviewed [37]. Initial studies on lung cancer proteomics were first published in the early 1990s. These early studies focused on the relationship between histopathological characteristics and 2D-PAGE reproducibility [38]. A few years later, the first differentially expressed proteins in lung cancer were identified in small cell lung cancer (SCLC), including β-tubulin, heat-shock proteins 73 and 90, lamin B, and proliferating cell nuclear antigen (PCNA). This report demonstrated for the first time that 2D-PAGE combined with protein identification was an effective approach to identify biomarkers in cancer [39]. Later on, with improvements in MS technology, it was possible to identify about 20 potential biomarkers in lung cancer tissue [40]. Recently, a SELDI study in early lung cancer stages and premalignant bronchial lesions analyzed LCM specimens of normal lung, atypical adenomatous hyperplasia,

and malignant tumors taken from patients participating in a screening program. Protein profiles were generated in each epithelial cell type and found to be highly reproducible in identifying populations at high risk for lung cancer [41].

When serum samples from lung cancer patients vs healthy controls were analyzed by SELDI technology, a decision classification tree yielded a diagnostic sensitivity and specificity of 93 and 97%, respectively [42]. Another study compared serum samples from lung cancer and healthy controls. Five protein peaks in a blinded test achieved a sensitivity of 87%, a specificity of 80%, and a positive predictive value of 92%. Sensitivity was even significantly better (91%) for detection of non-small cell lung cancers (NSCLC) [43]. Evaluation of circulating autoantibodies in lung cancer patients revealed antibodies against annexins I and II, recoverin, protein gene product 9.5, and α-enolase [44].

Lung cancers are traditionally classified into various subtypes on a histological basis. Squamous lung cancer, the most common histological subtype, is followed by adenocarcinoma, and other histologies. These various subtypes were classified on the basis of 2D-PAGE [45] and MS profiles [46].

5.1.1.1. *Squamous Lung Carcinoma.* Forty-three differentially expressed proteins were characterized by 2D-PAGE and MS in squamous lung cancer. Some were found to be related to oncogenes, whereas others were involved in cell cycle and signal transduction regulation [47, 48]. When 2D-PAGE profiles of normal, metaplasia, dysplasia, and carcinoma tissues of human bronchial epithelia were used to investigate successive steps of carcinogenesis in squamous carcinoma, 23 protein spots were identified in dysplasia and invasive carcinoma groups, including various kinases and kinase inhibitors, metalloproteinase receptors, and tumor antigens [49]. Using proteomic technologies, heterogeneous nuclear ribonucleoprotein (hnRNP) B1 was found to be specifically overexpressed in squamous cell carcinoma, stage I lung cancer, dysplasia, but not in normal bronchial epithelium [50]. It was suggested that overexpression of hnRNP B1 occurred in the early stage of carcinogenesis and inhibited DNA-PK activity. This interaction resulted in subsequent accumulation of erroneous rejoining of DNA double-strand breaks, thus causing tumor progression [51].

5.1.1.2. *Lung Adenocarcinoma.* 2D-PAGE and MS were used to identify 9 protein isoforms that were overexpressed in lung adenocarcinoma, including antioxidant enzyme AOE372, ATP synthase subunit d (ATP5D), β1,4 galactosyltransferase, cytosolic inorganic pyrophosphatase, glucose-regulated 58-kDa protein, glutathione-*S*-transferase M4, prolyl 4-hydroxylase β-subunit, triosephosphate isomerase, and ubiquitin thiolesterase [52]. Proteomic approaches also showed that napsin A, a member of the aspartic proteinase family, was expressed at various levels in primary lung adenocarcinomas and

that this protein might be useful in degree of differentiation of primary lung adenocarcinoma as well as distinguishing primary and metastatic adenocarcinoma [53]. In another study, systematic identification of lung adenocarcinoma proteins by 2D-PAGE and MS uncovered numerous cytokeratins isoforms. For example, 14 of 21 isoforms of cytokeratin 7, 8, 18, and 19 occurred at significantly higher levels in tumors compared to uninvolved adjacent tissue. Specific isoforms of four cytokeratins correlated with either clinical outcome or individual clinical–pathological parameters. Interestingly, all five of the CK7 isoforms associated with patient survival represented cleavage products [54]. Overexpression of truncated forms of cytokeratins 6 and 8 was also found by others, thus confirming proteolytic processing steps in tumor material [55]. Taken together, these studies suggest that specific isoforms/cleavage products of individual cytokeratins may have utility as diagnostic or predictive markers in lung adenocarcinomas.

5.1.2. *Colorectal Cancer*

CRC is the second most common cancer worldwide. About 10 years ago, our group published the first 2D-PAGE map of purified colorectal epithelial cells [56]. At that time, we could identify about 50 polypeptides, most of them by N-terminal sequencing—since MS technology was only emerging. In the meantime, expression proteomic studies were carried out with cell lines, whole tissue biopsies, and purified epithelial cells of colorectal origin. 2D-PAGE reference maps, protein, and membrane protein databases are available on the internet (reviewed in [57]). It was possible to synthesize translational research results obtained in CRC in a quasi-meta-analysis [58]: eight large-scale proteomic studies on CRC were retrieved. Out of 408 differentially expressed proteins, 83% were found to be differentially expressed only in a single study, 16 proteins in 3 studies, 10 in 4 studies, 3 in 5 studies, and only a single protein in 8 studies. Confirmation at proteome level using large-scale transcriptomic studies was possible in only 25%. This proportion was higher (67%) for confirming proteome results using transcriptomic technologies. Obviously, reproducibility and overlap between published gene expression results at proteome and transcriptome level are low in human CRC. Importantly, the total number of patients involved in the proteomic studies was only 11, a surprisingly low figure.

Using SELDI technology, α-defensin isoforms were found to be elevated in serum from colon cancer patients and in protein extracts from CRC [59]. This result was confirmed by expression analysis of microarray data obtained from 283 tumors and normal tissues followed by serum analysis of colon cancer patients and controls by ELISA. This study yielded a diagnostic sensitivity of 70% and specificity of 83% for α-defensin in colon cancer [60]. Although these figures appear too low for developing a screening test, this

result is an interesting proof of concept for integrating tissue transcriptomic data with serum protein analysis as a means to discover serum biomarkers. Another study in CRC tissue combined 2D-PAGE with SELDI-MS. This study demonstrated that PACAP protein, hnRNP A1, flavin reductase, calgizzarin, NDK B (NM23-H2), cyclophilin A, and smooth muscle protein 22-α showed significantly different levels. Subsequent immunohistochemical analysis of tissue distribution and subcellular localization of some of the differentially expressed proteins demonstrated alterations in subcellular protein distribution [61].

AACT technology (see above) was applied to identify differentially regulated protein isozymes in a CRC cell line (DLD-1). For this purpose, deuterium-labeled (heavy) amino acids were incorporated into the proteome of p53-induced DLD-1 cells, whereas DLD-1 vector cells (controls) were grown in the unlabeled medium. In high-throughput LC-ESI-MS/MS analyses, the AACT-containing peptides were paired with their unlabeled counterparts, and their relative spectral intensities, reflecting the differential protein expression, were quantified. The p53-regulated proteins were associated with several distinct functional categories: cell cycle arrest and p53 binding, protein chaperoning, plasma membrane dynamics, stress response, antioxidant enzymes, and anaerobic glycolysis. This result suggested that p53-induced apoptosis involves the systematic activation of multiple pathways that are glycolysis-relevant, energy-dependent, oxidative stress-mediated, and possibly mediated through interorganelle cross talks [62].

5.1.3. *Gastric Cancer*

Primary biomarker screening was performed in gastric cancer using 2D-PAGE on purified gastric epithelial cells obtained with an epithelial cell enrichment technique from gastrectomy specimens. One hundred and ninety-one differentially expressed protein spots were identified by MS. Overexpression of cathepsin B was observed in most cancer tissue samples, as was serum level of cathepsin B, compared to healthy controls. Elevated serum levels were associated with a reduced survival rate, enabling the classification of some gastric cancer patients into a subgroup that should undergo aggressive therapy [63]. Then, using SELDI-TOF-MS, our group identified a single best mass that could separate gastric cancer from patients without cancer, with a sensitivity of 89% and a specificity of 90%. This peak was identified as thrombin light chain A, a proteolytic fragment of prothrombin, indicating that disturbances in the coagulation system are early events in gastric cancer biology and that a decrease or loss of thrombin light chain A may contribute to the diagnosis of cancer patients [64]. Importantly, cancer diagnosis was possible by a *loss* of protein expression.

5.1.4. *Pancreatic Cancer*

Because differential diagnosis of pancreatic cancer and chronic pancreatitis is not possible without surgery, there is an important medical need for novel biomarkers in pancreatic diseases. Global proteomic approaches in pancreatic cancer have been reviewed [65]. For example, 2D-PAGE and MS were used to compare cancer vs normal and pancreatitis protein patterns. Differentially expressed proteins included antioxidant enzymes, chaperones, and/or chaperone-like proteins, calcium-binding proteins, proteases, signal transduction proteins, and extracellular matrix proteins. Among these proteins, annexin A4, cyclophilin A, cathepsin D, galectin-1, 14-3-3 χ, α-enolase, peroxiredoxin I, TM2, and S100-A8 were specifically overexpressed in tumors [66]. Using SELDI technology, serum samples from patients with resectable pancreatic adenocarcinoma were compared with samples from matched patients with nonmalignant pancreatic diseases as well as healthy controls. Two peaks of interest were identified in this study and were found to provide improved diagnostic sensitivity (78%) and specificity (97%) when compared to the traditional CA19-9 serum marker [67]. Another SELDI-based study compared serum obtained from pancreatic cancer patients. This study found a panel of six unidentified peptides that provided a diagnostic sensitivity of 89% and a specificity of 74% [68]. One additional pancreatic cancer study found a circulating antigen identified as DEAD-box protein 48. Interestingly, this protein is highly similar to eukaryotic initiation factor 4A that plays a role in pre-mRNA processing [69].

5.1.5. *Liver Cancer*

Expression proteomic analysis of human hepatocellular carcinoma (HCC) was conducted using 2D-PAGE coupled with MS. A panel of proteins of interest were identified from well-differentiated and poorly differentiated tumor samples, including methionine adenosyltransferase, glycine *N*-methyltransferase, and betaine–homocysteine *S*-methyltransferase. These enzymes, involved in the methylation cycle in the liver, influence the level of *S*-adenosylmethionine (AdoMet), and chronic deficiency in AdoMet in the liver results in spontaneous development of HCC in knockout mice deficient in methionine adenosyltransferase [70]. This study is providing a good example of successful functional confirmation in the animal model of an expression proteomic result obtained in the human being. Analysis of human liver samples with 2D-PAGE was also used to identify proteins that could be molecular targets for diagnosis and treatment of Hepatitis C virus-related hepatocellular carcinoma. One of the numerous spots that showed stronger intensity in tumorous samples was identified as α-enolase, a key enzyme in the glycolytic pathway. Expression of this protein increased with tumor

dedifferentiation, suggesting that α-enolase is a biomarker for tumor progression [71]. α-Enolase has been reported as a protein associated with lung and pancreatic cancer (see above).

ICAT and 2D LC-MS were used to investigate the qualitative and quantitative proteomes of HCC, following LCM. A total of 644 proteins were identified, out of which 261 proteins showed differential expression [72]. In another ICAT study, hepatocytes labeled with different reagents were stimulated with interferon-α or Hepatitis C virus and compared with corresponding controls. Samples were combined, trypsinized, and subject to cation exchange and avidin affinity chromatographies. The resulting cysteine-containing peptides were then analyzed by microcapillary LC-MS/MS. More than 1200 proteins or related protein groups could be identified [73]. Direct glycoproteins targeting allowed, in combination with 2D-PAGE, to discover higher levels of α-1,6-linked fucose in rat HCC and hyperfocusylation of a glycoprotein, Golgi Protein 73 (GP73) in the serum of patients suffering HCC [74]. SELDI-TOF-MS was used for protein fingerprint of 106 serum samples form subjects with liver cancer, cirrhosis, and healthy individuals, and patterns analyzed with an artificial neural network: the sensitivity and specificity were 88 and 95%, respectively, which represents an improvement compared with the traditional methods [75].

5.1.6. *Breast Cancer*

Several proteomic technologies have been used to uncover biomarkers and molecular mechanisms associated with breast carcinoma, the commonest cancer in women (reviewed in [76]). For example, 2D-PAGE combined with MS analyzed changes in the proteome of infiltrating ductal carcinoma compared to normal breast tissue. Twenty-five differentially expressed proteins could be identified, comprising cell defense proteins, enzymes involved in glycolytic energy metabolism and homeostasis, protein folding and structural proteins, and proteins involved in cytoskeleton and cell motility. Further proteins were also mapped to establish a 2D-PAGE reference map of human breast cancer [77]. Another proteomic study, combining 2D-PAGE, MS, immunoblotting, and antibody arrays analyzed the proteome from adipose cells and interstitial fluid collected from mastectomy specimens of high-risk breast cancer patients to detect factors present in the tumor microenvironment and responsible for tumor growth and progression. A total of 359 unique proteins were identified, including numerous signaling molecules, hormones, cytokines, and growth factors involved in a variety of biological processes such as signal transduction and cell communication; energy metabolism; protein metabolism; cell growth and/or maintenance; immune response; transport; regulation of nucleobase, nucleoside, and nucleic acid

metabolism; and apoptosis [78]. This proteomics study provided a unique phenotypic overview of tumor microenvironment in human epithelial cancer.

Using SELDI-TOF, it was shown that combined measurement of serum complement component C3a(desArg) and a C-terminal-truncated form of C3a(desArg) significantly differentiates breast cancer patients from noncancer controls [79]. In a confirmatory study on independent samples, C3a (desArg) appeared to lack specificity among patients with benign diseases [80]. This work could be partially validated in an independent prospective study where some peaks of interest could be recovered, but the sensitivity for cancer detection was only between 33 and 45% [81]. SELDI-TOF was also applied to the analysis of breast ductal lavage and was found to enhance the potential of cytology [82]. In another study looking for circulating autoantibodies in breast cancer patients, 15 proteins were repeatedly immunodetected in breast cancer patients and controls, some isoforms being preferentially immunodetected by breast cancer sera [83].

Proteomic studies could also invalidate putative results obtained with transcriptomic technologies. For example, using a proteomic approach complemented by immunohistochemical analysis, it was demonstrated that levels of expression of 14-3-3 sigma were similar in matched malignant and nonmalignant breast epithelial tissue. Besides its biological significance, the methodological relevance of this finding should be emphasized, since transcriptional expression of the sigma isoform of 14-3-3 is frequently impaired in human cancers, including breast, which has led to the suggestion that this protein might be involved in the neoplastic transformation of breast epithelial cells [84].

5.1.7. *Ovarian Cancer*

Ovarian cancer continues to be the leading cause of death from gynecologic malignancies because of the inability to identify disease at a stage when surgical therapy could be curative. This has driven a search for newer methods of detection of early ovarian cancer. For example, a 2D-PAGE study determined protein patterns associated with a predisposition to develop ovarian cancer in ovarian surface epithelium obtained during prophylactic oophorectomy in high-risk female patients. Eight proteins altered in high-risk patients were identified: three were already known as ovarian tumor-associated proteins, providing a proof of concept, whereas five were novel findings, representing potential early markers for the evaluation of the risk of developing ovarian cancer [85]. Numerous groups are pursuing similar serum-based approaches to ovarian cancer diagnosis (reviewed in [86]): characteristic SELDI-TOF and MALDI-TOF mass spectral patterns could be identified in sera of patients that may yield a sensitive and specific signature for ovarian cancer [87]. With refining analysis by quadrupole tandem LC-MS/MS, the

sensitivity and specificity results of models now being generated are consistently 100 and 100%, respectively. Over 1000 proteins and peptides that may be selective or specific to ovarian cancer have now been sequenced from serum samples from women with early and advanced stage ovarian cancer [88]. Reverse-phase microarrays were applied for proteomic mapping of phosphorylation end points in the metastatic tissue of ovarian cancer patients, and a reference standard based on a mixture of phosphorylated peptides was developed [89].

5.1.8. *Cervical Cancer*

In cervical cancer of the uterus, a 2D-PAGE study focused on the viral carcinogenesis characterized tumor-related proteins that were identified as oncogenes products, cell cycle-regulating proteins, genome-stabilizing, telomerase-activating, and cell-immortalizing proteins. The data have been synthesized from a human cervix cancer proteome database [90].

5.1.9. *Endometrial Cancer*

SELDI technology and peptide mass fingerprinting have also been applied in endometrial cancer. Two proteins, EC1 and EC2, were altered during carcinogenesis of the human uterine endometrium [91]. By improving analysis by quadrupole TOF-MS, it was possible to resolve two further potential biomarkers for endometrial cancer, chaperonin 10 and calgranulin A, in tissue homogenates [92]. Nine potential markers for endometrial cancer have been similarly discovered using a combination of differentially labeled tags, iTRAQ and cICAT (see above) with multidimensional LC and tandem MS. These include chaperonin 10, pyruvate kinase M1 or M2 isozyme, calgizzarin, heterogeneous nuclear ribonucleoprotein D0, macrophage migratory inhibitory factor, and polymeric immunoglobulin receptor precursor; underexpressed proteins were α-1-antitrypsin precursor, creatine kinase B, and transgelin. All these markers are known to be associated with various forms of cancer [93].

5.1.10. *Prostate Cancer*

The use of proteomics in prostate cancer, the most common cancer in men, was reviewed [94]. *In vitro* studies on prostate cell lines provided positive proof-of-principle results in the identification of novel biomarker when proteomics was utilized to query prostate tissue specimens. 2D-PAGE followed by MS was used to investigate protein profiles in voided urine after prostatic massage in patients with prostate cancer or with benign prostatic hyperplasia [95]. In this study, a potential novel cancer biomarker, calgranulin B/MRP-14, was identified. In another study, agarose 2D-gel

electrophoresis followed by LC-MS was applied to differentiate high molecular mass and alkaline protein patterns of androgen-dependent and -independent prostate cancers. A total of 295 proteins were identified representing 91% of excised spots. Eighteen proteins were regulated when the tumor became androgen-independent, including radical scavenger enzymes such as antioxidant protein 2, superoxide dismutase 1, thioredoxin peroxidase; GTP-binding protein β-chain homologue; and the ha1225 gene product [96].

2D-DIGE technology (see above) was applied to analyze the phosphoprotein fractions in prostate cancer cells transfected with prostate collagen triple helix (PCOTH), a growth-promoting gene. The phosphorylation level of oncoprotein TAF-Iβ/SET was significantly elevated in prostate cancer cells transfected with PCOTH. In combination with siRNA findings, this observation suggested that PCOTH is involved in growth and survival of prostate cancer cells and may, in part, be related to the TAF-Iβ pathway. This study implied that PCOTH might also be a drug target in prostate cancer [97], thus emphasizing importance of combining proteomic and siRNA technologies.

In prostate cancer, ICAT was used for assessing quantitative profile changes in the proteome of cultured cells in response to androgens. Changes in levels of 77 proteins in response to androgens were detected including spermine synthase, fatty acid synthase, and calreticulin precursor. A large number of proteins that had not been previously reported to be expressed in prostate cells were also quantitatively identified including members of the dual specificity protein phosphatase subfamily, "similar" to hypothetical protein DKFZp434B0328.1, to 14-3-3 protein to hypothetical protein 458 and actin components [98].

MS-based mass profiling combined with multivariate analysis identified platelet factor 4, a chemokine with prothrombolytic and antiangiogenic activities, as a diagnostically predictive protein in depleted serum of prostate cancer patients [99]. SELDI-TOF-MS was applied to the discovery of serum markers of bone metastasis in prostate cancer. Unique isoforms of serum amyloid A were identified in these patients. Machine-learning algorithms were used to identify these patients with a sensitivity and specificity of 89% [100].

Differences in the expression of cell surface proteins between "normal" prostate epithelial and prostate cancer cells were investigated using combined affinity chromatography of biotin-tagged surface proteins with MS. This analysis identified 26 integral membrane and 14 peripheral surface proteins, including ALCAM/CD166, ephrin type A receptor, EGF-R and prostaglandin F2 receptor regulatory protein, the voltage-dependent anion selective channel proteins porins 1 and 2, ecto-5′-nucleotidase (CD73), and scavenger receptor B1. Costimulation with type I and II interferons had additive

or synergistic effects on the membrane density of several of the peripheral surface proteins [101].

5.1.11. *Bladder Cancer*

Bladder cancer is the fifth most common malignancy in the world and represents the second most common cause of death among genitourinary tumors. Adipocyte fatty acid-binding protein (A-FABP) has been identified as a marker of progression in bladder cancer in a large-scale proteomics-based study. In this study, tumor profiling showed a substantial downregulation of A-FABP in invasive lesions. Results were confirmed by using a tissue microarray containing over 2000 samples. This work provided strong evidence that deregulation of A-FABP may play a role in bladder cancer progression and suggested a significant prognostic value for this marker [102]. In another comparative proteomic analysis in bladder cancer, a significant downregulation of S100C was found in invasive tumors (vs superficial tumors) and was associated with poorer survival [103].

5.1.12. *Leukemia*

2D-PAGE combined with advanced MS was used to profile proteins in acute leukemic cells. In this study, heat-shock 27-kDa protein 1 as well as other proteins were found to be highly expressed and may play a role in distinguishing this cancer from acute myloid leukemia. Another set of upregulated proteins was restricted to granulocytic lineage leukemia. High-level expression of NM23-H1 was associated with favorable prognosis. Thus, the protein patterns obtained could be used as an analytical tool for facilitating molecular definition of human classification of acute leukemia [104].

5.1.13. *Lymphoma*

An application of ICAT technology in cancer was to study the activation of p38 mitogen-activated protein kinase (MAPK) in follicular lymphomas. MAPK is a key mediator of stress, extracellular-, growth factor-, and cytokine-induced signaling, and has been implicated in the development of cancer. Inhibition of MAPK results in dose- and time-dependent caspase-3-mediated apoptosis [105]. Differential proteomic analysis using ICAT-LC-MS/MS of inhibited MAPK cells identified about 300 proteins belonging to diverse biochemical pathways. These included IL-6/phosphatidylinositol 3-kinase, insulin-like growth factor 2/Ras/Raf, WNT8d/Frizzled, MAPK-activated protein kinase 2, and nuclear factor κB. The differential phosphorylation status of selected kinase active proteins could be validated by Western blot analysis.

5.1.14. *Melanoma*

The plasma peptide component from 10 melanoma and healthy individuals was examined by a combination of RP-HPLC, SELDI-TOF-MS, and tandem MS. Fibrinogen α and inter-α-trypsin inhibitor heavy chain H4 fragments were absent in tumor samples [106].

5.1.15. *Pheochromocytoma*

The hypothesis that pheochromocytoma stages can be reflected by low molecular weight biomarkers was tested using peptide-profiling pattern recognition algorithms. It was possible to identify combinations of molecules that could distinguish all metastatic from all benign pheochromocytomas in a separate blinded validation set [107].

5.2. Functional Proteomic Studies

Most functional proteomic studies have been performed in cancer cell lines, that is, after exposition to toxicants, RNA inhibition, differentiation agents, viral transfection, and so on. These studies covered several aspects of mutagenesis, tumor promotion, and progression. In the recent years, it has been shown that repeated analysis of the proteome at different tumor stages also deliver distinct patterns and thus a functional picture of disease progression at the molecular level.

5.2.1. *Signaling Pathways*

Reverse-phase protein array technology has been applied to analyze the status of key points in cell signaling involved in prosurvival, mitogenic, apoptotic, and growth regulation pathways in the progression from normal prostate epithelium to invasive prostate cancer. Using multiplexed reverse-phase protein arrays coupled with LCM, the states of signaling changes during disease progression from prostate cancer study sets were analyzed [108]. After differentiation with dimethylsulfoxide (DMSO), 2D-PAGE followed by MS was used to identify signaling proteins in differentiated vs undifferentiated cell lines. Differences in expression of GTP-binding/Ras-related proteins, kinases, growth factors, calcium-binding proteins, and phosphatase-related proteins were observed. Most signaling proteins were upregulated in differentiated cells, whereas only eight such proteins were observed in undifferentiated cells. The on/off switching profiles of several individual signaling proteins from different signaling cascades is likely the key to understanding biochemical mechanisms involved during the differentiation process [109].

5.2.2. *Cell Cycle*

Using 2D-PAGE, cell cycle entry has been shown to be associated with a significant increase in p27(kip1) phosphorylation in human primary B lymphocytes. Detailed analysis revealed that different cyclins and cyclin-dependent kinases interact with distinct posttranslationally modified isoforms of p27(kip1) *in vivo*. These results have to be interpretated in the context of overexpression of cyclin D3 in the presence of high levels of p27(kip1) in human B-cell lymphomas with adverse clinical outcome [110].

5.2.3. *Oxidative Stress*

A cellular prooxidant state promotes cells to neoplastic growth in part because of modifications to proteins and their functions. Reactive nitrogen species formed from nitric oxide (NO) or its metabolites can lead to protein tyrosine nitration that is elevated in some cancers. Using 2D-PAGE and MS in a lung cancer cell line exposed to NO, more than 25 nitrated proteins were identified, including metabolic enzymes, structural proteins, and proteins involved in prevention of oxidative damage. These alterations may contribute to the mutagenic processes and promote carcinogenesis [111].

5.2.4. *Chaperone Proteins*

Although chaperones play an important role in tumor biology, no systematic work on their expression patterns had been reported thus far. 2D-PAGE combined with MS was used for the concomitant determination of several chaperones in 10 human tumor cell lines. Human tumor cell lines of neuroblastoma, colorectal cancer, adenocarcinoma of the ovary, osteosarcoma, rhabdomyosarcoma, malignant melanoma, lung, cervical and breast cancer, promyelocytic leukemia were analyzed. The main chaperone groups included HSP90/HATPasC, HSP70, Cpn60_TCP1, DnaJ, Thioredoxin, TPR, Pro_isomerase, HSP20, ERP29_C, KE2, Prefoldin, DUF704, BAG, GrpE, and DcpS. The 10 individual tumor cell lines showed different expression patterns. These important results have served as a reference map of chaperones and isoforms in human cancers [112].

5.2.5. *Viral Oncogenesis*

Comparison of global patterns of protein expression can be achieved in cell lines through the use of cDNA microarray and proteomic techniques. This approach has provided in-depth information on the impact of HPV-16 E6 on gene expression and served as a valuable resource for investigation of the biochemical basis of uterine cervical carcinogenesis. In three cases (CDK5, Bak, and I-TRAF), expression was matched in both analyses of cDNA microarrays and proteomics [113].

5.2.6. *P53 Function*

The p53 gene is a transcription factor essential for DNA damage checkpoints during cellular response to stress. Mutations within this gene are the most common genetic alterations found in human cancer. Although most pathogenetic modifications are missense mutations that abolish the p53 DNA-binding function, some p53 mutations may determine different phenotypes in distinct cell types. 2D-PAGE analyses in a thyroid cancer cell line indicated that expression of a significant portion (up to 25%) of protein species were modified by p53 mutants. Several of these proteins were identified by MS procedures, including HSP90 and T-complex proteins. Interestingly, these proteins were already known to be related to p53 function [114].

5.2.7. *Mitochrondrial Dysfunction in Cancer*

Bioenergetic dysfunction of mitochondria has been reported as a hallmark of many types of cancers (i.e., downregulation of ATP synthase β-subunit expression in liver, kidney, colon, squamous esophageal, and lung carcinomas, as well as in breast and gastric adenocarcinomas). ATP synthase d-subunit was found to be associated with chemoresistance to 5-fluorouracil (5-FU) in CRC using 2D-PAGE. In a functional assay, suppressed ATP synthase d-subunit expression by siRNA transfection increased cell viability in the presence of 5-FU [115].

5.2.8. *Chromatin-Associated Proteins*

High-mobility group A (HMGA) proteins are nonhistone architectural nuclear factors that play a general role in chromatin dynamics. These proteins were analyzed by a combination of affinity chromatography, 2D-PAGE, and MS. About 20 putative HMGA proteins were identified and assigned to three different classes: mRNA-processing proteins, chromatin-remodeling related factors, and structural proteins. These experimental data indicated that HMGA proteins were highly connected nodes in the chromatin protein network. Because these proteins are strongly implicated with cancer development, the identification of molecules able to perturb the HMGA molecular network may provide an attractive tool to interfere with oncogenic activity [116].

5.2.9. *Cell Invasion*

Malignant gliomas (astrocytomas) are lethal invasive brain tumors. Invasive cell migration is initiated by extension of pseudopodia into interstitial spaces. In a DIGE technology study (see above), pseudopodia of glioma cells were harvested and their protein profile compared with the profile of whole cells. Increased pseudopodial constituents were identified as actin,

hepatocyte growth factor, hepatocyte growth factor receptor, isoforms of annexins I and II, as well as several glycolytic enzymes [117]. These proteins may represent potential therapeutic targets to suppress tumor invasion.

6. What Has Been Achieved in Cancer Proteomics?

6.1. Understanding Cancer

Large-scale proteomic studies have addressed on a molecular basis the complexity of the alterations associated with transition from benign to malignant tumor cells that lead to appearance and progression of cancer. For example, proteomic-based studies of the effects of transforming growth factor (TGF)-β in early and late stages of carcinogenesis have widened our knowledge of TGF-β-dependent regulation of cell proliferation, apoptosis, DNA damage repair, and transcription (reviewed in [118]). Another example of the utility of global protein expression approaches in understanding carcinogenesis is demonstrated by an extensive comparative survey of alterations found in breast epithelium during malignant transformation [119]. These include differences in levels of key regulators of the cell cycle, signal transduction, apoptosis, transcriptional regulation, and cell metabolism. The impact of proteomic approach was also demonstrated in a study that compared the similarities and differences between inflammation and cancer [120]. This unique study examined the expression of intracellular proteins in a regressive cancer cell line and a progressive inflammatory cell-promoting cancer cell line.

Thus, simultaneous monitoring of thousands of proteins in cancer has uncovered novel signaling mechanisms and unravelled a series of complex processes involving multiple changes in protein expression, abundance, and function. In addition to isolated proteins, protein–protein interactions are fundamental to carcinogenetic processes. A comprehensive determination of all important protein–protein interactions that occur within a given tissue (as an integrated system) is critical to ultimately providing a fundamental understanding of cancer biology. The availability of genome-scale sets of cloned open-reading frames has facilitated systematic efforts at creating proteome-scale datasets of protein–protein interactions that are represented as complex networks or ''interactome'' maps in normal and cancer patients. Although far from complete, currently available maps provide insight into how biochemical properties of proteins and protein complexes are integrated into normal and cancerous tissues [121]. Taken together, theses studies document a significant role for proteomics in basic research and for identifying mechanisms of carcinogenesis. It should be noted, however, that this approach is viewed as being complementary to others.

6.2. Screening and Diagnosis

On the basis of the numerous studies described above, it is reasonable to claim that the proteomic approach, using 2D-PAGE, SELDI-TOF, or other emerging technologies, has the potential to identify novel biomarkers in human cancer. For example, peptide biomarkers were overwhelmingly (88%) viewed as likely to have diagnostic application in oncology in a survey of experts from the pharmaceutical industry [122]. However, the numerous expression research results described above have not yet been translated into *in vitro* diagnostics tests. So far, it has only been demonstrated that a limited panel of protein or peptide biomarkers can improve the specificity of available tests and thus has the potential of facilitating early diagnosis of cancer.

This gap between expectations and actual practice might have technical reasons:

- There is a relative lack of experience with the technology of proteomics compared to transcriptomics and genomics,
- Only increasing understanding of complexity, dynamic range, incomplete sampling, false positive matches, and integration of diverse datasets for plasma and serum proteins will lay the foundation for validation of novel *in vitro* diagnostics in cancer [123],
- Concerns remain about SELDI-TOF technology because of the lack of sequences and consequently of a biological hypothesis, the discrepancy between the sensitivity of SELDI-MS compared to the concentration of known cancer biomarkers, and possible bioinformatics overfitting of MS data,
- Protein microarray assay or multichannel ELISA is still not widely established.

Indeed, detection and diagnosis of cancer can only rely on an integrated approach using clinical history, physical examination, imaging, and histopathology. Some bias in study design can also have prevented a large acceptance of diagnostic research results by the medical community:

- Most studies have been performed by biochemists and the clinical aspect has been neglected,
- For most biomarkers discovery studies, sample size was not large enough,
- Choice of control groups was inadequate or incomplete,
- No confirmatory studies were performed.

Moreover, regulatory requirements were not fulfilled by most research groups because they were not considered for study design. For regulatory agencies and pharmaceutical companies to be convinced that combining

biomarkers performs better than the conventional, single-marker approach, it appears essential to fulfill requirements for *in vitro* diagnostics development such as:

- Providing diagnostic tests that allow for definite and reliable diagnosis tied to a decision on interventions (prevention, treatment, or nontreatment),
- Meeting stringent performance characteristics for each analyte (in particular test accuracy, including both precision of the measurement and trueness of the measurement), and
- Providing adequate diagnostic accuracy, that is diagnostic sensitivity and diagnostic specificity, determined by the desired positive and negative predictive values which depend on disease frequency [124].

6.3. Prognosis

Although most follow-up tests in clinical oncology are based on the quantification of serum proteins, proteomic technologies have found only limited use for the development of novel prognostic biomarkers thus far. This is surprising since proteomic technologies appear particularly well suited for analyzing dynamic processes, for example, in clinical oncology where prognosis is changing all the time and particularly following surgery, but also after chemotherapy, depending on immune defenses, and so on.

Forecasting a particular patient's prognosis is best possible by defining tumor extension at the time of diagnosis, using the tumor node metastasis (TNM) staging system of the International Union against Cancer (UICC) [125]. This validated (but static) prognostic information is giving the framework for proteomic studies, and proteomics analysis (such as repeated measurement of serum biomarkers over the course of disease) is complementing this information. A proof of concept for the application of proteomics for prognostic purpose in oncology is available: patient survival has been shown to correlate with protein expression profiles in lung cancer [126]. Such dynamic information cannot be provided by transcriptomics studies in cancer tissue.

6.4. Therapy

About 40 small protein or peptide drugs are currently in clinical use with an estimated annual market of US$1 billion. These include insulin, parathyroid hormone (PTH), calcitonin, growth hormones, and so on [127]. In oncology, several antibodies directed against surface proteins have entered the market over the last years, including herceptin in breast cancer, cetuximab in CRC, imatinib in gastrointestinal stromal tumors, and so on.

Proteomic technologies have been increasingly applied by pharmaceutical companies as the object of significant investments for discovering and validating small protein drugs and targets for monoclonal antibodies at various stages of development.

6.4.1. *Target Discovery*

The main application of proteomics in drug development is clearly the identification of drug targets. Pathological processes are reflected by characteristic alterations in the proteome. Thus, most pharmaceutical companies active in oncology have recognized the need to implement proteomic discovery strategies to initiate the drug discovery process. In the meantime, large datasets have been generated that now need to be interpreted and prioritized. For this purpose, pharmaceutical companies increasingly rely on the integration with bioinformatics for data interpretation, and on further validation of proteomic technologies for their application in various areas of drug development.

Indeed, there are still many technological challenges to meet the needs for high sensitivity, reproducibility, and throughput required for successful target discovery in cancer proteomics, including:

- Current and partial view of the proteome,
- Limitations for the analysis of hydrophobic proteins—significant for analysis of receptors and ion channels,
- Large dynamic range of protein concentration in serum or plasma, thus preventing accurate separation of these complex biological mixtures,
- Low reproducibility and overlapping between studies.

However, it is the hope that the rapid progress in sample preparation procedures and in proteomic technologies particularly improved mass spectrometer sensitivity will solve these problems.

6.4.2. *Target Validation*

Given a trend to evaluate patterns rather than single biomarkers, it is unclear how to define an acceptable validation strategy to date. This problem might represent a unique opportunity for proteomic technologies because they allow analysis of large sets of molecules at the functional level. There are two ways in applying proteomic technologies for target validation:

- Functional studies in animal models for validation of proteins of interest in humans,
- Expression studies in humans at different steps of carcinogenesis for validation of proteins in animal or *in vitro* models.

For validation purposes, proteomics can be associated with other functional tools. For example, proteomics combined with small interfering RNA

(siRNA) elucidated the mechanism of action of paclitaxel, a potent drug of natural origin widely used in the treatment of ovarian, lung, and breast cancer. In a first step, 2D-PAGE and MS were used to investigate the mechanisms of action of paclitaxel on cervical carcinoma cell line carrying HPV. This approach demonstrated that treatment suppressed the expression of the mitotic checkpoint protein BUB3. In addition, this study identified several cellular proteins that were responsive to treatment in HeLa cells, namely apoptosis-related, immune response-related, and cell cycle checkpoint-related proteins. Paclitaxel treatment diminished growth factor/oncogene-related proteins and transcription regulation-related proteins. In a second step, functional proteomic analysis by siRNA targeting was used to investigate the role of mitotic checkpoint protein BUB3 in cell cycle progression in herpes virus positive and negative cervix cancer cell lines. Paclitaxel showed antiproliferative activity through the membrane death receptor (DR)-mediated apoptotic pathway involving activation of caspase-8 with a TRAIL-dependent fashion as well as the mitochondrial-mediated pathway involving downregulation of Bcl-2 by cytochrome *c* release. This study showed the power of proteomic profiling combined with siRNA technology for better understanding of the actions of cancer drugs [128].

6.4.3. *Analysis of Binding Sites*

Proteomic technologies have been applied for fine-tuning drug development studies in cancer. This approach has been used in analyzing the binding sites of alkylating agents (i.e., microtubule disrupters) and thus powerful anticancer drugs. β-Tubulin was separated using 2D-PAGE from B16 cells incubated with an alkylating agent. Alkylated β-tubulin had a lower apparent molecular weight and a more basic p*I* than the unmodified protein. Using MALDI-TOF-MS, it was possible to demonstrate that none of the cysteine residues of β-tubulin was linked to the alkylating agent, as previously supposed. In contrast, a glutamic acid at amino acid position 198 was identified as target for alkylation via an ester bond with ICEU. This site should play an essential role in the conformational structure necessary for the interaction in the microtubule [129].

6.4.4. *Response Prediction*

Chemoresistance remains an unresolved problem in clinical oncology. This issue highlights the need for identifying molecular factors that lead to drug resistance in cancer cells. The hope is that a tumor biopsy can be analyzed to generate a molecular description of the tumor in addition to standard histopathology, allowing the selection of a therapy targeting specific molecular defects. Proteomic technologies have been used for studying global protein expression in chemosensitive and chemoresistant cancer cells to find

candidate proteins that are associated with the drug-resistant phenotype. For example, molecular profiling of individual patient's tumors is currently being evaluated in clinical trials at the National Institutes of Health, National Cancer Institute for monitoring epidermal growth factor (EGF) cell signaling events for patients with breast and ovarian cancer [130], or at other institutions for patients with CRC [131]. Proteomic approaches have been proposed for selecting chemotherapy in neuroblastoma [132, 133], glioblastoma [134], for detecting proteins related to chemoresistance in cervix carcinoma [135], for discovering mechanisms of chemoresistance to 5-FU in CRC [136], or radioresistance-associated proteins in rectal cancer [137].

6.4.5. *Therapy Monitoring*

Following initiation of treatment, serum probes can be obtained to determine whether the tumor is responding to therapy, as well as determining whether the tumor has developed resistance mechanisms that may require modification of therapy, that is so-called responder profiling. Protein biomarkers such as CEA, CA 15-3, AFP, and PSA are already in clinical use for therapy monitoring, and it is reasonable that these biomarkers will be complemented by others in the future.

6.4.6. *Prevention of Side Effects*

The need for predicting side effects in drug development and the limitations of conventional tests in animal models have been recently highlighted by life-threatening complications that occurred in healthy human volunteers during a stage-I oncology drug trial in Great Britain. Proteomic technologies are expected to play an important role for identifying patients at risk for experiencing side effects. For example, 88% of pharmaceutical experts considered that proteomic technologies are likely or very likely to play an important role in this respect [138]. In one study, SELDI technology was used to evaluate serum protein patterns in breast cancer patients after docetaxel infusion. The relative expression levels of target proteins were monitored following docetaxel injection. Two proteins, kininogen and apolipoprotein A-II, were correlated with adverse effects [139].

6.4.7. *Toxicoproteomics*

Toxicoproteomics is a new scientific discipline that combines proteomic technologies with bioinformatics. This approach provides a means to identify and characterize mechanisms of action of toxicants in carcinogenesis. In contrast to toxicogenomics, a discipline that determines genetic suspectibility of a particular individual following exposure to a carcinogenetic agent, toxicoproteomics allow the monitoring of the body's response to a particular toxicant. The current regulatory toxicological approach usually includes

investigation of carcinogenicity, in generally lengthy (2 years) studies in rodents. This is especially true for detection of early protein biomarker signatures that precede neoplastic appearance [140]. Several examples demonstrate the potential of proteomic approaches to reduce time and expense of traditional carcinogenicity testing. For example, the liver carcinogen *N*-nitrosomorpholine (NNM) was investigated in rats to identify potential early protein biomarker signatures indicative of the carcinogenic processes. Analysis was performed 18 weeks following treatment revealed significant upregulation of stress proteins, including caspase-8 precursor, vimentin, and Rho GDP dissociation inhibitor. Interestingly, the proteins annexin A5 and fructose-1,6-bisphosphatase were found to be deregulated early after exposure. This finding may indicate their potential use as predictive biomarkers for early liver carcinogenicity. Another toxicoproteomics study was performed in municipal incinerator plant workers exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD is a chemical compound known to induce severe reproductive and developmental problems. Seven overexpressed proteins were identified in this study. Interestingly, the most overexpressed protein was identified as AFP, the classical serum marker for HCC. In cultured HepG2 cells, TCDD treatment resulted in increased mRNA and protein expression of AFP, but reduced albumin expression. This toxiproteomics study provided strong evidence that TCDD may induce liver cancer [141].

7. Conclusion

The study of cellular protein expression by proteomics has relied on technologies that first appeared in the 1970s and have been continuously improved. In combination with advanced bioinformatics and large databases, proteomics now provide a powerful tool for basic research, clinical diagnostics, and drug development applications.

The future challenge for cancer proteomics is, however, enormous. Individual cells have the potential to generate several hundred thousand to millions of proteins. Because of the complexity of the biological systems in general, proteomic studies result in the generation of extremely large datasets that make interpretation extraordinarily difficult. Thus, the most significant contribution of proteomics research in cancer is expected to derive not from the analysis of single experiments, but from comparison studies obtained from various cancer types and stages. In addition, results must be interpreted with respect to those obtained in normal individuals and from those with benign disease. As can be appreciated, there is an increasing need to integrate proteomics with genomics and metabolomics in order to provide the most comprehensive and functional interpretation of both clinical and pathological data.

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PARANEOPLASTIC NEUROLOGICAL SYNDROMES AND ONCONEURAL ANTIBODIES: CLINICAL AND IMMUNOLOGICAL ASPECTS

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1. Abstract

Paraneoplastic neurological syndromes (PNS) are infrequent disorders that are associated with cancer. The syndromes are highly heterogeneous and often affect several areas of the nervous system. Among the most well-known syndromes are paraneoplastic encephalomyelitis, cerebellar degeneration, sensory neuronopathy, and Lambert–Eaton myastenic syndrome. There are various associated tumors, in particular small cell lung cancer, cancer of the breast and ovary, and thymoma. The onset of neurological symptoms often precedes the cancer diagnosis, and the recognition of a paraneoplastic syndrome should lead to immediate search for cancer. The etiology of the paraneoplastic syndromes is believed to be autoimmune. Antibodies to onconeural antigens, expressed in the tumor of the affected individual and in normal neurons, are found in many of the patients. These antibodies are useful markers for paraneoplastic etiology. The pathogenesis of the PNS is uncertain, but cellular immune responses are thought to be the main effector mechanism.

The cornerstone of therapy is the identification and treatment of the underlying malignancy. In some of the disorders, immunosuppressive therapy is of additional benefit. The prognosis of the different PNS varies depending on the level of affection and the degree of neuronal death.

2. Introduction

Cancer patients often develop symptoms from organs remote from the primary tumor. The symptoms are usually caused by metastasis or toxic effects of therapy and less often by such secondary factors as nutritional deficiency, metabolic disturbances, opportunistic infections, and effects of critical illness. Other systemic diseases such as diabetes or amyloid may become manifest or aggravated during the course of the cancer disease and cause symptoms from remote organs. Paraneoplastic neurological syndromes (PNS) are a rare cause of remote symptoms [1]. The PNS affect less than 1% of all cancer patients [2]. Even in small cell lung cancer (SCLC), the tumor type most commonly associated with PNS, these disorders occur in less than 5% of the patients [3, 4].

The PNS are interesting as excellent examples of naturally occurring tumor immunity [5, 6]. In PNS, the relevant tumor antigens belong to the group of antigens normally expressed in immunoprivileged sites only, such as the nervous system, and trigger both cellular and humoral responses [7]. These immune responses may limit tumor growth [8] and improve the response to oncological therapy [9, 10]. PNS are sometimes even associated with tumor eradication [11]. However, the immune response also targets normal neurons expressing the same antigens, thereby causing autoimmune neuronal damage.

The clinical characteristics, response to therapy, and prognosis of the different PNS are heterogeneous, but some clinical features are common. First, in most patients, the nervous system symptoms appear before cancer is diagnosed. Second, at this stage, the tumor is small and sometimes undetectable. Third, the usual onset of PNS is subacute and the progression rapid, although the opposite case does not exclude a paraneoplastic etiology. Finally, the neurological symptoms are often very serious, and the patient may die from the effects of the PNS and not cancer itself [12].

Some of the PNS affect multiple elements of the nervous system (paraneoplastic encephalomyelitis, PEM), whereas others involve one group of neurons only (Purkinje cells in paraneoplastic cerebellar degeneration, PCD), or one area (hippocampus in limbic encephalitis, LE). PNS and associated tumors overlap considerably, as some tumors are associated with several different PNS and vice versa (Table 1). Many of the disorders are associated with antibodies directed to antigens shared by tumor and nervous tissue (onconeural antigens). The onconeural antigens are normally expressed in immunoprivileged sites only as cells of the nervous system and germ cells [13].

The term ''classical PNS'' is reserved for the PNS in which the association with cancer is common and includes encephalomyelitis, limbic encephalitis, paraneoplastic cerebellar degeneration, and paraneoplastic opsoclonus–myoclonus (OM), as well as sensory neuronopathy (SN), chronic gastrointestinal pseudo-obstruction, Lambert–Eaton myasthenic syndrome (LEMS), and dermatomyositis [14]. This chapter does not include dermatomyositis.

Detection of a well-characterized onconeural antibody (Table 1) in a patient with suspected PNS definitely diagnoses the condition as paraneoplastic [14]. Thus, the antibodies, when present, are highly useful markers of causation. The various antibodies are also valuable as guidance on the most likely site of malignancy (Table 2). However, investigation is complicated by the fact that many PNS are associated with different onconeural antibodies, and patients often harbor several types of onconeural antibodies [15]. Several antibodies are also associated with heterogenous clinical manifestations [16]. This complexity implicates that the antibodies are markers of cancer more than markers of a specific PNS [16].

TABLE 1
MAJOR PARANEOPLASTIC SYNDROMES, ASSOCIATED ANTIBODIES, AND TUMORS

Clinical syndrome	Associated antibody	Frequently associated tumors
Paraneoplastic encephalomyelitis	Hu	SCLC
	CRMP-5	SCLC, thymoma
	PCA-2	SCLC
	Amphiphysin	SCLC, breast
Limbic encephalitis	Hu	SCLC
	Ma2	Testis
	CRMP-5	SCLC
	ANNA-3	SCLC, thymoma
	VGKC	Thymoma, SCLC
Brain stem encephalitis	Ri	Breast
	Yo	Breast, gynecological tumors
	Hu	SCLC
	Ma2	Testis, SCLC
Extrapyramidal syndromes	Hu	SCLC
	Ma/Ma2	Miscellaneous
	ANNA-3	Testis
	CRMP-5	SCLC, thymoma
Paraneoplastic cerebellar degeneration	CRMP-5/CV2	SCLC
	Yo	Ovary, breast
	Hu	SCLC
	Ri	Breast
	Tr	Hodgkin's lymphoma
	Zic4	SCLC
	mGluR	Hodgkin's lymphoma
	ANNA-3	SCLC
	Ma/Ma2	Miscellaneous
	CRMP-5/CV2	SCLC
Paraneoplastic opsoclonus-myoclonus	Ri	Breast
	Hu	SCLC
	Ma	Testis
Visual syndromes	Recoverin	SCLC
	CRMP-5	SCLC
Stiff-Person syndrome	Amphiphysin	Breast
Sensory-motor peripheral neuropathy	Hu	SCLC
	Amphiphysin	SCLC
	CRMP-5	SCLC, thymoma
Subacute sensory neuropathy	Hu	SCLC
	Amphiphysin	SCLC
	ANNA-3	SCLC
	CRMP-5	SCLC, thymoma
Lambert–Eaton myastenic syndrome	VGCC	SCLC

TABLE 2
MAJOR ONCONEURAL ANTIBODIES AND ANTIGENS AND ASSOCIATED NEUROLOGICAL FINDINGS AND TUMORS[a]

Antibody	Antigen	Neuronal reactivity	Common neurological features	Associated tumors
Anti-Yo (PCA-1)	Cdr2	Cytoplasm of Purkinje cells	Cerebellar degeneration, brain stem encephalitis	Ovary, breast, gastrointestinal tumors
Anti-Hu	HuD, HuC, Hel-N1	Nucleus > cytoplasm (all neurons)	Encephalomyelitis, SSN, LE, brain stem encephalitis, cerebellar degeneration, myelitis, gastrointestinal pseudo-obstruction, extrapyramidal affection	SCLC, neuroendocrine tumors
Anti-Ri (ANNA-2)	NOVA	Nucleus > cytoplasm (CNS neurons)	Brain stem encephalitis, cerebellar degeneration	Breast
Anti-amphiphysin	Amphiphysin	Presynaptic vesicles	Stiff-Person syndrome	Breast, SCLC
Anti-CRMP5/CV2	CRMP5	Cytoplasm of oligodendrocytes	Encephalomyelitis, cerebellar degeneration, LE, chorea, sensory neuronopathy, sensorimotor neuropathy, optic neuritis, gastrointestinal pseudo-obstruction	SCLC, thymoma, gynecological tumors
Anti-Ma	Ma1–5	Neurons (nuclei/nucleoli > cytoplasm)	Cerebellar degeneration, brain stem dysfunction	Miscellaneous
Anti-Ma2/Ta	Ma2	Neurons (subnucleus)	LE, brain stem encephalitis, extrapyramidal affection	Testis, lung
Anti-recoverin	Recoverin	Retinal inner and outer nuclear layers, inner and outer segment layers	Visual disturbances	SCLC

[a]Other antibodies include PCA-2, mGluR, Zic4, and ANNA-3; these antibodies are reported in smaller numbers of patients. VGCC antibodies are associated with both LEMS and cerebellar degeneration, but are not diagnostic for a paraneoplastic etiology.

The scope of this chapter is to present the clinical features of the major PNS and their associated antibodies, diagnostic procedures and updated aspects of the pathogenesis, and treatment of patients with PNS.

3. Onconeural Antigens

When Posner *et al.* [17, 18] discovered that PNS patients harbor high titres of autoantibodies in the serum and cerebrospinal fluid (CSF) in the 1980s, this represented a new method of identifying PNS. In the following two decades, antibodies from affected patients have been used to screen complementary DNA (cDNA) expression libraries to identify autoimmunity-related target antigens [19]. This strategy has led to the identification of most of the onconeural antigens associated with PNS.

The onconeural antigens are expressed in the tumor of the affected patient as well as in the normal cells of the nervous system. The neural target antigens of PNS are often proteins participating in vital functions of the intracellular environment (Table 2). Musunuru and Darnell [19] have proposed a classification of onconeural antigens into four categories according to their biological charactistics: neuromuscular junction proteins, neuron-specific RNA-binding proteins, neuronal signaling proteins, and vesicle nerve terminal-associated proteins. Antibodies to neuromuscular junction proteins, such as those directed to the voltage-gated calcium channel (VGCC antibodies), are known to be pathogenic, whereas antibodies of the other three groups are directed to intracellular targets, and their pathogenic role is thus uncertain.

Two onconeural antigens in the RNA-binding protein group are the Nova antigens and the family of Hu antigens, targets for the Ri and the Hu antibodies, respectively [19]. These proteins are located mainly in the neuronal nuclei. Probing cDNA expression libraries with Ri antisera has defined two antigens, Nova-1 and Nova-2 [20]. The Nova proteins probably function as splicing regulators [21]. The Nova proteins are expressed only in neurons of the central nervous system (CNS). The Hu antigen family consists of four different proteins, but HuD is the major antigen [22]. Hu proteins probably function in neuronal development and survival. Another family of onconeural antigens, those belonging to the Ma family, is also located in the neuronal nucleus. There are three Ma antigens: Ma1, Ma2, and Ma3; Ma2 is the major antigen [23]. The function of the Ma proteins is unknown, but it is suggested that the proteins participate in phosphorylation-dependent RNA processing [24].

The cdr2 antigen, the target antigen of the Yo antibodies in paraneoplastic cerebellar degeneration, is located in the cytoplasma. The cdr2 protein is a neuronal signaling protein, and is, like the Ma proteins, normally expressed

only in certain parts of the brain and testes [25]. In contrast to Ma, however, the cdr2 antigen is unique among the onconeural antigens because its expression is restricted at a posttranslational level. Recoverin, the cancer-associated retinopathy antigen, is another protein engaged in neuronal signaling. Recoverin is a calcium-binding protein, a key player in the modulation of retinal phototransduction and essential in the survival of retinal neurons [26, 27]. The antigens of the collapsin response mediator protein (CRMP) family seem to be involved in multiple cellular and molecular processes including differentiation, migration, and apoptosis. Their expression is normally downregulated in adult brain but is altered in such diseases as PNS [28]. Antibodies to the CRMP-5 antigen are associated with several PNS.

The expression of onconeural antigens is highly restricted in some instances. Recoverin is found in the retinal cells only, and cdr2 expression is restricted to the Purkinje cells and brain stem. Other antigens such as Nova are widely distributed in the CNS, whereas the HuD and CRMP-5 antigens are expressed by neurons of both the CNS and the peripheral nervous system. The extensive immunoreactivity is reflected by the heterogenous clinical manifestations in patients with Ri, CRMP-5, Hu, and amphiphysin antibodies [15, 29, 30].

4. Paraneoplastic Syndromes of the CNS

4.1. Paraneoplastic Encephalomyelitis

PEM is one of the most frequent cancer-associated syndromes. This complex disorder usually affects several areas of the CNS. Cerebellar and brain stem disorders, as well as limbic encephalitis, are the most common clinical presentations of PEM [31, 32]. Focal involvement of the sensorimotor cortex has been described in a few cases [33], and PEM may manifest as epileptic seizures or epilepsia partialis continua [33, 34], or as extrapyramidal symptoms [35]. Two-thirds of the patients are affected in both the CNS and the peripheral nervous system. The predominant feature in more than half of these is SN [32, 36], hence the commonly used term is PEM/SN. Autonomic dysfunction is common in PEM/SN patients [36], often presenting as gastrointestinal dysmotility [37].

The most common malignancy associated with PEM is SCLC [32], but several other tumors have also been associated with PEM [32, 38, 39]. The Hu antibody is the most common associated antibody, but several other antibodies are found in isolated PEM (Table 1). In the majority of cases, PEM precedes the tumor diagnosis, but when the cancer is already acknowledged, PEM often predicts tumor progression or relapse [32].

The main pathological features of PEM/SN are inflammation, nonapoptotic neuronal loss, gliosis and cellular infiltrates [40, 41]. The distribution of

lesions usually reflects the clinical symptoms, but the pathological features are often more widespread than would be predicted based on the clinical symptoms [41, 42].

4.2. Focal Encephalitis

4.2.1. *Limbic Encephalitis*

The main features of LE are epileptic seizures and mental symptoms such as depression, personality changes, and cognitive dysfunction. The onset is usually subacute and progressive [43]. LE is the predominant feature in about 9% of PEM cases, and LE patients often exhibit additional symptoms from other CNS locations [44]. Not surprisingly, SCLC is found in about half of the LE patients, and in this group, most are Hu antibody positive [43].

The second most common tumor in LE is testicular germ cell cancer [43], and these patients usually harbor antibodies to the Ma2 protein. Antibodies to Ma2 antigen alone are usually associated with testicular cancer. Similar to many other onconeural antibodies, Ma2 is associated with clinical multifocality. Many patients have symptoms of brain stem and hypothalamic dysfunction, and only one-fourth of Ma2 positive patients exhibit classical LE [45]. Patients with antibodies to the major antigen Ma2 and antibodies to Ma1 or Ma3 have different tumors [43, 46], and are more likely to have additional neurological signs [45].

Another tumor that is associated with LE is thymoma [47]. Some thymoma patients and some patients with SCLC have antibodies to voltage-gated potassium channels (VGKC) [48, 49]. VGKC antibodies are, however, more commonly associated with nonparaneoplastic LE, and the prompt response to immunosuppressive treatment in these patients certainly indicates that the VGKC antibodies are pathogenic [50, 51]. The distinction between LE patients with and without paraneoplasia is of great therapeutic and prognostic importance, and warrants careful investigation among patients with subacute dysfunction of the temporal lobe(s).

LE is sometimes also associated with CRMP-5 antibodies [52], and new antibody targets are still being identified [53].

Apart from VGKC antibody positive patients, subgroups of patients with paraneoplastic LE have a better prognosis than patients with other paraneoplastic CNS disorders, in particular, Ma2 antibody positive patients often benefit from immunotherapy [23].

4.2.2. *Brain Stem Encephalitis*

Brain stem encephalitis is characterized clinically by gaze palsy, diplopia, reduced consciousness, and central sleep apnea [54]. This disorder is associated with breast cancer and tumors of the female genital organs [55],

thymoma [56], SCLC [36], and testicular cancer [45]. Brain stem encephalitis is sometimes an isolated PNS manifestation [57], but more often, there are signs of associated cerebellar, limbic, or cortical affection or there is a coexisting OM syndrome [58, 59].

There is considerable antigen diversity in brain stem encephalitis, with a number of associated antibodies, in particular the Ma, Hu, and Ri antibodies [60]. The Ma2 antibody is associated with brain stem dysfunction, isolated or in combination with diencephalic encephalitis or LE [45]. Vertical gaze palsy is common in these patients [58]. If testicular cancer is not found, lung cancer should be suspected in these patients [61]. One-third of the patients with Ma2 antibodies have narcoleptic-like symptoms, and immune-mediated hypocretin dysfunction has been suggested in such cases [61]. OM with Ri antibodies has traditionally been associated with breast cancer, but the most common tumor in these patients is actually SCLC [62]. SCLC is also the predominant tumor in Hu antibody positive patients with brain stem encephalitis.

The various antibodies are associated with affection of distinct levels of the brain stem. In patients with Ma2 antibodies, the cranial mesencephalon is usually affected, whereas patients with Ri antibodies usually have inflammation in more caudal parts of the mesencephalon, resulting in visual disturbances secondary to involvement of the nuclei of the third, fourth, and sixth cranial nerves (OM). In Hu antibody positive patients, brain stem encephalitis is often combined with medullary symptoms.

The prognosis of brain stem encephalitis is generally poor, although Ma2 positivity is associated with a better outcome [61].

4.3. Extrapyramidal Disorders

There are several individual and serial case reports of patients with tumors and extrapyramidal features such as chorea, ballistic movements, and dystonia [35, 63–65]. Again, SCLC is the most common associated tumor, but renal cancer and lymphoma are also reported [65]. Patients with Hu or Ma antibodies can exhibit extrapyramidal symptoms as part of the multifocal CNS involvement [65, 66], whereas CRMP-5 is probably the most common antibody associated with paraneoplastic chorea. Coexisting neuronal antibodies are found in 50% of the CRMP-5 positive cases, and accompanying symptoms such as vision loss, LE, loss of smell or taste, and peripheral neuropathy are often present [65]. Movement disorders are uncommon in CRMP-5 positive patients in general, being a clinical feature in only 15% (subacute chorea in 11%) [30]. In some patients, paraneoplastic causation is strongly suspected even when no known onconeural antibody is detected [64, 65]. Individual patients have shown clinical improvement and decline in antibody levels after chemotherapy or methylprednisolone [65, 67].

4.4. Paraneoplastic Cerebellar Degeneration

The clinical features of classical PCD are severe pancerebellar involvement, with ataxia of the limbs and trunk, dysarthria, dysphagia, and nystagmus, progressing for as much as 12 weeks and stabilizing within 6 months [14].

About half the PCD patients have antineuronal antibodies, and the antibody diversity is considerable [58]. In postmenopausal women, PCD is usually associated with tumors of the ovary or breast, and more than 90% harbor an onconeural antibody, most often the Yo antibody [55]. The clinical signs are most often consistent with isolated involvement of the cerebellum [16] or cerebellar and brain stem involvement in combination [68]. The Hu antibody is the second most common antibody in PCD, usually associated with SCLC, and in these patients, PCD is often a part of a clinical PEM/SN syndrome [52]. Other regions of the CNS are quite frequently involved in PCD patients, especially those harboring other antibodies than the Yo antibody [68]. Forty-one percent of the patients with PCD and SCLC harbor P/Q-type VGCC antibodies, even without symptoms or signs of LEMS [69]. Finally, PCD may present as part of the OM syndrome associated with Ri antibodies [32, 36, 68]. Dysarthria and nystagmus are less common symptoms in this group.

The Tr antibody is associated with PCD among patients with Hodgkin's disease. The Tr antibody positive group differs from the Yo antibody positive patients in several aspects. The patients tend to be younger, there is a male preponderance, and the onset of cerebellar symptoms usually follows rather than precedes the cancer diagnosis. Tr antibodies can be detectable in CSF alone, and usually disappear after oncological therapy [70]. The tumor does usually not express the Tr antigen, in contrast to other onconeural antigens [70, 71].

The pathological hallmark of PCD with Yo antibodies is degeneration and loss of Purkinje cells and often of granule cells as well. $CD8^+$ cells are found in the cerebellum and sometimes in the cerebral cortex, and there is diffuse microglial activation [72]. In some cases, there are changes in the corticospinal and spinocerebellar tracts and dorsal columns [42].

The irreversible neuronal loss probably accounts for the dismal functional outcome in PCD patients: 53% die from nervous system causes [68]. The median survival in PCD patients with Yo antibodies has been reported to be 13 months [68] to 22 months [73], although the survival is longer in cases associated with breast cancer than in tumors of the female genital organs (100 months vs 22 months) [55]. The Hu antibody associated cases have a particularly dismal prognosis, with a median survival of only 7 months [68]. Ri and Tr antibody positive patients usually have longer survival [68]. Patients with PCD and cancer of the female genital organs are reported to

have smaller tumor volumes than cases without PNS, but the cancer is still often advanced at diagnosis [73].

4.5. Paraneoplastic Opsoclonus–Myoclonus

Opsoclonus is a disorder of eye movement characterized by multidirectional saccades. Opsoclonus is usually associated with myoclonus, constituting OM, and there are often coexisting signs of cerebellar dysfunction and encephalopathy. The causation of OM is complex, including viral, metabolic, toxic, and structural disorders. Paraneoplastic OM was first described in children. Neuroblastoma is found in 50% of the pediatric OM cases, but on the whole, only 2–3% of all children with neuroblastic tumors have paraneoplastic OM [74, 75].

Paraneoplastic OM in adults has been described in association with SCLC, non-small cell lung cancer and carcinomas of the lung, breast, kidney, and gastric ventricle as well as malignant melanoma [62, 76]. Some patients harbor Ri antibodies, which may coexist with other onconeural antibodies [15].

Paraneoplastic OM patients without the Ri antibody frequently have heterogenous immunity to other neuronal autoantigens [77]. No associated antibody has been identified in children with paraneoplastic OM [78], but CD5+ B-cell expansion has been found in the CSF, correlating with disease severity and duration, suggesting that a humoral response takes place in these children [79].

Postmortem findings of patients with Ri antibodies include perivascular and interstitial lymphocytic aggregates in the tegmentum and basal pons, and Purkinje cell loss are found in some cases [80].

4.6. Visual Syndromes

Cancer-associated retinopathy is characterized by acute or subacute, progressive visual disturbances that affect both cone and rod function, and usually with symptoms from both eyes. The symptoms usually precede the cancer diagnosis. SCLC is by far the most common associated tumor, but cancer-associated retinopathy has also been reported in patients with non-small cell lung cancer [81], and various types of adenocarcinoma [82]. The most commonly found antibody is directed against the 23-kDa photoreceptor protein recoverin and is not specific to cancer-associated retinopathy [82, 83]. Several other antibodies have been described, and some patients are seronegative. Metastatic malignant melanoma is sometimes associated with visual loss years after the tumor diagnosis (melanoma-associated retinopathy). The serum of such patients shows various patterns of retinal antibody activity [84]. Some patients respond transiently to high doses of

intravenous methylprednisolone. The visual loss is usually not improved by oncological therapy.

Finally, visual disturbances may be a result of paraneoplastic optic neuritis, often in combination with retinitis as well as effects in other parts of the nervous system. This entity is associated with CRMP-5 antibodies and SCLC [85].

4.7. Spinal Cord Disorders

Isolated affection of the spinal cord in PNS is rare. Most often, myelitis is part of the PEM syndrome, with accompanying symptoms from other parts of the nervous system and signs of intrathecal inflammation, such as elevated protein and moderate pleocytosis in the CSF. The myelitis can involve both anterior and posterior columns and can be localized to a few segments or extend to the whole cord [42].

Acute necrotizing myelopathy is a very rare disorder characterized by low back pain, followed by subacute, ascending flaccid paraplegia, segmental loss of all sensory modalities, and sphincter involvement. Autopsy findings include, destruction of both gray and white matter, usually affecting extensive cord segments. Acute necrotizing myelopathy has been described in connection with different carcinomas, and the prognosis is poor. Patients with Hodgkin's disease may develop a similar clinical picture with intense vasculitis of the spinal cord.

Whether motor neuron disease may occur as a paraneoplastic disorder is debated. Small series show that motor neuron disease can coexist with lymphoproliferative disease, and elevated CSF protein or serum paraprotein in patients with motor neuron disease should prompt further investigation [86, 87]. In patients with lymphoma and pure lower motor neuron symptoms, the neurological course is usually relatively benign [88]. Motor neuron disease resembling amytrophic lateral sclerosis has been reported in patients with carcinoma, but in most reports, treating the malignancy has not improved the neurological symptoms, and the association is probably coincidental [89]. One exception is rapidly progressive motor neuron disease in patients with Hu antibodies, in whom subtle signs from other parts of the nervous system may be found [90, 91]. Another possible exception is women with breast cancer and symptoms resembling primary lateral sclerosis, where no antibodies have been identified [89].

4.8. Stiff-Person Syndrome

Stiff-Person syndrome is characterized by progressive rigidity, predominating in paraspinal and abdominal musculature, and superimposed, painful spasms [92]. Most cases are idiopathic, and 70% of these patients have

diabetes. Most harbor antibodies to glutamic acid decarboxylase [93]. Variants of the syndrome, involving limbs or with additional CNS signs [94], suggest a paraneoplastic background. Paraneoplastic Stiff-Person syndrome is particularly associated with breast cancer [55, 95]. Amphiphysin I, a presynaptic vesicle-associated protein, is the major autoantigen in paraneoplastic Stiff-Person syndrome [96]. The function of this protein is unknown. Patients with amphiphysin antibodies can have several different PNS but very often harbor other onconeural antibodies as well, and Stiff-Person syndrome is actually only found in a minority of amphiphysin antibody positive patients [29].

5. PNS of the Peripheral Nervous System

5.1. Sensory Neuronopathy

The SN often associated with the Hu antibody is characterized by primary damage to the nerve cell body. The patient suffers from progressive, painful sensory disturbances evolving subacutely, usually with a Rankin score of 3 within 12 weeks of the onset of symptoms [14]. Presenting symptoms include paresthesia, hypoestesia, and very often proprioceptive loss in the affected areas; sensory ataxia is common. The upper limbs are often involved [97]. The distribution of the SN is often atypical for peripheral neuropathy. The involvement is usually asymmetrical, especially at the onset of symptoms, and may affect the face or upper limbs only. SN is often the presenting symptom of the PEM/SN syndrome, but signs of CNS involvement usually evolve, and autonomic disturbances are common [36].

Some SN patients exhibit motor symptoms only, but predominant SN with accompanying motor signs is much more common [32]. This is reflected by the electrophysiological results, where sensory nerves are almost always found to be abnormal, but some degree of motor nerve affection is evident in half the patients [98, 99]. Sensory nerve action potentials should be absent in at least one of the nerves studied to fulfill the diagnostic criteria of SN [14]. An axonal and euronal pattern is the most common, in particular in the sensory nerves, but demyelinating features is detected in one-fourth of the patients [98, 100]. SN usually precedes the diagnosis of a tumor by weeks or a few months [98]. SCLC is the most common malignancy, but other tumors of the lung, or extrapulmonal cancers, are reported as well [101].

Although the sensory ganglia and roots are the primary affected area in SN, demyelination and axonal degeneration combined with inflammatory infiltrates consisting of CD8+ cells and macrophages also extend to peripheral nerves [102]. Simultaneous affection of the CNS and autonomic ganglia is common [42].

5.2. Autonomic Neuropathy

Paraneoplastic autonomic neuropathy is primarily seen with SCLC [103]. Lymphoma, non-small cell lung cancer, and ovarian cancer are also associated with autonomic disturbances [104]. Autonomic dysfunction affects 23–30% of Hu antibody positive patients [36, 98] and is the predominant symptom at presentation in up to 9% of the patients [90]. The onset of symptoms is usually subacute. A prominent clinical manifestation in patients with paraneoplastic autonomic neuropathy is gastrointestinal dysmotility and intestinal pseudo-obstruction, which can occur as part of the PEM/SN syndrome or as the sole symptom of Hu antibody related PNS. Ortostatic hypotension and erectile dysfunction are other common features [37, 105, 106]. Autonomic neuropathy is also commonly associated with the CRMP-5 antibody and have been detected in more than 30% of CRMP-5 antibody positive patients [30].

Inflammation in autonomic ganglia and infiltration of B and T cells have been demonstrated at autopsy [107], and Hu antibodies have been shown to induce neuronal apoptosis in cultured myenteric neurons [105].

Antibodies specific for neuronal nicotinic acetylcholine receptors in the autonomic ganglia have been identified in some patients with both idiopathic and paraneoplastic autonomic neuropathy, and high levels of these antibodies were correlated with more severe disease, suggesting that the antibodies may be of functional importance [108].

Finally, autonomic dysfunction is a common feature of LEMS with and without malignant disease. In LEMS patients, the most frequent symptoms are cholinergic, including dry mouth, erectile failure, constipation, blurred vision, and impaired sweating, suggesting that major involvement is the parasympathetic nerves [109].

5.3. Acute and Chronic Sensory and Motor Neuropathies

Polyneuropathy with both sensory and motor involvement is much more common among cancer patients than pure SN [83, 110, 111]. SCLC is the most common associated tumor, although other solid tumors may be found [112]. Sensory-motor neuropathy is a quite common paraneoplastic feature in patients with onconeural antibodies, especially Hu and CRMP-5 antibodies. The CRMP-5 antibody is particularly associated with SCLC and thymoma [30]. The CRMP-5 antibody binds to oligodendrocytes as well as to neurons in specific brain regions and the retina and Schwann cells of the peripheral nervous system. In accordance with this, the clinical characteristics are heterogeneous. Many patients exhibit mixed axonal and demyelinating sensory-motor neuropathy, optic neuritis, or cerebellar dysfynction [85, 113], as well as extrapyramidal symptoms (Chapter 5.3).

Onconeural antibodies are identified in only 30% of suspected paraneoplastic neuropathies [100, 114]. The clinical characteristics of seronegative

patients are heterogeneous. The clinical course of cancer-associated neuropathy can be acute, subacute, chronic progressive, or relapsing-remitting, and can thus sometimes even mimic Guillain–Barré syndrome or chronic inflammatory demyelinating polyneuropathy. This heterogeneity represents a challenge to the clinician in terms of follow-up. Antoine *et al.* [100] investigated a group of patients with carcinoma-associated seronegative paraneoplastic neuropathy and divided the patients in two groups depending on the temporal relationship between onset of neuropathy and cancer [100]. The group with onset of neuropathy occured within 2.5 years of the carcinoma (mean 7.8 months) had severe symptoms that often corresponded to a well-defined inflammatory disorder (SN, mononeuritis multiplex, Guillain–Barré syndrome, or chronic inflammatory demyelinating polyneuropathy). The group of patients with a time delay of more than 2.5 years (mean 8.4 years) had slowly progressive neuropathy and minor disability minor. In most of these cases, the association was regarded as coincidental [100]. Of the patients with idiopathic neuropathy, 7.5% have been found to be paraneoplastic [115].

Seronegative sensory-motor neuropathy with additional features such as involvement of the face and trunk, sensory ataxia, CNS signs, and inflammatory changes in the CSF have been reported among patients with breast cancer [116]. In such distinct patient populations, new target antigens will probably be identified in the future. Antibodies to gangliosides were described in patients with progressive neuropathy and malignant melanoma [117] and SCLC [118]. In the latter case, the SCLC expressed the ganglioside antigens, and there was a clonal expansion of Vδ 1 positive T cells in peripheral blood as well as in the tumor. In addition, the neurological symptoms stabilized after tumor therapy. Gangliosides are, however, not a frequent target in paraneoplastic neuropathy [119].

In lymphoproliferative disease, sensory-motor neuropathy may be a result of diffuse malignant nerve infiltration or immune-mediated mechanisms [120]. Syndromes similar to Guillain–Barré syndrome sometimes occur among patients with lymphoproliferative disease, but in this group, it is uncertain whether cancer by itself favors the development of Guillain–Barré syndrome by suppressing immunosurveillance mechanisms [121].

Cancer-associated Guillain–Barré syndrome is generally infrequent. However, some case reports describe acute onset of paraneoplastic neuropathy with bulbar and respiratory affection quite similar to the common features of Guillain–Barré syndrome. There is a male preponderance in cancer-associated Guillain–Barré syndrome, sensory impairment is often minor, and the acute mortality is reported to be much higher than in Guillain–Barré syndrome patients in general [122].

Chronic inflammatory demyelinating polyneuropathy is sometimes associated with malignancy [123]. The reported incidence of carcinoma in chronic inflammatory demyelinating polyneuropathy varies from 4 to 10% [100, 124].

Elderly patients with chronic inflammatory demyelinating polyneuropathy and a subacute onset and poor response to treatment are particularly prone to harboring an underlying malignancy [125].

5.4. Neuropathy Associated with Paraproteinemic Disorders

Neuropathies associated with monoclonal gammopathy or paraproteinemia constitute a very heterogenous group that comprises patients with Waldenström's macroglobulinemia, cryoglobulinemia, multiple myeloma, osteosclerotic myeloma, and monoclonal gammopathy of unknown significance (MGUS) [126]. A monoclonal protein has been found in as many as 10% of patients with idiopathic peripheral neuropathy [127]. The relationship between the monoclonal protein and the peripheral nerve damage is uncertain in many of these diseases, and paraproteinemic neuropathy is often not regarded as paraneoplastic. We will, however, briefly mention two subgroups of specific importance.

Peripheral neuropathy occurs in about 16% of patients with MGUS, being particularly common in those with an IgM monoclonal gammopathy [128]. The IgM fraction of such patients includes antibodies to myelin-associated glycoprotein (MAG antibodies) that bind to determinants in peripheral nerve myelin, and, as shown by animal models, the antibodies mediate the myelin injury and secondary nerve damage [129]. The patients usually have a distal symmetric neuropathy, usually with predominant sensory affection, and the neurological symptoms frequently precede the detection of gammopathy [130].

Peripheral neuropathy is particularly common in osteosclerotic myeloma, affecting one-third to one-half of the patients [131]. These patients often have unusual combinations of symptoms affecting multiple organs, the POEMS syndrome. The acronym POEMS refers to the dominant clinical features (*p*olyneuropathy, *o*rganomegaly, *e*ndocrinopathy, *M* protein, and *s*kin changes). The peripheral neuropathy and the monoclonal plasma cell-proliferative disorder are the major criteria of POEMS, whereas combinations of the other three main features, as well as others, may present during the course of disease [132]. The neuropathy is usually distal, symmetric, and progressive with a proximal spread, and involves both motor and sensory nerves. The response of POEMS patients to oncological therapy is often very good and the prognosis is much better than in multiple myeloma. It is thus of great importance to distinguish POEMS with osteosclerotic myeloma not only from multiple myeloma but also from MGUS neuropathy and CIDP, as the therapy differs considerably [131]. Specific antibodies to nerve or myelin components have not been identified in POEMS, but as the neuropathy often improves within 3–6 months after cancer therapy, a causal relationship seems highly likely.

6. Paraneoplastic Nervous System Syndromes of the Neuromuscular Junction

Myasthenia gravis, LEMS, and neuromyotonia affect the neuromuscular junction. These disorders are not necessarily associated with malignancy but are sometimes associated with tumors and are regarded as PNS in these patients.

Eighty-five percent of patients with myasthenia gravis have antibodies to the acetylcholine receptor [133], but only 10–15% of patients with myasthenia gravis have a thymoma. LEMS is much more commonly associated with cancer than myasthenia gravis. LEMS affects individuals in mid to late life, begins insidiously and typically presents as proximal muscle weakness and generalized fatigue, with a sparing of the ocular and bulbar muscles. Autonomic dysfunction is more frequent than in myasthenia gravis, and dry mouth is a particularly common symptom. Cancer is found in about 60%, usually diagnosed within 2 years of the onset of neurological symptoms [134]. The most common malignancy is SCLC. Smokers who are HLA-B8 negative are particularly prone to harboring SCLC, but interestingly, the HLA-B8 haploltype is also correlated with an improved prognosis when present in LEMS patients with SCLC [135, 136]. LEMS patients with SCLC sometimes have other PNS, in particular PCD [137]; in some patients, the neuromuscular symptoms respond to therapy, whereas the other PNS does not [31].

Neuromyotonia, or Isaacs' syndrome, can occur as a paraneoplastic manifestation [138]. In a study of 60 patients with neuromyotonia, 8 individuals developed lung cancer or malignant lymphoma [139]. Importantly, and in contrast to most other PNS, the tumors presented after the symptoms of nerve hyperexitability, in some cases up to 4 years later. This long latency has also been reported in LEMS with SCLC [140].

VGKC antibodies can be present in paraneoplastic neuromyotonia. However, VGKC antibodies are probably much more common in nonparaneoplastic disease (nonparaneoplastic LE, neuromyotonia, and Morvan's syndrome) [51, 139, 141].

7. Diagnosis of Paraneoplastic Nervous System Syndromes

7.1. Diagnostic Criteria

The diagnostic considerations in PNS are complex and hardly straightforward. Problematic issues include antibody positivity vs negativity, patients with no detectable tumor and the ongoing characterization of new syndromes as well as new antibody specificities. Guidelines of the diagnostic criteria of PNS have been established by an international network of

neurologists, in an effort to clarify and outline the nomenclature and criteria for PNS [14]. According to these guidelines, nervous system syndromes can be defined as definite or possible PNS. The guidelines recognize certain syndromes as classical PNS (PEM/SN, PCD, limbic encephalitis, opsoclonus–myoclonus, SN, chronic gastrointestinal pseudo-obstruction, LEMS, and dermatomyosisits) and specific antibodies as well characterized (Hu, Yo, Ri, CV2, Ma, and amphiphysin antibodies), and base the definitions of definite or possible PNS on this.

The diagnosis of PNS is regarded as definite among:

1. Patients with a classical syndrome and cancer that is diagnosed within 5 years of onset of neurological symptoms
2. Patients with a nonclassical syndrome that resolves or significantly improves after cancer treatment without concomitant immunotherapy (unless spontaneous remission is known to occur)
3. Patients with neurological symptoms and onconeural antibodies (well characterized or not) and cancer developing within 5 years of the onset of symptoms
4. Patients with a neurological syndrome (classical or not) and a well-characterized onconeural antibody, but no cancer

This classification allows for seronegative patients to be classified as having PNS (no. 1). In addition, the set of definitions takes into consideration that new antibody specificities are still being characterized (no. 3). Different clinical subtypes are still being described in association with well-known antibodies, and such cases are also included. Finally, a negative screening for malignancy does not exclude PNS, as spontaneous remission is known to occur. As cancer is ultimately detected in the majority of patients with symptoms and a well-characterized antibody, the number of false positives is probably low (no. 4).

The criteria also include three groups in whom PNS diagnosis is regarded as possible:

1. Individuals at high risk of cancer with a classical syndrome, but without onconeural antibodies
2. Patients with a neurological syndrome and a less well-characterized antibody but no cancer
3. Patients with and nonclassical neurological symptoms, no onconeural antibodies, but where cancer is detected within 2 years of the onset of symptoms

Thus, the clinically important group comprising seronegative patients with peripheral neuropathy and an associated cancer can be regarded as possible

PNS. The time delay of 2 years is based on studies showing that an underlying malignancy will usually be manifested within this period [100, 115].

The guidelines aim to increase the accuracy with which PNS are classified. However, the guidelines also emphasize the clinical impact of antibody positivity, as well as the importance of the time span between neurological symptoms and cancer. These are aspects of great practical interest for clinicians handling patients where PNS is a possibility.

7.2. Paraclinical Investigations

Numerous different diagnoses should be considered in the clinical management of a patient with suspected PNS [12]. This suspicion is usually raised if the patient has symptoms that are consistent with a classical PNS or if there are unusual clinical nervous system manifestations. Weight loss, persistent cough, or abnormal blood tests in a patient with unexplainable neurological disease are other signs that should alert the clinician. As PNS manifests after the cancer diagnosis in about 30% of the patients, the onset of neurological symptoms in an individual with an acknowledged cancer disease that are not attributable to therapy effects or metastasis should lead to investigation for PNS. These patients usually undergo multiple diagnostic procedures: analysis of serum and CSF, radiological examinations, neurophysiological tests, and targeted investigations for a primary tumor (gynecological examination, mammography, and so on).

CSF abnormalities are very common in PNS (slight–moderate pleocytosis, elevated protein, and oligoclonal bands on isoelectric focusing) [142] and are reported in most patients with paraneoplastic LE [44, 143], paraneoplastic neuropathies with onconeural antibodies [100], and PCD [144]. Normal CSF does not exclude the diagnosis of PNS.

Magnetic resonance imaging of the CNS may show signs of pathology in some of the syndromes, such as in LE, in which 80% of the patients have an increased T2 signal [43, 44], or atrophy on T1-weighted sequences [143] of one or both medial temporal lobes. Magnetic resonance imaging abnormalities have been detected at onset of PNS in brain stem encephalitis [57] and in cortical encephalitis [33]. In other syndromes, as PCD and POM, radiological investigations are often normal at onset of disease and are therefore of limited diagnostic value [62, 145, 146].

Electroencephalography usually shows focal or generalized pathology and epileptic activity among patients that have LE [44] or encephalomyelitis with epileptic manifestations [33, 34]. Electrophysiological studies are important in investigating paraneoplastic peripheral neuropathy, including the degree of sensory vs motor nerve involvement, axonal or demyelinating features, and neuronopathy [98, 100, 147].

Neurological symptoms and signs consistent with PNS in a patient without known cancer should catalyze a thorough and individualized search for malignancy. Tumors of the lung or abdominal organs that are derived from tissues of neural crest cell origin are more likely to be associated with PNS. In adults, the most common are tumors of the lung, female genital organs, and lymphoreticular system, whereas in children, the dominant tumor types are neuroblastoma and ganglioneuroblastoma. As the tumors are often very small, negative results do not exclude PNS, and investigations should be repeated at regular intervals (we suggest 3–6 months) in patients with definite or possible PNS without a tumor.

When conventional imaging fails to identify the cancer, [18F]Fluorodeoxyglucose positron emission tomography (FDG-PET) is highly useful. FDG-PET has 80–90% sensitivity in detecting tumors among patients with PNS and an onconeural antibody vs 30% using computer tomography [148, 149]. If available, the FDG-PET is thus the preferred mode of investigating patients with suspected PNS, in particular in identifying small lung tumors or mediastinal lymphadenopathy [150] and when a suspected tumor is not available for biopsy [148]. False negative results are rare in FDG-PET but may correspond to tumors with a diameter of less than 6–8 mm. Thus, a negative FDG-PET scan in a patient with an onconeural antibody or strongly suspected PNS should lead to repeated investigation [151]. The sensitivity and specificity is lower in patients without onconeural antibodies [148, 150, 151]. Complementing FDG-PET by computer tomography scanning further increases the sensitivity and accuracy of tumor diagnosis [149, 152]. High-resolution computer tomography is an alternative diagnostic procedure, but this technique is less sensitive and specific than FDG-PET [149].

In some patients, explorative procedures may be warranted to ensure cancer diagnosis; one example is explorative lapratomy in postmenopausal women with PCD and Yo antibodies [153]. Sometimes, the tumor is only found at autopsy or not at all. The fact that the latter may represent an example of successful tumor immunity is intriguing, emphasizing the possible importance of PNS as a model to understand immune mechanisms in cancer [154].

7.3. Detection of Onconeural Antibodies

Most PNS antibodies can be detected using immunohistochemistry performed on cryostat sections of rat nerve tissue, usually snap frozen in isopenthane [52, 155, 156] or fixed in formaldehyde for detection of CRMP-5 antibodies [52]. If the patient serum produces a staining pattern in nervous tissue consistent with an onconeural antibody at a dilution of 1:500 or more, this is usually regarded as positive [157] (Fig. 1). The specificity of

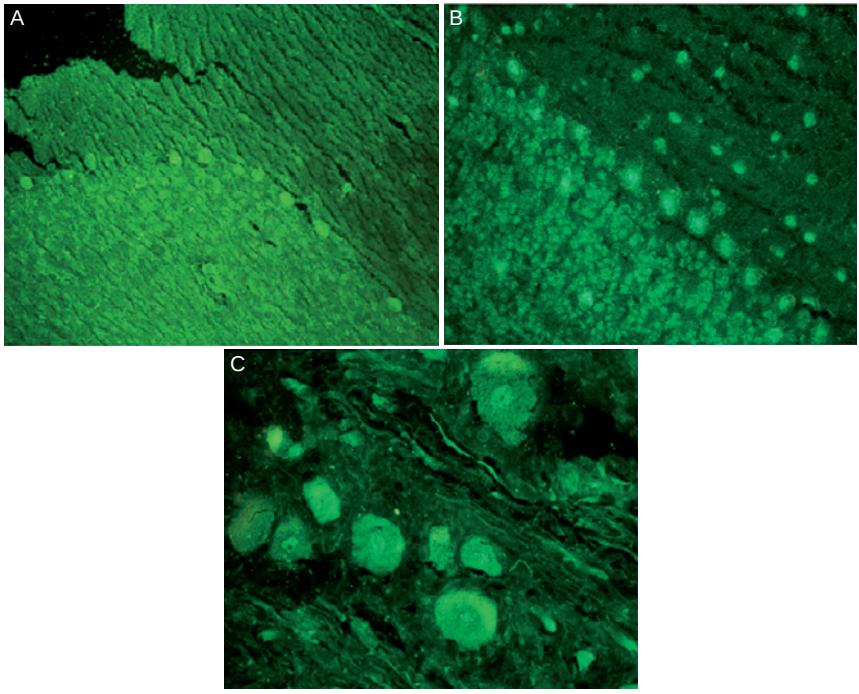

FIG. 1. Examples of positive immunostaining. (A) Cytoplasmic staining of rat Purkinje cells by human serum diluted 1:1000, from an individual with paraneoplastic cerebellar degeneration and Yo antibodies. (B) Serum from an individual with paraneoplastic encephalomyelitis and Hu antibodies, diluted 1:500, showing nuclear staining of rat Purkinje cells. (C) Serum from the same individual as in (B), staining the neuronal nuclei of dorsal ganglion from rat, with nucleolar sparing.

the antibodies detected by immunohistochemistry must be confirmed by immunoblotting against neuronal extracts, or, preferably, recombinant proteins [157, 158]. The Tr antibody is detectable by immunohistochemistry alone and not by immunoblotting [71], possibly because this antibody is directed to a conformational epitope that is only present in tissue sections.

The antibodies are usually found in the CSF as well as in the serum, and there is often evidence of intrathecal antibody production, that is a CSF: serum antibody ratio exceeding 1 [159, 160], and CSF oligoclonal bands [144]. Serum analysis of onconeural antibodies is regarded as sufficient for practical clinical purposes [157], except for the Tr antibody, which is sometimes detectable in the CSF only [70].

Positive immunohistochemistry must be confirmed by immunoblotting, as immunohistochemistry alone will result in an unacceptable number of

false positive as well as false negative results. Antinuclear antibodies may in particular produce neuronal staining that can be misinterpreted as positivity for Hu or Ri antibodies [158]. The collaboration of different laboratories to ensure quality assurance in analyzing onconeural antibodies is strongly recommended [161].

Enzyme-linked immunosorbent assay is used for detecting antibodies to recoverin [162] and has also been employed to detect Hu antibodies [163, 164].

The use of an immunoprecipitation method has been reported for detecting Hu, Ri, and Yo antibodies [165, 166]. This method is highly specific and sensitive, as it can detect low levels of antibodies not demonstrated by immunohistochemistry or immunoblotting, and is useful in prevalence studies. However, low levels of antibodies are often not correlated with clinical disease and must be regarded with caution in patients without neurological symptoms. For instance, 25% of patients with SCLC have low levels of Hu antibodies [167]. There are also case reports of individuals with cancer and high antibody levels without PNS, even after prolonged observation [166, 168].

The most common onconeural antibody in patients with PNS is Hu, followed by CRMP-5 and Yo, whereas PCA-2, amphiphysin, ANNA-2, and ANNA-3 seem to be quite rare. Apart from Yo antibody positive sera, 31% of PNS serum samples have been found to have more than one onconeural antibody, which emphasizes the importance of a broad diagnostic antibody screening [16].

7.4. Seronegative Paraneoplastic Nervous System Syndromes

In all the PNS, some patients are seronegative for onconeural antibodies. The percentage of seronegative individuals varies but is particularly high in heterogenous subpopulations such as patients with sensory-motor neuropathy. Some patient groups with more homogenous features almost always harbor an onconeural antibody, such as postmenopausal women with PCD, where Yo antibodies are found in 90% of the patients [55]. The practical implication of seronegativity is that a PNS cannot be excluded even if testing for onconeural antibodies is negative. As discussed previously, if a patient exhibits the features of a classical PNS and cancer is diagnosed within 5 years of the onset of neurological symptoms, the PNS is regarded as definite even in the absence of an onconeural antibody [14]. However, identification of distinct subpopulations of patients with similar symptoms and screening of cDNA expression libraries by patient sera still leads to the identification of new types of antibody reactivity [169], and this suggests that the seronegative group will diminish further in the years to come.

8. Pathogenesis of Paraneoplastic Nervous System Syndromes

8.1. Tumors

With the exception of the Tr antigen, onconeural antigens are invariably expressed in the tumors of patients with antibody-verified PNS, and failure to find the relevant antigen expressed in a tumor from such patients should prompt investigation for a second tumor [14]. However, onconeural antigens are also expressed in tumors from patients without onconeural antibodies and with no signs of nervous system disease. In particular, tumors derived from neuroendocrine cells express the Hu antigen. Thus, 100% of SCLC tumors [170] and 50–78% of neuroblastomas [170, 171] express the Hu antigen. Tumors of non-neuroendocrine origin can also express the Hu antigen, as demonstrated by a study of extrathoracic non-small cell lung cancer associated with PEM/SN [32]. Cdr2 is expressed in 60% of ovarian tumors and 25% of breast tumors [172, 173].

Many SCLC patients mount an antitumor immune response without signs of PNS [9, 167, 174]. Nevertheless, most SCLC patients do not have this immune response. Thus, the rarity of PNS cannot be attributed to infrequency of antigen expression. This discrepancy suggests that additional factors, perhaps related to tumor major histocompatibility complex (MHC) expression, contribute to the initiation of the PNS immune response. A study of Hu antigen and MHC class I expression in SCLC and neuroblastoma supports this theory. Seventeen of 20 tumors from Hu antibody positive patients expressed both proteins, but only 4 of 30 specimens from seronegative individuals expressed both proteins [171]. Altered expression and/or down-regulation of MHC molecules is a common immune-evasive strategy of tumor cells [175].

The onconeural antigen is not necessarily expressed uniformly throughout the tumor. The cdr2 antigen has been detected in only 10% of the cells in tumors from PCD patients [176]. In one PCD case, the cdr2 antigen was expressed by a primary tumor, but not by its metastasis [177].

Cellular infiltrates are found in the tumors from patients with PCD [153, 178] and PEM/SN [90]. These infiltrates may contain CD8+ T cells [114] but are often predominantly composed of plasma cells [153]. Otherwise, the features of tumors from patients with PNS as determined by histological investigation do not differ from those of individuals with a normal nervous system. Moreover, the Hu antigen is not mutated in tumors from PEM patients [179].

The tumors found in PNS are generally small and sometimes occult. Several reports indicate that PNS patients have prolonged survival compared

with other cancer patients [11, 74], and this suggests that the immune reponse in PNS may benefit the cancer prognosis. Improved survival in SCLC patients with low titers of antibodies is probably not related to the presence of Hu antibodies alone [9, 167], and this warrants further studies of the immune responses, especially cellular activation, in such populations.

8.2. Humoral Immune Response

Several criteria have to be fulfilled for an autoimmune disorder to be regarded as antibody mediated: demonstration of a specific antigen; the reproduction of disease in recipient animals on transferral of antibodies; the induction of autoimmune disease by active immunization with antigen; and clinical improvement or stabilization of the symptoms when antibodies are removed by immunotherapy or plasmapheresis [114]. Most antibodies found in PNS only satisfy the first criterion. However, there are some exceptions. The prime example is LEMS, where the VGCC antibodies interacting with presynaptic calcium channels are pathogenic. However, these antibodies are not markers of a paraneoplastic background for LEMS [180]. Another example is the recoverin antibody that is present in some patients with CAR. This antibody is incorporated into rod photoreceptor cells and is pathogenic *in vitro* as well as *in vivo* [181]. Antibodies to mGluR1 that are found in some patients with Hodgkin's disease and cerebellar ataxia also have functional effects when transferred to mice but do not yet fulfill all the criteria above [182].

Antibodies directed to antigens that are components of cell surface receptors are often pathogenic *in vivo* (such as ion channel antibodies and, possibly, mGluR1 antibodies) [95, 182, 183]. The pathogenic relevance of antibodies to intracellular targets is much more controversial, but antinuclear antibodies are reported to be pathogenic in some diseases such as systemic lupus erythematosus [184].

Numerous studies have tried to elucidate the role of onconeural antibodies in other PNS. Immunoglobulin G (IgG) containing Hu antibodies penetrated viable neurons expressing the Hu antigen in *in vitro* experiments [185]. When the IgG fraction of Yo antibody positive sera is administered intraperitoneally in laboratory animals and the blood–brain barrier is disrupted, intracellular IgG can be demonstrated in Purkinje cells [186]. Intrathecal antibody production takes place in many of the PNS [159, 187], and autopsy studies of affected individuals show deposits of IgG in neurons [80, 188, 189] and in the tumor [189]. These results all favor a pathogenic role for the onconeural antibodies.

Passive transfer of the IgG fraction from a patient with breast cancer, Stiff-Person syndrome and amphiphysin antibodies resulted in stiffness and

spasms in Lewis rats primed with activated T lymphocytes to open the blood–brain barrier [190]. Binding of IgG was demonstrated in CNS of symptomatic animals, strongly suggesting that, in this case, antibodies alone were sufficient to cause disease [190]. As amphiphysin is presumed to be located in the cytosol and not at extracellular locations, the study favored a pathogenic role of antibodies directed to intracellular targets. However, one cannot conclude whether the disease was caused by amphiphysin antibodies or by other antibodies of the IgG fraction. This question remains unanswered in another report of IgG-mediated cytotoxicity by PNS sera in cell cultures [191], as well as in a study in which PNS IgG induced the expression of cell adhesion molecules and accelerated neuronal differentiation in mouse brain-derived neurons *in vitro* [192].

Yo antibody positive PCD sera bind the cdr2 leucine zipper domain and abrogate the ability of cdr2 to interact with c-myc, an interaction that may trigger neuronal apoptosis [193]. This model provides a pathway by which the Yo antibodies may interfere directly with essential intracellular functions, but their relevance in the human disease is still uncertain.

However, there are several arguments against a primary pathogenic role of the onconeural antibodies that are directed to intracellular antigens. Neither passive transfer of Hu or Yo antibodies [194, 195] nor active immunization with HuD or cdr2 [196, 197] have resulted in clinical or pathological signs of disease in animals, but no animal model has succeeded in reproducing the continous intrathecal synthesis of antibodies that are characteristic of the human syndromes [198]. Serum and purified IgG containing Hu antibodies had a toxic effect on Hu-expressing cell lines, but the Hu antibodies did not mediate this toxicity [199]. In a mouse model of neuroblastoma, HuD DNA immunization resulted in tumor growth inhibition but did not induce neurological disease [200]. Another report stated that Hu antibodies evoked neuronal apoptosis in cultured myenteric neurons, and this apoptosis could contribute to enteric nervous system impairment underlying paraneoplastic gut dysmotility [106]. Removal of the antibody response by plasmapheresis does not usually ameliorate symptoms in affected individuals [198], whereas this therapy improves symptoms in proven antibody-mediated diseases as LEMS [201].

Finally, other factors but the onconeural antibodies may predispose an individual to neuronal damage or contribute to inflammation. We have reported lack of expression of the complement regulator protein CD59 on the surface of Purkinje cells in individuals with PCD, indicating vulnerability to the effects of complement activation [202]. Complement depositis have been found in the brain stem of a patient with paraneoplastic OM [80]. Others have found absent or weak complement deposition in postmortem studies of the brain of PEM/SN patients [41, 203, 204], suggesting that

complement activation is not a major factor contributing to the pathogenesis in this subgroup.

8.3. Cellular Immune Response

Various lines of evidence support T-cell activation as an important part of the pathogenesis of PNS. First, the pathological hallmark of PNS is neuronal degeneration in combination with inflammatory infiltrates [203, 205]. Perivascular infiltrates contain mainly CD4+ T cells, whereas CD8+ T cells dominate in the parenchymal infiltrates [41, 80, 203]. Even in PCD patients, among whom T-cell infiltrates have been difficult to demonstrate, these have been detected in the early stages of disease [72, 178]. CD20+ B cells, however, have only been observed in the perivascular infiltrates in PEM/SN [41]. In addition, white blood cells usually increase in the CSF of affected individuals in the first stage of disease [153, 198]. In PCD, most of these cells are $\alpha\ \beta$ T cells, and 40% of the T cells are activated, indicating an ongoing T-cell-mediated immune response [206].

Second, expanded populations of cdr2-specific T cells with the capacity to lyse target cells presenting the Yo antigen *in vitro* have been found in the blood and CSF of PCD patients [206–208]. Hu-specific cytotoxic T cells have been identified in the blood of patients with PEM/SN [209]. T-cell receptor analysis in brain tissue from Hu antibody positive patients have revealed oligoclonal expansion of cytotoxic T lymphocytes, but their antigen specificity has not been determined [210]. Although reproducing PNS in animal models has been very difficult, subclinical encephalomyelitis by Ma-specific T cells has been successfully induced in rats [211].

How the onconeural antigens are presented to the immune system is still unknown. Outside the CNS, dendritic cells are thought to play an essential part in the presentation of tumor antigens [7]. Antigen presentation within the CNS is necessary for reactivation of T cells that have crossed the blood–brain barrier. Neurons have been thought to lack expression of MHC class I molecules, necessary for recognition by CD8+ T cells, but this dogma has been challenged [212]. The normal level of MHC I expression is nevertheless low or absent, but MHC class I upregulation by local cytokines could result in presentation of intracellular peptides and engage PNS-specific T cells effectively [19, 213], and few MHC class I molecules are required to sensitize a target cell for lysis by class I-restricted cytotoxic T lymphocytes [214].

Autoantigen-specific CD8+ T cells may mediate autoimmune injury in the CNS [215], and cytotoxic T lymphocytes can attack and injure neurons [208, 210, 216, 217]. In a study of the effects of T cells specific for the onconeural antigen PNMA1 of the Ma family, transfer of CD4+ T cells to rats resulted in a perivascular inflammatory response that involved CNS regions affected

in human disease. However, the animals had no neuronal degeneration and no clinical signs of disease, and the authors suggested that selective neuron loss involves interplay between CD4+ and CD8+ T cells specific for an onconeural antigen [211].

Experimental evidence suggests that the role of cytotoxic T-lymphocyte-mediated tumor immunity in patients with PNS and Yo antibodies is limited, as CD8+ CTLs were found in only two of nine tumors from PCD patients [218]. Suppression of the T-cell response in PCD by tacrolimus has not led to tumor recurrence, but only short cycles of treatment have been administered [206].

The Darnell group [7] has proposed an interesting theory regarding the pathogenesis of PNS. CD4+ T cells and CD8+ T cells specific for a tumoral antigen, in this context an onconeural antigen, are activated by the mechanism of cross-presentation (Fig. 2). Systemic B cells are activated by the CD4+ T cells, initiating production of onconeural antibodies, some of which cross the blood–brain barrier. Antibodies to PNS antigens have been found inside neurons in animal models [195], in cultured neurons exposed to PNS sera [185] and in autopsy of affected individuals [188], indicating that

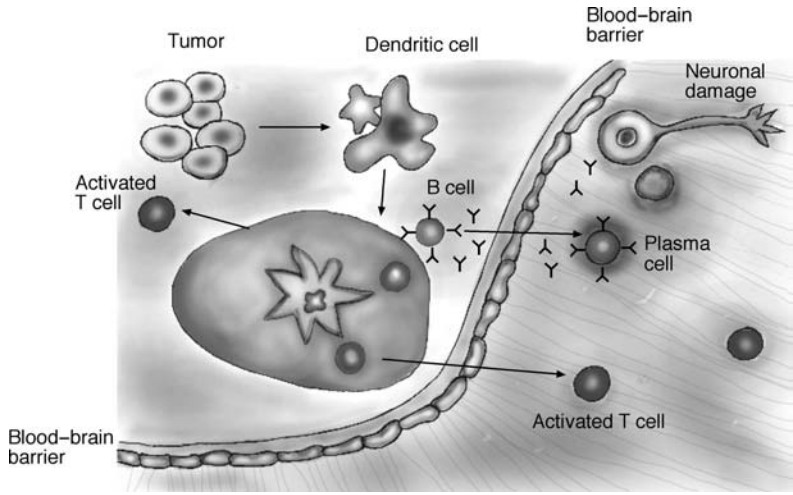

FIG. 2. The proposed mechanism of PNS pathogenesis. A growing tumor expresses onconeural antigens. Dendritic cells engulf apoptotic tumor cells and migrate to the local lymph node, where they present the tumoral antigens to the immune system. This mode of activation is termed cross-presentation and leads to activation of T and B cells. Activated B cells differentiate into plasma cells and produce onconeural antibodies. Antibodies and cytotxic T lymphocytes act back on the tumor, perhaps limiting tumor growth. Cytotoxic T lymphocytes and plasma cells also cross the blood–brain barrier and target normal neurons, causing inflammation, neuronal damage, and ultimately cell death.

antibodies are internalized. Once internalized, antibodies that target functional epitopes on intracellular PNS antigens may trigger neuronal apoptosis by interfering with essential intracellular processes [7]. This mechanism is probably insufficient to cause the widespread neuronal loss in many of the PNS. However, resident antigen-presenting cells in the CNS could then cross-present antigens of apoptotic neurons, stimulating CNS T cells. Cytokine production by the responding T cells can amplify the process by promoting blood–brain barrier permeability, upregulation of MHC class I expression, and recruitment of additional T cells [7, 19].

9. Therapy for Paraneoplastic Nervous System Syndromes

There are two approaches to treating PNS: eradicating the source of the antigen, that is, the tumor, through surgery, radiotherapy, or chemotherapy; and modulating the immune response such as by administering corticosteroids, intravenous immunoglobuline or azathioprine, or depleting IgG (plasma exchange) [219, 220]. The single most important factor in managing patients with PNS is identifying and treating the associated tumor as early as possible, but even with complete tumor remission, the PNS respond to varying degrees [12, 221]. Nevertheless, case reports indicate neurological improvement following chemotherapy and radiotherapy, and complete regress of tumor is associated with a more favorable course of PEM [10] and longer survival in PCD [68]. Patients with syndromes affecting the CNS usually benefit less from oncological therapy than patients with peripheral nerve syndromes as SN, possibly due to the fact that the CNS syndromes evolve rapidly, and at the time the PNS is correctly diagnosed and treatment is instituted, the neuronal damage is already irreversible. There are some notable exceptions to this rule. Patients with LE sometimes improve after cancer therapy, regardless of antibody and type of associated tumor [43]. Some patients with Ma antibody-associated syndromes respond well to oncological therapy and immunomodulation [23], and in patients with Tr antibodies, cerebellar dysfunction may resolve after tumor therapy [70, 222].

Whereas idiopathic OM often resolves, spontaneously or after administration of intravenous immunoglobuline or corticosteroids [58], the outcome of paraneoplastic OM is more variable and depends on the tumor response to therapy. Children with paraneoplastic OM frequently respond to chemotherapy, adrenocorticotropic hormone, or immunomodulation [223]. The response to immune therapy in adults with paraneoplastic OM is very modest, but prompt tumor therapy, immunomodulation, and depletion of IgG may be of some benefit [62, 224]. Without antineoplastic therapy, the prognosis is usually poor, and symptoms often progress, ultimately causing death [62].

The evidence of an autoimmune pathogenesis supports the use of immunomodulatory therapy in PNS. If therapy is started at an early stage of the disease, the chances of neurological improvement are better, both with intravenous immunoglobuline alone [225] and with plasmapheresis and chemotherapy in combination even if no tumor has been diagnosed [226].

The anti-CD20 monoclonal antibody rituximab induces a long-lasting depletion of B cells in peripheral blood and has been used for other autoimmune disorders. The effect of this therapy in PNS patients has solely been investigated in uncontrolled trials. Although some patients have been reported to respond, most do not [227]. In general, there is no decline in antibody levels after rituximab therapy [68, 227]. The contribution of rituximab to the improvement in some patients is uncertain, as the importance of B-cell activation in the pathogenesis of PNS has not been proved. Inhibition of T-cell activation might hold more promise, and tacrolimus, a specific inhibitor of activated CTL, has been used in treating a few PCD patients. Tacrolimus effectively reduced the number of cdr2-specific cytotoxic T lymphocytes in the blood and CSF but did not influence the neurological symptoms [206].

The prognosis is somewhat better for PNS of the peripheral nervous system. This may be due to differing susceptibility of autoreactive B and T cells, differences in the permeability of the blood–brain and blood–nerve barriers, and a higher degree of regeneration of axons and glial cells in the peripheral nervous system than in the CNS. Some patients with SN respond to steroids or intravenous immunoglobuline [228] and to chemotherapy or radiotherapy [229] by stabilizing or sometimes even improving their neurological symptoms. In patients with a combination of peripheral and central involvement, the former sometimes responds to therapy, whereas the latter do not [222]. Even taking these aspects into consideration, the majority of patients with peripheral disorders do not respond much to therapy. The frequent association with PEM and the occurrence of Hu antibodies suggest a clinical continuum and shared pathogenic mechanisms. Individuals with less severe disablement at the onset of therapy have a better chance of improvement than severely disabled ones [230].

As a general rule, patients with PNS of the neuromuscular junction benefit considerably from cancer therapy. Immunotherapy of paraneoplastic LEMS, such as prednisone or plasmapheresis, can improve the symptoms for a while as the pathogenic VGCC antibodies are removed, but effective treatment of the underlying tumor is the cornerstone of therapy [201, 231].

Some PNS cases run an indolent natural course [232], or the PNS may improve spontaneously [233]. Spontaneous remission should always be considered when patients seem to improve by oncological therapy or immunomodulation.

10. Future Aspects

PNS research has progressed considerably during the past 20 years. The classification of separate disease entities and the improved diagnostic possibilities offered by antibody detection have contributed much to the increased clinical awareness of PNS. Progress made in the research of these diseases has not only elucidated the underlying mechanisms but has also brought new insight into tumor immunology in general. Nevertheless, many questions remain unanswered. The pathogenesis of the PNS and the effects of the immune reponses on a growing tumor are still uncertain. The PNS represents a therapeutic challenge, and new treatment strategies need to be designed. New antibody targets will be identified in the future, and the presence of ion channel antibodies in both paraneoplastic and nonparaneoplastic disease is of great interest. Finally, an unresolved issue is why so few patients develop PNS, although tumors frequently express onconeural antigens. Hopefully, future research can expand knowledge of the probable dysregulation of the immune system in these individuals and yield insight that can be applied not only to PNS but also to other autoimmune diseases.

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PATHOPHYSIOLOGICAL MECHANISMS OF ANGIOGENESIS

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Abbreviations

Ang	angiopoietin
Bcl-2	B-cell leukemia/lymphoma 2
CCL	chemokine ligand
CGRP	calcitonin gene-related peptide
CRLR	calcitonin receptor-like receptor
COX-2	cyclooxygenase-2
CTGF	connective tissue growth factor
eNOS	endothelial nitric oxide synthase
Erk	extracellular receptor kinase
FAK	focal adhesion kinase
FGF	fibroblast growth factor
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
NK_1	neurokinin-1
PD-ECGF	platelet-derived endothelial cell growth factor
PDGF	platelet-derived growth factor
PEDF	pigment epithelium-derived factor
PI3K	phosphatidylinositol 3′-kinase
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKB	protein kinase B
PLA_2	phospholipase A_2
PLCγ1	phospholipase C-γ1
PKC	protein kinase C
RAMP	receptor activity-modulating protein
SH-PTP1	protein tyrosine phosphatase
TIMP	tissue inhibitor of metalloproteinase
TK	tyrosine kinase
VEGFR	vascular endothelial growth factor receptor
VEGF	vascular endothelial growth factor

1. Abstract

The growth of new blood vessels may be either beneficial or harmful. The angiogenic process may be measured by a variety of techniques, although it may often be the quality rather than quantity of resulting blood vessels that determines function. Endothelial cells play a key role in the initiation of angiogenesis, and vascular endothelial growth factor (VEGF) may be viewed as a prototypical direct-acting angiogenic factor. VEGF acts through

multiple cell surface receptors and signaling pathways to stimulate endothelial cell proliferation, survival, and migration. By inducing other growth factor expression, VEGF stimulates a cascade of angiogenic activity.

Different tissues may utilize various angiogenic pathways that are modulated by diverse host tissue responses. Furthermore, a single tissue may progress through a sequence of angiogenic pathways, for example, as acute injury progresses to chronic inflammation. The phenotype of the resulting neovasculature is critically dependent on the context in which it is formed.

Biomarkers of angiogenesis are being developed as an aid to assessing human disease. Histological assessment of vascular density and angiogenic factor expression, *in vivo* imaging, Doppler ultrasound, and biofluid assays each may have clinical utility. Therapeutic targeting of angiogenesis will depend both on the generation of acceptable pharmacological agents and on the identification of patients who may and do gain benefit from such treatments.

2. Introduction

Blood vessels are essential for the life of higher organisms, transporting nutrients and waste products, communicating endocrine signals, and conveying the cellular components of the immune response. Failure of vascular growth is embryonically lethal, and robust mechanisms have evolved to ensure that blood vessels grow appropriately and adequately. Given the ancient origins of the blood vasculature in evolution, it is not surprising that mechanisms for the regulation of vascular growth have diverged between species and tissues, creating layer on layer of molecular pathways that enhance or inhibit vascular growth and survival. Some mechanisms of vascular growth may be common to all tissues and species at all times, whereas others may be unique to a specific tissue or a particular physiological or pathological process. In order to take full advantage of this highly complex system, we must not only understand what may cause vessels to grow but rather what does and when and where.

2.1. Angiogenesis, Vasculogenesis, and Lymphangiogenesis

Angiogenesis has been defined as the growth of new blood vessels from preexisting ones. This distinguishes it from vasculogenesis where new blood vessels develop *de novo*, and lymphangiogenesis which represents the growth of new lymphatics. Vasculogenesis traditionally has been seen as uniquely occurring in the embryo. Pluripotential mesenchymal stem cells are coordinated in the early embryo to form the endothelium of dorsal vascular structures prior to the development of blood circulation. More recently, the

existence of stem cells even in adult blood has received much research attention due to their potential capacity to regenerate tissues that would otherwise be terminally differentiated and nonreplaceable. Recognition of the potential of these albeit scarce cells raises again the possibility that vasculogenesis may occur in the adult [1].

Bone marrow-derived endothelial progenitor cells are mobilized through the activation of metalloproteases and the remodeling of extracellular matrix within the bone marrow. High plasma levels of the vascular endothelial growth factor (VEGF) family of growth factors promote this process [2]. Studies with transplanted or grafted tissues confirm that vessels that are detached from the circulation may link up with perfused blood vessels to reestablish circulation [3]. This raises the possibility that isolated islands of vascular tissue arising by vasculogenesis may contribute to expansion of the perfused vascular bed. This contribution of endothelial progenitor cells to blood vessel growth in the adult has been elegantly revealed in lethally irradiated mice reconstituted with bone marrow from mice whose Tie-2 receptor has been labeled with lacZ or green fluorescent protein [4]. Bone marrow-derived endothelial progenitor cells were initially localized to proliferative clusters at a distance from preexisting vasculature, then coalesced into vascular cords, and, by day 21, became functional vessels.

Lymphangiogenesis and its dysregulation may contribute to inflammation and to metastatic spread of tumors. The growth of new lymphatics may share common mechanisms with blood vessel angiogenesis, although other aspects are divergent [5]. Many of the reagents used to study blood vessel endothelium and the molecules that have been targeted to manipulate angiogenesis may cross-react with lymphatics. Caution may therefore be required in determining whether studies of angiogenesis or its inhibition specifically refer to blood vessel growth or, in addition, to lymphangiogenesis.

2.2. Beneficial and Harmful Angiogenesis

Blood vessel angiogenesis is conveniently classified as either physiological or pathological, although the boundary between the two can be somewhat indistinct. Blood vessel growth during embryogenesis and the normal menstrual cycle can be seen as clearly a good thing that we would not normally wish to inhibit. At the other extreme, vascularization of a solid tumor facilitates its growth and metastasis, and is therefore seen to be harmful. Angiogenesis driven by inflammation or hypoxia may variously be harmful or helpful depending on its context. Inflammation is a normal process during tissue repair, and failure of angiogenesis during wound healing or in peptic ulcers may contribute to poor outcome. On the other hand, excessive angiogenesis driven by inflammation in arthritis may contribute to joint damage.

Angiogenesis driven by myocardial hypoxia may permit collateral formation, relief of angina, and minimize tissue damage during myocardial infarction. On the other hand, hypoxic drive to retinal neovascularization can contribute to retinal hemorrhage and blindness.

The situation becomes even more complex in organs composed of heterogeneous tissues where angiogenesis in different parts of the organ may have different implications. For example, in the osteoarthritic knee, angiogenesis in synovium is associated with synovitis, while osteochondral angiogenesis leads to blood vessels invading the articular cartilage, associated with cartilage degradation and abnormal new bone formation [6, 7]. The synovium and articular cartilage are bathed in a common pool of synovial fluid, and yet synovial and osteochondral angiogenesis appear to be differentially regulated [7]. Vascularity in the synovium and the articular cartilage each is linked to osteoarthritis in the joint, but otherwise they appear to develop independently of each other.

3. Measuring the Angiogenic Process

Angiogenesis is a multistage process rather than a single event. Endothelial cell activation and basement membrane degradation permit migration of endothelial cells which are replaced by proliferation. Migrating cells organize into solid cords which then luminate to form tubular structures and subsequently anastomose. This neovasculature permits blood flow but lacks vasoregulatory capacity. Pericyte proliferation and migration, and their association with these immature vessels, are essential to permit vascular survival [8, 9]. Subsequently, these new vessels differentiate through the acquisition of a smooth muscle wall (arteriolarization), expression of vasoregulatory molecules and their receptors, and, eventually, over a period of weeks, invasion by fine, unmyelinated sensory, and sympathetic nerves.

The temporal boundaries of angiogenesis are difficult to define such that no single event can be regarded as entirely specific or adequate to define that angiogenesis has occurred. For example, demonstration *in vitro* that a factor can stimulate endothelial cells to produce proteases, migrate, proliferate, or associate into capillary tubes need not necessarily indicate that those molecules can sustain the creation of functional new blood vessels *in vivo*. Endothelial cells lining large vessels, such as the aorta, may migrate and proliferate in response to luminal damage, for example, in atherosclerosis. This may lead to reendothelialization of a preexisting vessel without necessarily forming new vessels. Double-staining techniques for endothelial cells and, for example, α-smooth muscle actin can reveal vascular structures that are deficient in

pericytes, although these may variously indicate either new vessel formation or the loss of pericytes during vascular regression [10].

Increases in vascular density, tissue blood volume, or blood flow may be useful outcome measures of angiogenesis. However, vascular density is also modulated by vascular regression and changes in the perivascular tissue such as edema; matrix synthesis; and cellular proliferation, infiltration, and apoptosis. Blood flow is particularly confounded by vascular tone, and many angiogenic factors are also vasodilators. It may be difficult to distinguish angiogenesis from the increased blood flow that is associated with inflammation.

In summary, the complexity of the angiogenic process has led to a wide range of indexes of angiogenesis which may be best considered together rather than each in isolation in order to gain a deep understanding of vascular growth.

3.1. Quantity Vs Quality of Angiogenesis

Regulation of the later stages of angiogenesis may be particularly important in highly differentiated tissues and the close relationship between hypoxia and angiogenesis provides a neat mechanism by which perfusion can be matched to the oxygen requirements of the tissue [11]. However, proliferation of blood vessels may itself impair the function of some tissues. For example, vascularization leads to opacification in the cornea. Vascularization of the articular cartilage impairs its biomechanical characteristics and leads to endochondral bone formation [6].

Tissues that are normally avascular, such as articular cartilage, have evolved the ability to survive with a very low metabolic rate. Cartilage depends on the blood flowing through adjacent structures for its nutritional support. Cartilage normally impedes its vascular invasion by its generation of antiangiogenic factors and a matrix environment that is hostile to vascular growth despite its normally low oxygen tensions [12–14].

The synovium slides over the surface of the articular cartilage and provides for ~90% of its metabolic requirements. In contrast to the avascular cartilage adjacent to its surface, synovium is highly vascular [15]. Indeed, vascular densities of the normal synovial lining may be as high as can be sustained in any resting tissue. The synovial blood vessels are tightly regulated in health by vasomotor systems, and their structure is highly organized to provide oxygenated blood to the synovial surface, while minimizing shunting through the deeper synovial tissues [6]. Angiogenesis in the synovium leads to increases in deep vessel numbers, rather than any increased perfusion at the synovial surface, with paradoxical persistence of joint hypoxia [16].

Any increase in angiogenesis in articular tissues may disturb an already precarious balance between perfusion, metabolic demand, and structural

integrity. The quality and distribution rather than the quantity of blood vessels appear to be important in maintaining the healthy joint. The questions here, rather than being what makes blood vessels grow, may be more appropriately put as what makes them grow in a particular direction, what makes them branch and anastomose, and what determines the particular distribution of arterioles, capillaries, and venules. In addition, the growth of fine unmyelinated sensory nerves along newly formed blood vessels in tissues that are normally avascular may contribute to the chronic pain of some inflammatory and angiogenic diseases such as arthritis and spondylosis [6, 17].

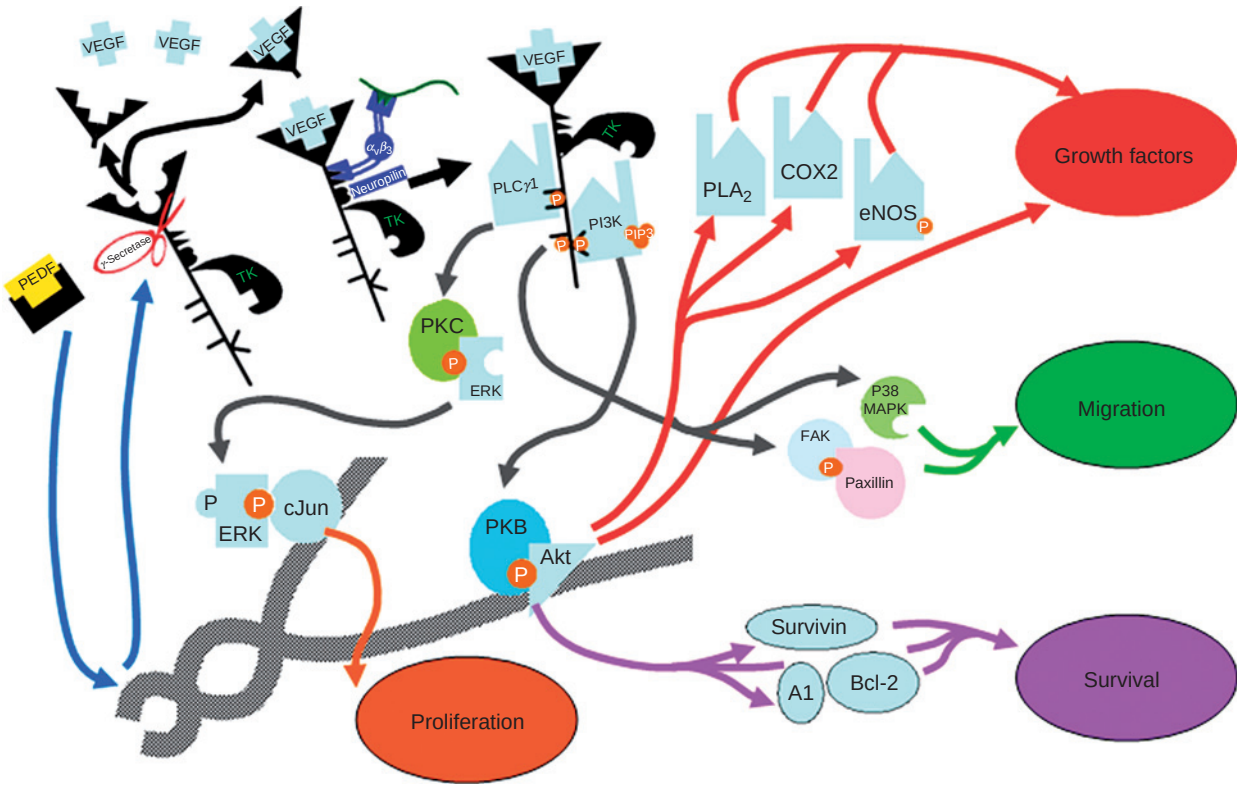
4. Endothelial Cells and the Initiation of Angiogenesis

Angiogenesis may be better regarded as a cascade rather than a linear process, with any of a variety of factors able to push the vessel through any single stage of its growth. Divergent consequences may result from stimulation by any one factor. Effects on blood vessel growth depend on what other angiogenic or antiangiogenic factors are available, on the precise expression of receptors and second messenger systems by the vessel itself, and on whether its matrix environment is either permissive or hostile to vascular invasion.

The initial stages of angiogenesis conveniently can be investigated using cultured endothelial cells, although differences are apparent between endothelial cells derived from different sources. The expression of receptors for angiogenic factors may differ between arterioles, capillaries, and venules such that angiogenic activity may be observed in some endothelial cell types but not in others [18]. Furthermore, culture conditions may themselves change endothelial cell gene expression, enhancing angiogenic responses where the resting endothelium may show none. Indeed, demonstrating angiogenic activity on endothelial cells *in vitro* is only one step in determining whether a factor may and does stimulate vessel growth *in vivo*.

4.1. VEGF as a Prototypical Direct-Acting Angiogenic Factor

The upregulation of VEGF by resident fibroblasts and its expression by migrating macrophages, and the upregulation of VEGF and its receptors by endothelial cells in the growing vessel, each indicate the importance of this factor in maintaining pathological vascular growth. This view is now strongly supported by interventional studies in both man and animals, which points to an almost ubiquitous role for VEGF in both physiological and pathological angiogenesis [19].


VEGF
VEGF
VEGF
VEGF
VEGF
$\alpha_v\beta_3$
Neuropilin
TK
TK
TK
γ-Secretase
PEDF
PLCγ1
P
PI3K
P
P
PIP3
PKC
P
ERK
PLA2
COX2
eNOS
P
Growth factors
P38
MAPK
Migration
FAK
P
Paxillin
P
P
cJun
ERK
PKB
P
Akt
Survivin
A1
Bcl-2
Survival
Proliferation

VEGF is sufficient to induce angiogenesis in a variety of animal models, although it is unlikely that it ever does so alone in physiological or pathological conditions. The complexity of cellular events that follow exposure of isolated endothelial cells to VEGF illustrates the processes that can initiate angiogenesis (Fig. 1), many of which are shared by other angiogenic factors. The interested reader is directed to other reviews of the molecular biology of VEGF for more detail [19, 20]. This section attempts to illustrate how the initial responses of endothelial cells to angiogenic factors indicate diverse molecular targets through which this process could be pharmacologically manipulated.

4.2. VEGF and Its Receptors

VEGF-A is a member of a family of growth factors that also includes VEGF-B through F, placental growth factor, and, more distantly, platelet-derived growth factor. VEGF-A exists as at least seven different homodimeric isoforms that arise from alternative splicing of mRNA from a single gene. VEGF-A_{121}, VEGF-A_{145}, and VEGF-A_{165} have clearly demonstrated biological effects on blood vessel endothelial cells.

VEGF acts through receptors on the cell surface of which three with diverse functions and activities have been most clearly defined on blood vessel endothelial cells. VEGFR1 (vascular endothelial growth factor receptor-1) (Flt-1) and VEGFR2 (KDR/Flk-1) are receptor tyrosine kinases related to the platelet-derived growth factor family of receptors, whereas neuropilin-1 lacks a cytoplasmic kinase domain and may function to facilitate VEGF interaction with other VEGFRs. Angiogenesis can be inhibited either by inactivating VEGF (e.g., using anti-VEGF antibodies) or by blocking its receptor tyrosine kinases. Both these approaches have led to encouraging clinical trials in man, particularly in renal tumors where mutations in the von Hippel–Lindau tumor suppressor gene lead to overproduction of VEGF [21, 22].

VEGFR2 appears to mediate most of the biological activities of VEGF on endothelial cells. VEGFR1 may contribute to the angiogenic activity of VEGF by enhancing the production of proteases and growth factors.

Fig. 1. Angiogenic signaling by vascular endothelial growth factor. VEGFR activation follows binding of VEGF and is facilitated by neuropilin and $\alpha_v\beta_3$-integrin. Tyrosine kinase activity of active receptors phosphorylates at several sites, activating PKC/Erk, PI3K/Akt/PKB, and FAK/paxillin pathways. These in turn stimulate endothelial cell proliferation, survival, and migration. In addition, the production of both polypeptide and low molecular weight growth factors cascades the angiogenic response. VEGFRs may be cleaved by γ-secretase to yield inhibitory, soluble forms. For more detailed explanations, see text.

Activated VEGFR1 alone, however, has relatively weak tyrosine kinase activity and may be insufficient to stimulate endothelial cell proliferation [20]. On the other hand, inhibition of angiogenesis by factors such as pigment epithelium-derived factor (PEDF) is associated with reduced VEGF-induced phosphorylation of VEGFR1 [23].

VEGFR1 is also produced in a soluble form, overexpression of which inhibits VEGF effects on endothelial cells, suggesting that VEGFR1 may function primarily as a negative regulator of VEGF activity at VEGFR2 [24]. PEDF increases endothelial cell γ-secretase activity, increases cleavage and intracellular translocation of the transmembrane domain of VEGFR1, and inhibits angiogenesis [23]. The precise balance between pro- and antiangiogenic actions of VEGFR1 may depend on pathophysiological context and the balance between the production of membrane-bound and soluble forms.

In mature tissues, VEGFR3 is primarily localized to lymphatic endothelial cells, where binding of VEGF-C stimulates lymphangiogenesis [25]. Some inflamed tissues, such as from arthritic joints, display VEGFR3 colocalized with other blood vessel markers on endothelial cells, although a role in the accelerated blood vessel angiogenesis in this tissue has not been clearly demonstrated [26].

4.3. VEGF Signal Transduction During Angiogenesis

Activation of VEGFR2 results in several parallel intracellular signaling events. A protein kinase C (PKC)/extracellular receptor kinase (Erk) pathway appears to predominantly mediate proliferative actions of VEGF on endothelial cells. A separate phosphatidylinositol 3′-kinase (PI3K)/protein kinase B (Akt/PKB) pathway predominantly enhances endothelial cell survival. Several pathways may mediate endothelial cell migration, involving PIP_3, the p38 mitogen-activated protein kinase (MAPK), and focal adhesion kinase.

4.3.1. *The PKC/Erk Pathway and Endothelial Cell Proliferation*

Receptor tyrosine kinase activation leads to autophosphorylation of specific tyrosine residues in the intracellular domain of VEGFR2. A proliferative signaling cascade is initiated, inter alia, by autophosphorylation of Tyr1175, creating a binding site for phospholipase C-γ1 which, in turn, activates PKC [27]. Erk translocates to the nucleus where it phosphorylates transcription factors such as c-Jun, inducing transcription of c-fos and leading to cell proliferation. PKC-dependent activation of Erk leads to downstream activation of phospholipase A_2 and consequent production of prostacyclin by endothelial cells, and also induces cyclooxygenase-2 (COX-2) expression [28, 29]. Decreasing COX-2 expression or the administration of

COX-2-specific inhibitors can decrease VEGF-induced endothelial cell proliferation, indicating that COX-2 mediates some of VEGF's angiogenic actions.

The Erk pathway may be subject to negative feedback through increased production of protein tyrosine phosphatase (SH-PTP1). Anti-SH-PTP1 antibodies increase VEGF-induced endothelial cell proliferation, suggesting that upregulation of SH-PTP1 damps down the angiogenic effects of VEGF [28].

4.3.2. *The PI3K/Akt/PKB Pathway and Endothelial Cell Survival*

Survival signaling pathways include VEGFR2-dependent PI3K activation, leading to accumulation of phosphatidylinositol (3,4,5)P_3 (PIP_3) and activation of Akt/PKB. Akt/PKB enhances endothelial cell survival by inhibiting caspase activity, in part through survivin; by upregulating the antiapoptotic proteins A1 and Bcl-2; and by inhibiting B-cell lymphoma 2 (Bcl-2)-associated death promoter homologue. Endothelial cell survival may also be facilitated by Erk and MAPK pathways, for example, through downregulation of Rho-kinase [30].

4.3.3. *Pathways Mediating Endothelial Cell Migration*

Endothelial cell migration is mediated by two additional pathways that stimulate cytoskeletal reorganization. These involve the p38 MAPK and focal adhesion kinase with paxillin phosphorylation, respectively [31]. PIP_3 also activates the small GTP-binding protein Rac which facilitates endothelial cell migration. The PI3K pathway may also contribute to VEGF-enhanced endothelial cell proliferation [32].

4.3.4. *Other Modulators and Mediators of VEGF-Enhanced Angiogenesis*

Increased nitric oxide generation mediates some angiogenic effects of VEGF, including endothelial cell proliferation and migration. Endothelial nitric oxide synthase (eNOS) activation results from several events downstream of VEGFR2 activation. Increased intracellular Ca^{2+} mobilization results in rapid nitric oxide production. Akt/PKB-dependent phosphorylation of eNOS in response to VEGF also rapidly increases eNOS activity by removing the need for Ca^{2+} to stimulate nitric oxide generation [33]. In addition, VEGFR2 stimulation enhances eNOS expression via PKC/Erk, resulting in more sustained endothelial activation [34].

VEGF enhancement of VEGFR2 activity may be facilitated by the association of VEGFR2 with other endothelial cell surface molecules such as $\alpha_v\beta_3$-integrin and neuropilin-1 [19, 35]. Integrin facilitation of VEGFR2 activation depends on its association with ligands such as vitronectin in the pericellular matrix. Coexpression of neuropilin-1 with VEGFR2 more than doubles the

VEGFR2 binding to VEGF-A_{165} and endothelial cell migration. VEGFR1 and VEGFR2 activities may also be enhanced by their upregulated expression in neovascular endothelial cells [19].

4.4. Autocrine Production of Angiogenic Factors by Endothelial Cells

VEGF stimulation of endothelial cells also induces their autocrine production of a cascade of other growth factors, including platelet-activating factor [36]. Increased platelet-activating factor expression stimulates endothelial cell migration and further promotes the expression of fibroblast growth factor (FGF)-1, FGF-2, and macrophage inflammatory protein 2 [19].

Autocrine expression of VEGF by endothelial cells has also been demonstrated in response, for example, to hypoxia, growth factors, and oxidative stress [37, 38]. Induced endothelial cell expression of VEGF itself may be dependent on a number of second messenger systems, and can be inhibited by blocking MAPK, PI3K, and PKC [37].

Other angiogenic factors may also induce the production of autocrine growth factors by endothelial cells, including FGF-2, CCL2 and CCL3 chemokines, and connective tissue growth factor (CTGF/CCN2) [39–42]. Induction of FGF-2 expression by endothelial cells has been found to be necessary for the angiogenic activity of a number of angiogenic molecules such as substance P and eicosanoids [40, 43]. For example, blocking antibodies to FGF-2 will inhibit the apparently direct, neurokinin-1 (NK_1) receptor-mediated stimulation of endothelial cell proliferation by substance P.

5. Differential Angiogenic Pathways

Complex angiogenic pathways involve the discrete regulation of each stage in this multistep process. Tissue- and disease-specific expression of angiogenic factors and inhibitors may vary through the different phases of a pathological process. For example, an angiogenic switch may turn slow growing neoplasms into aggressive, metastasizing tumors [44]. Sequential activation of various angiogenic and inhibitory pathways may coordinate the beneficial progression from hemostasis through successful repair during wound healing, or the arguably less desirable transition from acute to chronic inflammation.

5.1. Angiogenic Factor Expression in Malignant Tumors

The expression of angiogenic factors by tumor cells varies between different tumor cell types and with time in any particular tumor or its subclones. In cross-sectional studies, malignant tumors of increasingly advanced stage

display higher expression of angiogenic factors. For example, VEGF expression increased from metaplasia through advanced esophageal adenocarcinoma, and similarly varied between adenomas through various grades of colorectal carcinoma [45, 46].

There is no single angiogenic factor that is expressed by all tumor cells. For example, platelet-derived endothelial cell growth factor (PD-ECGF, thymidine phosphorylase) was found in one study to be expressed by more than 50% of tumor cells in only 32% of squamous cell carcinomas and 42% of adenocarcinomas of the lung, and in none of 22 small cell carcinomas [47]. Individual tumor types may variously express different angiogenic factors, any one or combination of which may be responsible for vascular growth in that tumor. For example, VEGF and PD-ECGF are each independently associated with high microvascular density within pancreatic carcinomas; although, in one study, only 56% of pancreatic carcinomas were found to be positive for VEGF and 32% for PD-ECGF [48]. A variety of factors including VEGF, PD-ECGF, and the chemokine CXCL8 may correlate with vascular densities within some tumors, whereas in other tumor types the extent of expression of, for example, VEGF may not show any clear relationship with tumor vascularization [48–51].

As well as differing between tumors of different types, angiogenic factor expression may vary between individual tumor cells within a tumor. This heterogeneous production of factors between subclones of tumor cells within a primary tumor is reflected by variations in factor production between primary tumors and their metastases [52]. Indeed, inconsistent relationships between matched individual tumors and their metastatic deposits in their levels of vascularity and endothelial cell proliferation may indicate that angiogenic factor production by the metastasizing clone differs from that in the primary tumor [53].

5.2. Consequences of Differential Angiogenesis in Tumors

The hypothesis that an ''angiogenic switch'' can explain progression of malignant tumors was eloquently put forward by Folkman over a decade ago [44]. As originally described, tumor growth may be restricted by the inadequacy of its blood supply until it ''switches on'' a production of angiogenic factors that in turn stimulates its neovascularization and facilitates tumor growth and subsequent metastasis. The molecular entities that may be expressed by tumor cells to induce this angiogenic switch have been more difficult to pin down, and, indeed, the expression of several or any of a variety of angiogenic factors may be responsible for such a switch.

It is difficult to determine from cross-sectional studies whether new induction of angiogenic factors leads sequentially to tumor progression, as implied

by the angiogenic switch hypothesis, or whether angiogenic factor expression identifies a subgroup of tumors that were likely to progress from the start. The potential for tumor cells themselves to drive an angiogenic switch has been complemented by the recognition of host cells within the tumor environment such as fibroblasts and macrophages, which may express a variety of angiogenic factors and thereby induce angiogenesis, as it were, from the outside.

Differences in angiogenic factor expression explain some of the differences in appearance and behavior between different tumor types. For example, higher VEGF expression by breast carcinoma cells to some extent distinguishes ductal tumors from those of a lobular type [54]. Basal cell carcinomas of the skin are locally invasive but have very low metastatic potential compared with squamous cell carninomas. VEGF expression is localized to the invasive edge of basal cell carcinomas, whereas it is expressed throughout the tumor in squamous cell carcinomas [55]. Similarly, CXCL8 is more strongly expressed and associated with high vascularity in diffuse rather than in intestinal-type gastric carcinomas [50].

Variations in the expression of angiogenic factors may modulate tumor vascularization and therefore oxygenation. This, in turn, may contribute to differing responsiveness to treatments between different tumor cell types. For example, increased radioresistance in pancreatic carcinoma cell lines was associated with an increased expression of angiopoietin (Ang)-2, which plays an important role in vascular maturation [56].

5.3. Host Tissue Angiogenic Responses

Cells in the environment of tumors also express angiogenic factors and may be no less important than the tumor cells themselves in influencing tumor vascularization. Whereas the tumor cells appear to be the main source of VEGF in supraglottic squamous cell carcinomas of the head and neck, the majority of VEGF positive cells in non-small cell lung carcinomas and invasive ductal breast carcinomas may be macrophages and fibroblasts [57]. Indeed, some angiogenic factors such as FGF-2 and transforming growth factor $\beta 1$ may be predominantly expressed by macrophages and fibroblasts in most tumor types.

The importance both of the tumor cells and their environment as sources of angiogenic factors is supported by their independent associations with tumoral vascular density in human tissues [58]. This principle has been elegantly demonstrated using species-specific reagents in mice bearing human xenografts. Antibodies that block both human and mouse VEGF inhibited equally the growth of three tumor cell types, HM-7, A673, and HPAC [59]. Avastin is a monoclonal blocking antibody that binds and inhibits human but not mouse

VEGF. It was found to be almost completely effective in inhibiting HM-7 and A673 tumor cell growth, but less than 50% effective in inhibiting HPAC growth. In the context of HPAC tumor growth, it appears that VEGF produced by the mouse is sufficient to support the growth of the human tumor cells.

5.4. Vascular Phenotype

Different patterns of vascularization may also suggest differential regulation of angiogenesis in different tissues. Corrosion casts of the vasculatures of bladder carcinomas revealed two types of capillary systems, dense flat networks, and tightly packed tortuous loops [60]. The distinction between flat and tortuous microvascular systems has also been observed directly by arthroscopy in patients with inflammatory arthritis [61]. Patients with seronegative arthritis associated with psoriasis typically display more tortuous vascular patterns than do patients with the more common rheumatoid arthritis. The fact that the creation of different vascular patterns may reflect regulation by different angiogenic factors has been suggested also in carcinoma *in situ* of the breast [62]. Two vascular patterns have been described in this condition: first, a diffuse increase in stromal vascularity and second, a dense rim of microvessels close to the basement membrane of involved ducts. PD-ECGF expression correlates with the presence of a dense vascular rim but not with increased stromal vascularity.

In addition to differences between tumors and their environment, the neovascular phenotype itself may differ between tissues. A variety of vascular morphologies has been discussed above, and specific molecules expressed by the neovasculature may also vary. For example, binding of the antiangiogenic factor endostatin was found to almost all bladder tumor vessels, three quarters of the vessels in prostatic carcinomas, and only 11% of renal tumor vessels [63]. VEGFR3, which, in most tissues, is restricted to lymphatics, has been identified in the new blood vessels of inflamed synovium [26]. These and other characteristics of different neovascular beds may contribute to heterogeneous responses to therapies that target the vasculature.

5.5. Plasticity of Angiogenic Pathways

Angiogenesis requires that endogenous agonists and receptors are sequentially expressed from the initiation of vascular growth through the assembly of a mature and functional blood vessel. This is illustrated by the VEGF and angiopoietin systems. VEGF stimulates endothelial cell migration, proliferation, and capillary tube formation. VEGFRs are upregulated by the new endothelium, and VEGF facilitates the survival of these newly formed blood vessels [64]. Recruitment of pericytes and other ancillary cells results in the

local expression of Ang-1 that can interact with its Tie-2 receptor on endothelial cells [8, 65]. These and other changes shift the new vessel toward a maturing phenotype. Expression of Ang-2, a naturally occurring antagonist of Tie-2, further buffers the angiogenic response, thereby stabilizing the neovasculature [66, 67].

This normal, sequential, coordinated expression of complementary factors which leads from blood vessel growth to establish a mature, resting vasculature, is observed during embryonic development and during physiological angiogenesis in the adult. It is distinct from the sequential expression of angiogenic factors that can lead from the initiation of angiogenesis to sustained vessel growth over months or years, for example, during chronic inflammation. Persistent angiogenesis, far from resulting in a functional, normally regulated vascular bed appropriate to the tissue's metabolic demands, instead leads to a disorganized, dysregulated, leaky, and fragile network of blood vessels [68].

Several vasoactive peptides provide examples where one angiogenic pathway gives way to another, thereby maintaining angiogenesis. This plasticity of angiogenic pathways emphasizes the need to extend studies in normal tissues by undertaking research on pathological samples, since factors that are important in one may be redundant in the other.

5.5.1. *Neuropeptides*

Substance P is present in fine, unmyelinated nerves around resting blood vessels, whose endothelia express the NK_1 tachykinin receptor [69]. The release of preformed substance P therefore is well placed to initiate angiogenesis immediately following tissue injury [70]. Sensory nerves are, however, depleted during chronic inflammation [71]. Nerve growth progresses much more slowly than does that of blood vessels, and the neovasculatures of, for example, tumors, skin grafts, and arthritic joints are often relatively noninnervated, despite expressing NK_1 receptors [17, 72–74].

The recent discovery of endokinins and hemokinins provides an alternative pathway that may take over from neuronally derived substance P as an increasing proportion of vessels are noninnervated. These novel tachykinins are expressed by bone marrow-derived inflammatory cells and endothelial cells themselves [75]. Both hemokinin-1 and endokinins A and B are selective and full agonists at NK_1 receptors in man, rat, and mouse, inducing acute plasma extravasation and vasodilation [76]. It is likely that these novel tachykinins will also, like substance P, induce angiogenesis.

Such a "handover" of angiogenesis from nerves to inflammatory cells, using analogous signaling mechanisms, may also occur with calcitonin gene-related peptide (CGRP) and adrenomedullin. CGRP is a cotransmitter of substance P that also may induce angiogenesis [77]. Adrenomedullin is a

hypoxia-inducible angiogenic factor that is produced by, among other non-neuronal cells, macrophages [78–80]. Adrenomedullin stimulates endothelial cell proliferation, capillary tube formation, and vascular survival *in vitro* [81, 82] and enhances vascular growth *in vivo* [83, 84].

Biological effects of CGRP and adrenomedullin are mediated through cell surface, G–protein-coupled receptors, the CGRP receptor-1, and adrenomedullin receptors-1 and -2. The pharmacological selectivities of CGRP and adrenomedullin receptors depend on the heterodimeric association of a single calcitonin receptor-like receptor (CRLR) with one of three receptor activity-modifying proteins (RAMPs) [85]. CRLR is a seven-transmembrane G–protein-coupled receptor. CRLR association with RAMP1 confers selectivity for CGRP, whereas association of CRLR with RAMP2 or RAMP3 confers selectivity for adrenomedullin.

5.5.2. *Other Vasoactive Peptides*

Kinins are produced by enzymatic cleavage of kininogen during acute inflammation [86]. Normal tissues express B_2 receptors on their blood vasculature, which have relative selectivity for the intact bradykinin molecule. During inflammation, B_1 receptors are induced, and carboxypeptidase A cleaves kinins to produce their des-Arg fragments which have a relative selectivity for this receptor. B_2 and B_1 receptors each can mediate kinin-enhanced angiogenesis [87, 88]. Experiments using selective receptor antagonists have indicated a shift from B_2 receptor- to B_1 receptor signaling as inflammation evolves [89].

Angiotensin can also stimulate angiogenesis, despite being a vasoconstrictor peptide [90, 91]. Angiotensin displays equal affinity for each of two receptors, AT_1 and AT_2. Angiotensin-enhanced angiogenesis is mediated by AT_1 receptors, whereas upregulation of AT_2 in the granulation tissue damps down this angiogenic activity, providing a negative feedback loop [91]. Angiotensin is formed by enzymatic cleavage of angiotensinogen, initially involving the enzyme renin. The component of angiotensinogen that is cleaved from angiotensin I [des(angiotensin I)angiotensinogen] is a member of the serpin family of folded proteins, with structural similarity to PEDF. This fragment also displays antiangiogenic activity, suggesting an additional possible level of angiogenesis regulation by the rennin–angiotensin system [92].

In summary, factors that initiate angiogenesis may well differ from those that sustain it in chronic diseases. Some angiogenic factors and their receptors are present in normal resting tissues, or may be present in inactive forms rapidly released during tissue damage. This may be the case for kinins and FGF-2. Various other factors and their receptors may be upregulated during the early stages of the angiogenic process and become increasingly important as vascular growth continues. Such factors may either sustain angiogenesis,

or may ensure survival and maturation of newly formed vessels. Peptides that are released into tissues during an acute inflammatory response, including bradykinin, substance P, CGRP, and angiotensin II, may well initiate angiogenesis. Other vasoactive peptides and growth factors that are upregulated during chronic inflammation may maintain the angiogenic process once initiated. Different tumors may use any one or more of these pathways to facilitate their vascularization and subsequent growth and metastasis either by producing angiogenic factors themselves or by inducing an inflammatory response.

6. Impact of the Vessel's Environment on Angiogenesis

Blood vessel growth is profoundly influenced by the surrounding extracellular matrix. Protease expression or activation by endothelial cells is a key step in the initiation of angiogenesis. Indeed, endothelial cell expression of molecules, such as tissue plasminogen activator, is characteristic of growing blood vessels and plasminogen activator inhibitors impede endothelial cell migration and angiogenesis [93, 94].

The endothelial cell itself is responsible for depositing its own extracellular matrix, the basement membrane. Fibronectin, different laminins, and collagen IV are sequentially expressed by capillary endothelial cells during vascular maturation. Their relative roles in vascular survival are therefore likely to change as maturation progresses [95]. The deposition of laminin and collagen IV appears very rapidly following endothelial tube formation *in vivo* such that it is rare to find basement membrane-deficient endothelial cells even in tissues with very high rates of vascular growth [26, 57]. These matrix proteins enhance neovascular survival by their interactions with $\alpha_5\beta_1$- and $\alpha_v\beta_3$-integrins, thereby activating a variety of intracellular pathways [95–97]. These integrins are themselves selectively upregulated by neovascular endothelial cells and small molecular weight RGD-derived integrin ligands inhibit angiogenesis largely by impairing neovascular survival [98–100].

Extracellular matrix degradation not only clears the way for endothelial cells to migrate but also releases growth factors which are otherwise anchored in inactive forms within the matrix. FGF-2 and some VEGF isoforms (VEGF-A_{145}, VEGF-A_{189}, and VEGF-A_{206}) contain a heparin-binding domain through which they are bound within the extracellular matrix [19]. Other VEGF isoforms that lack this domain are more freely diffusible. FGF-2 and VEGF are released from the matrix following cleavage of heparan sulphate by heparanase [101]. High levels of heparinase expression by tumor cells are accompanied by increased tumor vascularization and vessel maturation [102]. Plasmin also contributes to the release of VEGF

from the matrix either directly by cleavage of diffusible bioactive fragments or by activating collagenases [103]. Tissue and urokinase-type plasminogen activators are produced by endothelial cells. These, in turn, generate plasmin from plasminogen. VEGF and FGF-2, therefore, by stimulating the release of tissue and urokinase-type plasminogen activators may contribute to further angiogenic factor release from the matrix in a positive feedback loop.

Matrix metaloproteases, either secreted or membrane bound, degrade collagens within the extracellular matrix, thereby facilitating endothelial cell migration and angiogenesis [104, 105]. As well as being a reservoir of angiogenic factors the extracellular matrix also contains inhibitors of proteases such as TIMPs, which may limit the potential for blood vessel growth. For example, the restraining role of TIMP-3 has been demonstrated by experiments on TIMP-3 nul mice in which malignant melanomas grow faster with increased vessel content than in wild-type mice [106].

Inhibitors of matrix metaloproteases also reside in normal hyaline cartilage, where they may inhibit vascular invasion [107]. Blood vessel invasion into cartilage induces endochondral ossification, a normal process in the growth of long bones, but which would be undesirable, for example, in the articular cartilage. Loss of angioinhibitory activity within articular cartilage may contribute to the vascular invasion and ossification, which characterizes the osteoarthritic joint [108, 109].

Tissue-specific expression of angiogenesis inhibitors, such as metalloprotease inhibitors produced by chondrocytes in hyaline cartilage or PEDF produced by the retinal epithelium, modulates any association between angiogenic factor expression and vascular growth [23, 110]. The expression of angiogenesis inhibitors by some tissues may be important in maintaining their normal structure and function, and may also contribute to their relative sparing in metastatic malignant disease.

7. Angiogenesis Biomarkers for Assessing Human Disease

Understanding the pathophysiological mechanisms of angiogenesis creates a number of clinical opportunities. Although most clinical attention focuses on the possibility of pharmacological treatment, another productive development leading from our increasing understanding of angiogenic pathways has been the design of biomarkers for use in human disease. Where angiogenesis contributes to pathology, biomarkers may usefully guide diagnosis, predict prognosis, demonstrate activity of pharmacological interventions, and demonstrate response or relapse after treatment. The need for robust biomarkers is well illustrated in breast cancer. Lymph node status and histological grade remain the best predictors of prognosis and metastasis

at present. However, these gold standards have limited predictive value, and it remains the case that a majority of women who currently receive chemotherapy may do equally well without it.

7.1. Vascular Density

High vascularity demonstrated histologically in primary tumors has been associated with high rates of metastasis, tumor recurrence, and poor survival in a variety of tumor types, in particular in the lung and breast [111, 112]. Other indexes of angiogenesis, such as vascular immaturity, may also predict poor prognosis independently of any association with high vascular density [112]. Angiogenesis may contribute to poor prognosis in cancer by facilitating hematogenous spread of tumor cells or by feeding the growing tumor.

7.2. Imaging

Attempts to translate histological indexes of vascular density to *in vivo* imaging, which can be performed serially and noninvasively, have thrown additional light on the potential role of angiogenesis in malignant disease.

7.2.1. *Magnetic Resonance Imaging*

Gadolinium-enhanced magnetic resonance imaging (MRI) can detect or differentiate tumors from adjacent normal tissues [113]. MRI findings correlate with vascular densities, particularly in malignant and inflamed tissues [113, 114]. However, these imaging techniques detect more than just blood vessel density. Indeed, perfusion, vascular permeability, and blood volume to an extent can be separately measured using enhanced MRI. Of these vascular parameters, permeability and perfusion appear to be more diagnostic of malignant disease than is overall vessel density [113]. Indeed, MRI estimates of microvascular permeability correlate with histological microvessel density in some tumors, and it may be that the high permeability of tumor neovasculature is key to the high enhancement demonstrated by MRI [115].

Enhanced MRI has been used to demonstrate that the decreases in tumor vascular permeability that follow short-term (2-day) administration of VEGFR tyrosine kinase antagonists precede the reduced vascular density and decreased tumor volume that follow longer term administration [116]. Correspondingly, in patients with advanced colorectal cancer and liver metastasis, decreased tumor vascular permeability and vascularity were demonstrated by dynamic contrast-enhanced MRI, both at day 2 and at the end of the first cycle of VEGFR tyrosine kinase antagonist treatment [117]. These early vascular responses discriminated between patients who would subsequently have stable disease and those who would progress following treatment.

7.2.2. *Doppler Ultrasound*

Doppler ultrasound has also been used to measure and visualize microvasculature *in vivo*. However, unlike enhanced MRI, Doppler ultrasound predominantly detects perfusion, a function of vascular density and vascular tone, rather than vascular permeability. Doppler ultrasound has some discriminative value for diagnosing malignancy and predicting prognosis, although experience has been less consistent than with MRI techniques [113]. In oncology, ultrasound techniques may be limited by their need for relatively superficial tissues. Indeed, Doppler ultrasound may have greater applicability in inflammatory rather than malignant conditions, for example in arthritis, where the technique appears to be sensitive to pathological change and responses to treatment [118]. Increased perfusion and vascular density may be seen as complimentary components of the inflammatory response.

7.2.3. *Nuclear Medicine Imaging*

Radiotracer-based techniques have taken advantage of markers that are relatively specific for the neovasculature [119]. Despite typically lower spatial resolution than with MRI, these techniques may have advantages in defining specific molecular pathways involved in angiogenesis. Three main targets have been utilized through $\alpha_v\beta_3$-integrin-binding ligands, matrix metalloproteinase (MMP) inhibitors, and single-chain antifibronectin antibody fragments [119]. In addition, radiolabeled antibodies to E-selectin, which is induced on vascular endothelium during angiogenesis, have been used for quantitative visualization of increased vascularity in inflamed joints [120]. The endothelial upregulation of E-selectin during inflammation may be unrelated to angiogenesis, and uptake of antibodies to E-selectin is a product not only of neovascular density but also of blood flow and endothelial cell activation.

The EDB domain of the fibronectin molecule may be a specific marker of new vessels [121]. Indeed, an [^{123}Iodine]-labeled anti-EDB single-chain antibody fragment (SCFvL-19) selectively localizes to a variety of tumor types in patients [119]. $\alpha_v\beta_3$-Integrin ligands, based on the RGD integrin-binding motif, have been characterized even more extensively. Radiolabeled RGD peptides can be relatively specific for $\alpha_v\beta_3$-integrin, their binding is blocked by nonlabeled RGD peptides, and they show selective tumor accumulation with high image contrast [122]. Radiolabeled RGD peptides have been used successfully also to image neovasculature during chronic inflammation in a mouse cutaneous, delayed-type hypersensitivity model [122]. In this context, the acute inflammatory response was not associated with specific uptake of the tracer, whereas chronic delayed-type hypersensitivity with angiogenesis was associated with increased uptake. This suggests a degree of

discrimination between the vascular changes of inflammation and those specific to angiogenesis. RGD-derived tracers can also be visualized by positron emission tomography [122].

7.3. Angiogenic Factor Expression

The expression of specific angiogenic factors drives angiogenesis within the pathological tissue and, not surprisingly therefore, factor expression may correlate with vascular density. Expression of VEGF and PD-ECGF by cancer cells may be associated with high tumoral vascularity and may predict poor survival [58]. Furthermore, the expression of angiogenic factors in intraepithelial neoplasia may contribute to progression to invasive tumors, for example, in the prostate [123].

The development of techniques detecting the expression of a wide range of factors in small samples of tumor tissues is being exploited in an attempt to determine angiogenic "expression signatures" that may predict which patients have a poor prognosis. For example, angiogenic factors contribute to a molecular expression pattern in primary breast carcinomas that may identify a subgroup of patients with a poor prognosis despite absent lymph node involvement [124].

Gene expression techniques may also be useful in determining response to systemically administered antiangiogenic therapies. Altered gene expression in peripheral blood cells may provide adequate evidence of responsiveness to pharmacological agents in dose-ranging studies without requiring serial tumor biopsy. For example, gene expression studies using peripheral blood mononuclear cells in patients receiving a VEGFR tyrosine kinase inhibitor revealed an altered expression of CD24, lactoferrin, lipocalin 2, and MMP-9 in the treated group [125].

7.4. Circulating Biomarkers

Circulating biomarkers for angiogenesis could have particular potential in clinical practice. Work in this area has focused on two classes of marker, angiogenic factors themselves and molecules that may be released from the neovasculature. Of the angiogenic factors, much effort has been focused on VEGF, although FGF-2, placental growth factor, interleukin-8, and the Angs have also been explored [126–129].

7.4.1. *Vascular Endothelial Growth Factor*

A number of studies have indicated that high levels of circulating VEGF may be associated with malignancy, and that they correlate with more extensive disease and poor prognosis [130–133]. Elevated circulating VEGF,

however, may also be a feature of chronic inflammatory disease, endometriosis, and benign ovarian cysts, limiting its potential utility as a diagnostic tool in situations where malignancy may not be the only disease present [134, 135].

An additional complicating factor is the large amount of VEGF in normal platelets, and the frequency with which thrombocytosis and coagulation accompany the angiogenic diseases under investigation. VEGF is released from platelet α-granules during coagulation and this is the predominant source of VEGF in serum. This confounding factor can be avoided by assaying plasma rather than serum [136]. Plasma concentrations of VEGF tend to be lower than those measured in serum, although high correlation between plasma and serum VEGF levels suggests that the latter may yet have some value. Platelet-derived VEGF may contribute to pathological angiogenesis, although this requires confirmation in specific diseases or tumors.

Circulating VEGF levels may have particular utility in determining response to treatment. Normalization of circulating VEGF levels may be associated with objective tumor response or stable disease and subsequently higher survival following treatment for malignant disease [137]. Correspondingly, increasing VEGF levels have been associated with tumor relapse, for example, in malignant melanoma [138]. High circulating VEGF levels may predict poor prognosis in early arthritis [139]. Furthermore, serum VEGF levels decrease in patients with rheumatoid arthritis who have been successfully treated [140, 141].

7.4.2. *Other Circulating Angiogenesis Biomarkers*

VEGF does not act alone in stimulating angiogenesis, and combinations of biomarkers may perform better than VEGF alone. For example, elevations of either FGF-2 or VEGF were associated with progressive disease during treatment for metastatic carcinoma, and high circulating levels of soluble VEGFR1 levels have been associated with poor prognosis in acute myeloblastic leukemia [130, 142].

One possible limitation to this approach may be the diversity of angiogenic factors that can be produced by different tumors. A common host response to malignant tumors may usefully circumvent this problem. For example, circulating angiogenic factors, such as FGF-2 and VEGF, may be derived from cells around or infiltrating the tumor, and therefore may be elevated irrespective of their expression by the tumor cells themselves.

A number of surface models may be shed by endothelial cells during angiogenesis, and therefore are available for immunodetection in plasma or serum. These include soluble forms of VEGF and Tie-2 receptors, CD31 and CD146, and endocan [142–144]. Although less thoroughly explored than

VEGF itself, the circulating levels of at least some of these factors may be elevated in patients with tumors and inflammatory diseases.

7.4.3. *Biomarkers in Other Biofluids*

Angiogenic factors may also be detected in other biofluids such as urine [145, 146] and synovial fluid [147]. For example, detection of angiogenin in a single-void urine displayed a sensitivity of 75% and specificity of 70% in discriminating carcinoma of the bladder from normal controls [146]. High levels of VEGF-like immunoreactivity in synovial fluid are associated with pathology [141, 148–150] and vascular morphology [61, 151], although synovial fluid is less accessible than blood for sequential analyses.

8. Therapeutic Targeting of Angiogenesis

Angiogenesis inhibition has been explored for its therapeutic potential in a variety of clinical areas. Initially driven by the hope that blocking new vessel growth will improve prognosis in solid tumors, drugs developed primarily for the oncology field have been applied to conditions as diverse as arthritis, psoriasis, and macular degeneration [152–154]. A potential drawback of the use of agents which inhibit all angiogenesis in nonmalignant disease is the likelihood that they would also impair physiological angiogenesis. The use of broad-spectrum angiogenesis inhibitors such as thalidomide in fertile women would seem inappropriate. However, concerns about teratogenicity need not absolutely deny access to such drugs. Concern about impaired wound healing from chronic angiogenesis inhibition may be circumvented by targeting antiangiogenic treatments to phases of active angiogenesis during the disease, perhaps, for example, during flares of arthritis.

A more sophisticated approach to angiogenesis inhibition would be to target disease-specific angiogenic pathways. For example, an angiogenic switch in solid tumors may be driven by tumor expression of a relatively small number of angiogenic factors. Identification of the angiogenic driver in an individual tumor, or in a particular pathology, may permit selective angiogenesis inhibition while permitting normal vascular growth during tissue repair.

An alternative to inhibiting angiogenesis itself would be to prevent survival of undesirable neovasculature. Some angiogenesis inhibitors, such as $\alpha_v\beta_3$-integrin ligands and endostatin, may function, at least in part, through inhibition of vascular survival. Alternatively, targeting of toxins or procoagulants to markers that are specifically expressed on the neovascular endothelium may lead to involution of new vessels. The well-established technology of laser ablation of retinal neovasculature to reduce the risk of

blindness in diabetic retinopathy is an example of the neovascular targeting principle, although its biological effect may extend beyond the destruction of the neovasculature [155].

Pharmacological neovascular targeting has been technically limited by restricted specificity of presumed neovascular markers. In particular, many markers, such as E-selectin, that are upregulated during angiogenesis may also be upregulated during endothelial cell activation in other contexts, or they may play important protective roles in the host response to injury or infection.

Therapeutic angiogenesis has achieved some success in situations of vascular insufficiency [156]. Gene transfer of angiogenic factors has shown early promise for revascularizing ischemic myocardium or calf muscles affected by peripheral vascular disease [157–160]. Topical application or gene transfer of angiogenic factors to wounds also has potential [161]. Although these techniques are currently limited by relatively low efficacy; therapeutic angiogenesis may have a place eventually in facilitating fracture repair or skin graft survival.

9. Conclusion

Angiogenesis contributes to physiological and pathological processes as diverse as the menstrual cycle and malignancy, wound healing, and arthritis. Angiogenesis may be either beneficial or harmful, depending on both the quantity and quality of the newly formed blood vessels. Endothelial cells play central roles in the initiation of angiogenesis, and VEGF may be viewed as a prototypical, direct-acting angiogenic factor. Its diverse effects on endothelial cell function are mediated by a multitude of signaling pathways and modulated by other angiogenic factors and inhibitors in the vessel's immediate environment. Different angiogenic pathways operate in and around various malignant tumors and other tissues, modulating disease outcome and tissue function. Plasticity within angiogenic pathways leads either to discrete episodes of angiogenesis concluded by the creation of a mature, functional vasculature or to sustained angiogenesis during chronic disease. Our deeper understanding of the roles and processes of vascular growth is leading the development of angiogenesis biomarkers for assessing human disease, using histological, molecular biology, imaging, and biochemical techniques. Ultimately, angiogenesis may be harnessed to improve therapies in man.

The complexity of angiogenesis provides both challenges and opportunities in the development of novel treatments. Substantial progress is now being made in identifying unique angiogenic pathways through different phases of differing physiological and pathological conditions.

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BIKUNIN (URINARY TRYPSIN INHIBITOR): STRUCTURE, BIOLOGICAL RELEVANCE, AND MEASUREMENT

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Abbreviations

AMBP	α-microglobulin/Bik precursor protein
Bik	bikunin
CAD	coronary artery disease
CRP	C-reactive protein
CVD	cardiovascular disease
EGFR	epidermal growth factor receptor
IL	interleukin
IαI	interleukin-α inhibitor
MAPK	mitogen-activated protein kinase activation
NF-κB	transcriptional nuclear factor κB
PαI	pre-α-inhibitor
PAR	protease-activated receptors
TNF	tumor necrosis factor
TLR	toll-like receptor
uTi	urinary trypsin inhibitor
Uri	uristatin

1. Abstract

Inflammatory processes, such as phagocytosis, coagulation, and vascular dilation, promote the release of serine proteases by neutrophils, macrophages, mast cells, lymphocytes, and the epithelial or endothelial cells. These proteases further facilitate the release of inflammatory cytokines and growth factors as well as take part in signal-cell proliferation through protease-activated receptors (PARs). Controlling the action of this cascade is necessary to prevent further damage to the normal tissues. One of the main anti-inflammatory response mediators is bikunin (Bik) that is responsible for inhibiting the activity of many serine proteases such as trypsin, thrombin, chymotrypsin, kallikrein, plasmin, elastase, cathepsin, Factors IXa, Xa, XIa, and XIIa. During the acute-phase response, Bik is released into plasma from proinhibitors primarily due to increased elastase activity. Bik is a glycoprotein, also referred to as urinary trypsin inhibitor, which in plasma inhibits the trypsin family of serine proteases by binding to either of the two Kunitz-binding domains. Bik also accumulates in urine. In conditions such as infection, cancer, tissue injury during surgery, kidney disease, vascular disease, coagulation, and diabetes, the concentrations of Bik in plasma and urine are increased. Several trypsin inhibitory assays for urine and immunoassays for both blood and urine have been described for measuring Bik. In addition to presenting the synthesis, structure, and pathophysiology of Bik, we will

summarize various diagnostic approaches for measuring Bik. Analysis of Bik may provide a rapid approach in assessing various conditions involving the inflammatory processes.

2. Introduction

Bikunin (Bik), a peptide excreted in the urine, is one of the primary inhibitors of the trypsin family of serine proteases. This peptide plays a key role in inflammation and innate immunity because of its two Kunitz-type binding domains [1, 2]. Bik suppresses proteolytic activity in a variety of tissues and can also exert localized anti-inflammatory effect [3–5]. Inflammation is an important indicator of infection, cancer, and tissue injury in acute and chronic states. In acute inflammation, fluids and plasma components accumulate in the affected tissues due to vascular dilation. Subsequent activation of platelets and increased presence of immune cells occur during repair. Long-standing inflammation may be present before the disorder is identified. Due to its inhibitory role and potential use as an early marker of inflammation, we will review the synthesis, structure, pathophysiology of Bik as well as the various approaches for its measurement in this chapter.

2.1. Protein Synthesis and Release

Bik is produced in two key proinhibitor forms, inter-α-inhibitor (IαI) ($\sim$220 kDa) and pre-α-inhibitor (PαI) ($\sim$120 kDa) [6–8]. Other names for Bik include urinary trypsin inhibitor (uTi), mingin, human inhibitor 30 (HI-30), urinastatin, ulinastatin, and Miraclid. Although uTi and Bik are generally interchangeable, the term Bik is exclusively used throughout this chapter. Biosynthesis of these compounds occurs primarily in the liver, but kidneys, pancreas, intestine, stomach, and cancer cells also produce these proinhibitors albeit at lower concentrations. The proinhibitors are released into the blood under normal conditions and are noninhibitory to most serine proteases [9]. During the acute-phase response, IαI expression is downregulated while PαI is upregulated [10]. IαI consists of three polypeptides (two heavy chains and Bik), whereas PαI consists of two polypeptides (one heavy chain and Bik) (Fig. 1). A chondroitin sulfate chain is the point at which the heavy chains are covalently linked to Bik.

Biosynthesis of IαI and PαI starts from α-1-microglobulin/Bik precursor protein (AMBP) ($\sim$43–63 kDa), consisting of three connected proteins (Bik, α-1-microglobulin, and a signal peptide) [8, 11, 12]. AMBP is an intracellular protein expressed constitutively and is generally suppressed during acute inflammation. Only trace amount of AMBP is found in blood or urine [13–15].

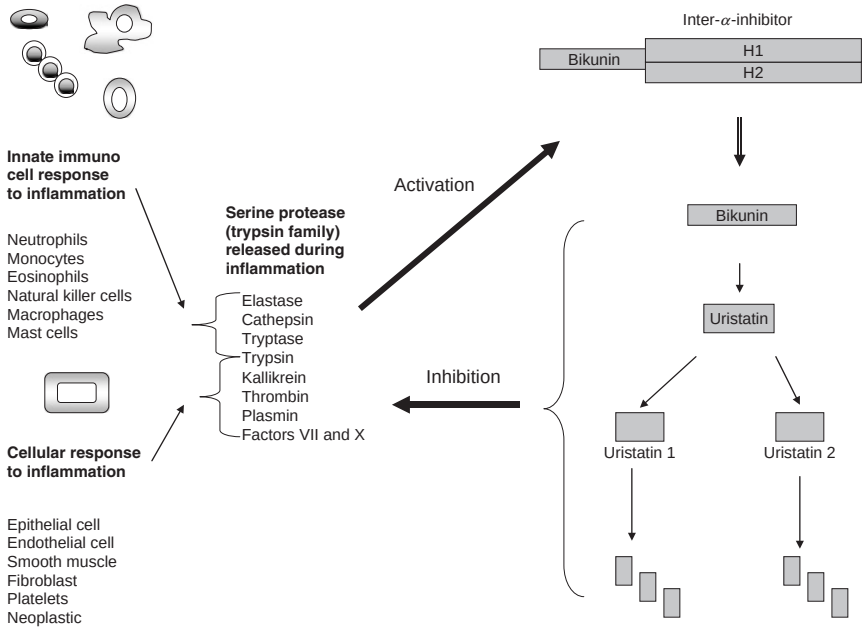

FIG. 1. Summary of the action of serine proteases on normal and abnormal cells, for example cancer cells. Pathogens activate inflammatory cells and foreign serine proteases mediate the invasion of healthy cells. Activated inflammatory cells release a serine protease to destroy pathogens and activate the inflammatory response of normal cells. The inflammatory response is shut down by Bik after release from the inter-α-inhibitor. Above dashed line: Bik is linked to H1 and H2 heavy chains or just an H3 chain; the two forms are found primarily in plasma. Serine protease splits Bik as shown in the figure to form Bik with N- or O-linked glycosides that are primarily found in urine. As Bik fragments further into Uri (various Bik without O-linked glycosides), the amount in urine increases over the amount found in blood.

Glycosylation and sulfation of AMBP occur during transport in the Golgi apparatus. The addition of chondroitin sulfate to Bik is initiated by xylosyltransferase (XT), an enzyme that transfers xylose from UDP-xylose [16]. Association of AMBP with its heavy chains occurs during intracellular biosynthesis in the liver, thus producing the IαI family of proinhibitors. In fact, four different heavy polypeptides chain genes have been identified [8]. The cleavage of α-1-microglobulin from AMBP occurs as the final step prior to IαI release from cell.

Increased serine protease activity is primarily responsible for releasing Bik from the IαI family of proinhibitors [6] (Fig. 1). Molecular weights ranging from 3 to 70 kDa have been observed for Bik depending on degree of fragmentation and glycoconjugation [9, 13–15]. The predominant Bik form

released by elastase has an average predicted molecular weight of about 30 kDa. More than 98% of Bik present in the circulation is bound to the proinhibitors (IαI and PαI) at a normal plasma concentration between 25 and 700 mg/liter [10, 14, 17]. During inflammation, degradation of the IαI by proteases occurs immediately and is correlated to increased white blood cells (WBC) and plasma elastase [10, 14, 17].

Once free from the complex, Bik passes readily into urine with an average half-life of 10 min. The amount of free Bik is $<$7.8 mg/liter in urine and $<$2.5 mg/liter in blood for $>$98% of healthy adults and children [14, 15, 17–19]. Given its potent anti-inflammatory properties, rapid elimination is expected to prevent a shutdown of repair and healing processes. Free Bik in blood up to 20 mg/liter and in urine up to 200 mg/liter correlate well with inflammatory conditions and biomarkers.

Rapid Bik renal clearance is partly due to a glomerular filtration rate that is 80-fold higher than that of albumin [20–22]. This phenomenon is thought to be due to its elongated shape and low molecular weight. Following passage through the glomerular basement membrane, Bik accumulates in the lysosomes of the proximal tubular epithelial cells and is not reabsorbed in the condensing vesicles.

Bik release also occurs when IαI heavy chains are covalently transferred to hyaluronan in the extracellular matrix [23]. This is a major component of cumulus cell–oocyte during fertilization and fibroblasts and mesothelial cells during inflammation. The heavy chains coupled to hyaluronan molecules bind to the cell surface through association with the hyaluronan receptor (CD44). The tumor necrosis factor-stimulated gene 6 (TSG-6) enhances the association of hyaluronan-linked heavy chains with CD44 and increases the release of free Bik [24, 25].

The trypsin family of serine proteases includes over 80 well-characterized enzymes having a minimum sequence homology of $>$27%. Two amino acid residues are absolutely conserved (Cys182, Gly196) within their active sites [26, 27]. These proteases have similar catalytic mechanisms that lead to hydrolysis of ester and amide bonds. This occurs via an acyl transfer mechanism that utilizes proton donation by histidine to the newly formed alcohol or amine group, dissociation and formation of a covalent acyl–enzyme complex.

Bik inhibits the trypsin serine proteases through binding of either of its two Kunitz domains. Depending on the serine protease and the Kunitz domain involved, dissociation constants (K_i) range from $\sim$0.03 to 800 μM [6, 28]. Bik fragmentation and glycation also effect strength and specificity of inhibition. For example, trypsin, chymotrypsin, kallikrein, plasmin, elastase, and cathepsin are inhibited at a K_i of 0.03–3 μM, whereas Factors IXa, Xa, XIa, and XIIa are less inhibited with a K_i of 15–800 μM. Protease inhibition is observed with both Kunitz domains except for Factors IXa and Xa that

are only inhibited by domain II. Bik is released into urine at 0.26–7.0 μM (7.8–200 mg/liter) during the inflammatory period. Bik studies on binding domain and K_i studies have not been done for tryptase or thrombin. Aprotinin has a similar inhibition profile and K_i except for kallikrein (stronger) and Factor XII (weaker) [28, 29].

2.2. Molecular Structure

The Bik glycoprotein has an isoelectric point (p*I*) of 2.1 and is composed of a peptide and two glycoconjugate portions [30]. The predicted peptide sequence of Bik is helpful to understanding protein function. The peptide has Kunitz-binding domains I and II attached to the N-terminal peptide tail [31–33] (Fig. 2). Protein mass spectrometry by surface-enhanced laser desorption ionization (SELDI) in combination with detection by Bik antibodies has demonstrated that substantial variation exists in the Bik molecule [13, 14].

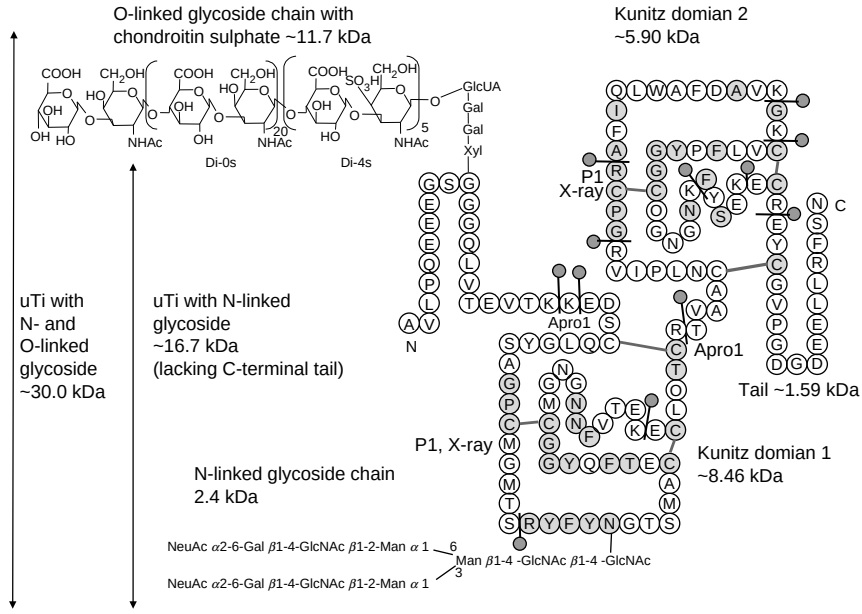

Fig. 2. Glycoside chains and the Kunitz domains. Amino acid and glycoside sequences for the two Kunitz-binding domains of Bik are shown. "C–C" across a chain indicates a cross-link. Predicted fragmentation points for Bik due to trypsin exposure are shown by lines with black dots. The aprotinin-matching peptides of each Kunitz domain are shown by a darkened circle. The first peptide of aprotinin is indicated as "Apro1." The X-ray structure-predicted touch points between Bik and trypsin are marked. The predicted P1 peptide of the active inhibitory site sequence is indicated as well.

Bik inhibitory strength and selectivity for serine proteases vary greatly with peptide sequence and posttranslational modifications such as fragmentation and glycoconjugation [6, 14, 15]. Predicted Bik fragmentation sites via trypsin binding and/or hydrolysis are shown (Fig. 2).

Aprotinin has been widely used to study Kunitz-binding domains including Bik (Fig. 2). The first peptide of aprotinin is labeled as "Apro1." Matching peptides of each Kunitz domain are shown (darkened circles). On the basis of the X-ray crystallographic structure analysis, predicted contact points of aprotinin bound to trypsin are labeled as "X-ray." Interestingly, these positions closely match the inhibitory active site amino acids known as the P1 peptide of aprotinin [34–36]. The active sites have arginine or lysine P1 amino acid peptides that lock into the trypsin pocket in noncleaveable conformation, thus causing inhibition.

The peptides associated with the inhibition site are defined as P3P2P1P1′. The sequence of GPCK or GPCR has been shown to be the strongest binding for trypsin [37]. The arginine or lysine nitrogen is bound into the trypsin active site at the Asp189 carboxylic acid, and the amide carbonyl is bound to the amide nitrogen of Gly193. This predicted peptide sequence is found in the Bik inhibition site for domain II, but not domain I. For domain I, the SI, S2, and S3 pockets of trypsin also play a role in substrate specificity and are thought to be best represented by trypsin peptides 189–195, 214–220, and 225–228, respectively.

The complete sequences of the carbohydrate chains of Bik are known to change during disease progression [31–33]. Both O- and N-linked glycoside chains are connected to Bik in domain I (Fig. 2). A key feature of the O-linked glycoside is the sulfated chondroitin chain. The O-linked chondroitin sulfate glycoconjugate is essential for cell membrane stability and is also involved in cell membrane binding.

Bik has been digested with various glycosidase enzymes to understand the role of the glycoconjugates [38]. Bik exposure to chondrotin ABC lyase removes the O-linked glycan forming uristatin (Uri) [4, 38–40]. Treatment of Bik with metalloendopeptidase generates the N-terminal tail with O-linked glycan from the Kunitz-binding domains. Exposure to neuraminidase effectively removes the sialyl groups on the N-linked glycan. The N-linked gylcan is removed by endoglycanase.

Bik through its O-linked glycoside chain binds with greater affinity to cells, calcium, and TSG-6 [41–43]. The O-linked glycoside core protein without N-linked glycoside is essential for the lysosomal membrane stabilization and urokinase activation by Bik. Interestingly, IαI and PαI are also structurally modified in their glycoside portions [44, 45]. The O-linked glycoside structure is preserved at the 4-sulfated galactose residue in the linkage region of Bik with IαI [46, 47].

The removal of N-linked glycan from Bik results in loss of protection of the kidney cell from necrosis [38]. This phenomenon is, however, not due to changes in protease activity as both O- or N-linked glycans only moderately impact the trypsin inhibitory strength of Bik [38]. Uri, Bik with the N-linked glycan, are a primary urine form [4, 14]. The N-linked glycan has a complex carbohydrate structure shown as a biantennary structure (Fig. 2). However, the true number of glycoforms is unknown. The N-linked glycans play a role in the body's defense mechanisms, have hemagglutination properties through sialic acids, and modulate membrane binding of endothelial cells to monocytes, granulocytes, and lymphocytes. Immunomodulatory activity is dependent on type of glycosylation as well as P- and E-selectin adhesion. Increased sialylation with inflammation or fucosylation with diabetes can additionally modify the glycan.

3. Roles in Biological Processes

The activity of Bik affects various biological functions, including inflammation, cell signaling, and apoptosis.

3.1. Role in Inflammation

As stated earlier, the primary function of Bik is to inhibit serine proteases [28]. These proteases are increased during inflammation and play key biochemical roles in the inflammatory process. Inhibition by Bik imparts anti-inflammatory activity. In all of these processes, the serine proteases initially exist as zymogens in their "pro" or enzymatically inactive forms (e.g., trypsinogen, plasminogen, proelastase, prothrombin, chymotrypsinogen). Enzymatic cleavage of blocking groups establishes their enzymatic activity.

Polymorphonuclear leukocyte elastase is released at sites of inflammation [48–50]. Key elastase homologues, including cathepsin G, proteinase 3, azurocidin, and mycolobastin, have Val-Xaa > Ala-Xaa cleaving affinity [9, 15, 51, 52]. Granules of neutrophils, monocytes, and macrophages contain elastase at high concentration ($\sim$1-pg enzyme per cell). Cytotoxic T lymphocyte, mast cells, and natural and killer cells release other serine proteases such as granzymes (A, B, H, M), tryptase 2 (K), and mast cell proteases 1 [53, 54].

These serine proteases are used to remove pathogens by their hydrolytic activity. They degrade cell membrane proteins and connective tissue matrices by hydrolysis of extracellular matrix proteins such as fibronectin, type IV collagen and laminin, or solubilizing fibrous elastins [55, 56]. Immune cell proteases also are capable of cleaving cytokines, growth hormone, neuropeptides, and procoagulant proteins such as Factors X and V.

During inflammation, degranulation of immune cells releases serine proteases that pass through and bind to the capillary wall. Increased levels of Bik suppress these immune cell proteases and protect the extracellular matrix in arterial walls and connective tissue [4]. Bik inhibits phagocytic destruction of cells. Bik has been shown to inhibit elastases, granzymes A and K [4, 57]. Mast cell tryptases (β- and α-tetrameric forms with a molecular weight of 134 kDa) are resistant to aprotinin [58]. Lymphocytes serine esterase TL2 is not inhibited by Bik [59].

Trypsin is typically considered an enzyme found in the pancreas and small intestine. However, trypsin, thrombin, and plasmin are also widely expressed in endothelium, including epithelial immune cells as well as neurons. Upregulated expression and release occurs during both acute and chronic inflammation [60]. Autocrine release of trypsin and thrombin causes activation of protease-activated receptors (PARs) reaction leading to cellular proliferation and inflammation [4]. This response includes release of proteins by all cells during chronic inflammation. Bik prevents PAR activation on cell surfaces.

Coagulation is part of tissue regeneration during inflammation. The vascular endothelium and muscle cells release a host of coagulation serine proteases such as plasmin, thrombin, and Factors VII and X. The role of proteases in the activation of the coagulation cascade is known to be a fundamental part of blood coagulation, fibrinolysis, complement activation, and extracellular matrix assembly. Factors VII and X cause cleavage of prothrombin to thrombin during coagulation. Thrombin forms insoluble fibrin from fibrinogen, forming a fibrous mesh (clot) in which blood cells are trapped. Bik inhibits blood coagulation through its action on plasmin, Factors IXa, Xa, XIa, and XIIa [61–63]. Plasmin indirectly causes platelet aggregation and endothelial cell proliferation, and is expressed during metastasis [64].

Muscle cells release kallikrein during inflammation causing formation of active kinin peptides (bradykinin and kallidin) from kininogen [65, 66]. Kinins are peptide hormones that produce vasodilation, increase capillary permeability, and cause pain and infiltration of neutrophils. There is a direct correlation between the amount of kinin in plasma or tissues and the degree of inflammation. Vascular dilation causes increased blood flow to infection [67, 68]. Bik inhibits formation of kinins and vascular dilation by kallikrein, thereby inhibiting smooth muscle contraction [69–71].

Finally, serine protease from parasites and pathogens can mimic the biological roles of proteases in normal cells. Release of protease from parasites and pathogens can activate cells and produce an inflammatory response. Many pathogens and parasites contain cathepsins and elastases along with many of other serine or serine-like proteases like *Streptomyces griseus A* or *B* and α-lytic protease that lead to the invasion of abnormal cells and tissue

destruction [4]. Additionally, gingipains or bacterial cysteine proteases have trypsin-like activity.

3.2. Bik in Cell Signaling

During acute infection and injury, PARs contribute to normal cell repair and the healing process. In persistent disease, PAR contributes to cellular changes as part of the chronic inflammatory defense [55, 56]. These receptors promote the hallmarks of inflammation such as swelling, redness, heat, pain, and tissue repair [4, 72]. This subgroup of the G–protein-coupled receptors uses proteases to initiate cell signaling via specific cleavage sites for thrombin, trypsin, and possibly other serine proteases [73]. Thrombin activates PAR1, PAR3, and PAR4, whereas trypsin activates PAR2 and PAR4. Tryptase, released from mast cells, activates PAR2. Any activated PAR leads to a cell signal cascade that affects cell shape, secretion, integrin activation, inflammatory response, transcriptional response, and increased cell motility. Trypsin also acts on PARs present on endothelial and epithelial cell surfaces [74–76].

PARs are single-use receptors that rely on proteolytic cleavage for activation. Trypsin and thrombin cleave PAR to expose the tethered ligand domain (SLIGRL-NH_2 or SLIGKV-NH_2) causing receptor activation [77]. Cleavage is dependent on the presence of associated proteins that anchor the proteases to the plasma membrane promoting localization on the cell surface. For example, tissue factor is needed for coagulation Factor VIIa to cleave PAR2. Following injury, cell proliferation and differentiation increase after serine protease exposure. Cell synthesis is activated through the p38 mitogen-activated protein kinase (MAPK) activation, protein kinase A and B (PKA, PKB) signaling [78, 80]. Signal transduction occurs through phospholipase C for MAPK and PKB and adenyl cyclase for PKA. Additionally, the intracellular C-terminus of PAR interacts with creatine kinase activating Rho kinase.

Aprotinin has been shown to prevent activation of PAR with reduction of cell proliferation and signaling [72]. In contrast to traditional receptors that sense through binding, proteolytic activation is irreversible and the cleaved receptors are degraded in lysosomes. Exposure to increased concentration of serine proteases will ultimately cleave all PARs on the cell surface [79, 80]. Cellular resensitization occurs with mobilization of PAR from Golgi storage and synthesis of new receptors. Continual destruction of PAR leads to unresponsive cells that are unable to produce new receptors. Given this mechanism of action, it is unlikely that PAR mediates routine intercellular signaling. Instead, they play important roles during less-frequent events such as coagulation or during mast cell degranulation. PARs are present on endothelial cells, epithelial cells, platelets, mast cells, neutrophils,

lymphocytes, neurons, astrocytes, and microglia [80]. Both intra- and extracellular serine proteases can signal these cell types by cleaving and activating PAR [55].

3.3. BIK IN APOPTOSIS

During chronic inflammatory disease, inflammatory cells (neutrophils, mast cells, macrophages, and lymphocytes) become increasingly more damaging to tissues. Anti-inflammatory action of Bik reduces cell death mediated by immune cell. Proinflammatory cytokine tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1) cause expression of multiple inflammatory and innate immunity genes for additional cytokines, chemokines, adhesion molecules, and enzymes. Aprotinin has been reported to cause a reduction in apoptosis *in vivo* by decreasing inflammatory cytokine expression (IL-1, IL-6, and TNF-α) thus preventing caspase-8 activation [81].

Serine proteases, released from immune cell granules, process cytokines and growth factors that control multiple cellular process [56]. Proteinase 3, cathepsin G, and elastase all cleave membrane-bound TNF-α, IL-1, and IL-18, and activate epidermal growth factor receptor (EGFR) and toll-like receptor-4 (TLR-4). These actions inhibit growth and lead to apoptosis with transcriptional nuclear factor κB (NF-κB) inactivation. Bik suppresses release of TNF-α, IL-1, and IL-18 and prevents EGFR and TLR-4 activation. Activation of NF-κB is a mediator of cell proliferation, whereas inhibition of NF-κB leads to apoptosis [82]. Overall, Bik inhibition of immune cell serine proteases increases cell proliferation and stability.

Bik also signals cells through changes to the intracellular electrolyte balance on binding to extracellular membrane walls. Calcium-activated potassium channels are inhibited through the aprotinin peptide of Bik [36, 42, 71]. Bik binding causes channel blockage via partial occlusion, repulsion, or allosteric interaction. The contractile state of smooth muscle cells is regulated by a negative feedback mechanism involving the contracting influence of calcium channels counterbalanced by the relaxing influence of the calcium-activated potassium channel. Lower intracellular calcium is known to reduce MAPK activation and cell proliferation. Intracellular prostaglandin synthesis is also reduced through calcium-dependent cytosolic phospholipase A_2 [80].

4. Diagnostic Testing

Various analytical approaches, including enzyme inhibition assays and immunoassays, have been described for Bik determination.

For enzyme inhibition assays, urine is the preferred specimen [4]. Interestingly, Bik can be measured by the inhibition of trypsin in urine but not in plasma. Urinary Bik analysis may also be performed by antibody staining, latex agglutination, and radioimmunoassay (RIA) [4]. Despite the analytical approach used, all Bik forms are measured together. The enzyme inhibition method involves adding known amounts of trypsin to the specimen and monitoring trypsin inhibition. Trypsin activity is assessed by detection of by-products from a cleavable substrate. Dipstick methods are available for the rapid detection of trypsin inhibitors in urine [15, 17–19].

Immunoassays for Bik, based on polyclonal antibodies (pAb), are affected by cross-reaction with Tamm–Horsfall protein (THP). This problem can lead to the generation of false positive results in cases of proteinuria [14]. In contrast, immunoassays that utilize plasma suffer from cross-reactivity to IαI [23]. The cross-reactivity with THP is due to complexed N-linked glycan, whereas cross-reactivity with IαI is due to bound Bik [14]. Cross-reaction with α-1-glycoprotein (AGP) also does not appear to be a significant factor in blood.

Monoclonal antibodies generated with purified Uri eliminated cross-reaction to THP and IαI in urine and plasma, respectively. Antibodies directed at the N-linked glycan allowed measurement of Bik in blood without IαI cross-reactivity. Antibodies directed at the peptide allowed measurement of urinary Bik without THP cross-reactivity. These antibodies do not cross-react with aprotinin. These antibodies allow estimating the IαI family in blood.

Despite its lack of specificity, Bik determination has been shown to correlate well ($p < 0.01$) with other indexes of inflammation, including C-reactive protein (CRP), WBC count, and erythrocyte sedimentation rate (ESR) [4]. The urine strip is an alternative to a blood CRP measurement. The Bik test was more predictive of upper respiratory and urinary tract infections as well as kidney diseases. Furthermore, it was more sensitive to bacterial and viral infection vs CRP. Because plasma proteins, that is CRP, are not cleared and circulate until hepatic metabolism, urinary Bik appears to be a better predictor of an abnormal WBC, ESR, and neutrophil degranulation.

Receiver operator curves (ROC) have demonstrated the superiority of urinary Bik vs CRP in predicting vascular inflammation, viral and bacterial infection. Bik determination by immunoassay is better able to separate patients with inflammation, that is fewer false positives and higher correlation to CRP and WBC, vs enzyme inhibition methods. Urinary IL-8 activity is also increased in acute and active inflammatory conditions and correlates positively with inflammatory markers.

5. Pathophysiology of Bik

5.1. Normal Populations

Bik analysis with dry chemistry strips has facilitated population studies for various groups and ages [15, 17–19]. Screening of presumed healthy school children and adults showed that Bik was associated with inflammation and/or infection (Table 1). Reference ranges for general and specific populations have been established for daily and hourly excretion rates [4]. In normal children, the interday excretion of Bik is fairly constant (Table 1). Approximately 50% of patients with fever were positive for Bik. Dividing the urine concentration of Bik with the urine creatinine value gives a good estimate of the basal Uri concentration in randomly collected urines [4]. These values agree well with those from a 24-hour urine collection. Immunosuppressed patients with AIDS or those on chemotherapy with suppressed WBC usually do not show increased Bik [4].

5.2. Pregnancy

Bik is normally elevated during pregnancy to prevent premature labor [4]. Clinically, Bik has been administered as a therapeutic agent to prevent premature labor. The expression of Bik decreases in preparation for labor as the quiescent uterine smooth muscle switches to a state of contractility. The mechanism by which Bik prevents premature labor is partly due to an inhibition of smooth muscle Ca^{2+} influx [83, 84].

5.3. Infection

Inflammation is a common component associated with sepsis, meningitis, as well as respiratory tract, urinary tract, viral, and bacterial infections (Table 1). Bik is elevated during bacterial or viral infection. The presence of urinary Bik correlates well with standard urinalysis tests for urinary tract infections [20]. Endotoxins released from infectious pathogens induce inflammation and immune cell activation. Macrophages release interleukins and cytokines (IL-1, IL-6, IL-12, IL-15, IL-18, TNF-α) on exposure to lipopolysaccharide (LPS) and lipoteichoic acid (LTA) endotoxins. These cytokines act as a chemotactic factors causing immune cell migration to the site of the infection followed by activation and release of proteases. Cytokines also induce increased vascular permeability in the endothelial. Bik suppresses further cytokine release by protease and intern additional migration and activation of immune cells. Additionally, a stabilization of the immune cell membrane prevents further release of proteases [4].

TABLE 1
CONDITIONS IN WHICH URINARY TRYPSIN INHIBITORS ARE INCREASED [a,b]

Conditions	Studied
Acute inflammation	Acute viral infections
	Kidney stones
	Preeclampsia
	Surgical trauma
	Transplant rejection
	Myocardial infarction
	CHF
	Pancreatitis
	Trauma
Cancer	Breast
	Colon
	Esophagus
	Leukemia, all types
	Lymphoma, all types
	Multiple myeloma
	Ovarian cancer
	Pancreas
	Stomach
Chronic inflammation	Acute coronary syndrome
	Crohn's disease
	Emphysema
	Hepatitis
	Inflammatory bowel disease
	Rheumatoid arthritis
	Systemic lupus erythematosus
Infection	Appendicitis
	Bacterial meningitis
	Bacterial sepsis or infections
	Pneumonia
	Upper respiratory tract infection
	Urinary tract infections
Kidney disease	Amyloidosis
	Tubular disease
	Glomerulonephritis

[a] Increased in bacterial infections such as pneumonia, upper respiratory tract infection, bacterial meningitis, tonsillitis, gastroenteritis, enterocolitis, streptococcal infection, mononucleosis, lymphadenitis, conjunctivitis, and whooping cough.

[b] Increased in severe viral infections such as mumps, varicella (chicken pox), influenza A and B, common cold, viral meningitis, infectious mononucleosis, measles (rubeola), or rotavirus-V enteritis. Severe viral infections are defined by increased lymphocyte count.

5.4. CANCER

During invasion and metastasis by malignant cells, proteolytic enzymes are required to disrupt the basement membrane [85–88]. The proteases plasmin and cathepsin are key enzymes used by invading cancer cells. Both proteases are directly inhibited by Bik. Cancer cells use cell-bound plasmin to activate the plasminogen signaling for urokinase. Bik binds to the cell wall and prevents cell-bound plasmin activation. Bik suppresses tumor invasion in the lungs, lymphatics, and ovaries [4]. Affected cells will express Bik and slow invasion by inhibition of cancer cell wall-bound plasmin. Bik is also released from the IαI by plasmin expressed on the surface of cancer cells. Increased levels of Bik have also been found in urine of patients with hematologic malignancies such as multiple myeloma, Hodgkin's and non-Hodgkin's lymphoma, and leukemia [4, 89]. In these cancers, the WBC count is elevated causing increased free elastase in the circulation. Urinary Bik correlates strongly with the presence of Bence-Jones protein in multiple myeloma. Bik can be formed directly by malignant cells or as the result of increased elastase. The former typically predominates since clinical time course shows increased WBC with reduced Bik. However, Bik levels in urine change in parallel with cancer cell number.

5.5. SURGERY

Inflammation due to surgery induces Bik in parallel with tissue damage [4, 90]. Bik usually continues to rise during the course of trauma. As an acute-phase indicator, Bik is generated at the site of cellular injury (Table 2). The rapid rate of Bik formation is due to the presence of its proinhibitor form at sites of inflammation. In organ transplantation, urinary Bik levels increase on the day of surgery and peak on or about the third day following surgery when liver function is normal. By the seventh day, urinary Bik levels usually decrease to basal levels. Following surgery, changes in the Bik are more gradual than traditional inflammatory serum markers.

5.6. KIDNEY DISEASES

Glomerulonephritis is the major cause of renal injury leading to failure and is typically associated with infection [4]. In glomerulonephritis, neutrophil polymorphonuclear leukocytes and macrophages cause capillary wall injury mediated by protease release [91]. The proteases elastase and cathespin are known to damage the basement membrane leading to proteinuria due to disrupted network structure and charge barrier [92]. Platelet coagulation and red blood cells (RBC) increase permeability of the basement membrane to proteins [93]. Stabilization of kidney cell membranes occurs on exposure to Bik, causing decreased N-acetyl-D-glucosaminidase (NAG) release due to

TABLE 2
BIK EFFECTS ON CELLULAR RESPONSE TO INFLAMMATION

Cells	Biological/pathological events	Events triggered by PAR activation
Endothelial cells	Chronic proinflammatory response	Leukocyte infiltration (rolling and adhesion), vascular dilation, inflammation mediator release (e.g., histamine, cytokines, eicosanoids)
Epithelial cells	Mucosal protection	Fluid and electrolyte balance, mucosal secretion, and protection
Fibroblast	Healing and repair (hemostasis)	Cell proliferation
Inflammatory cells: mast cells, lymphocytes, neutrophils	Acute proinflammation	Leukocyte infiltration (rolling and adhesion), vascular dilation, inflammation mediator release (e.g., histamine, cytokines, eicosanoids)
Neurons	Hyperalgesia	Formation of neuropeptides
Platelets	Clotting	Coagulation
Sensory nerve endings	Neurogenic inflammation	Formation of neuropeptides and calcitonin gene and related peptide leading to recruitment of granulocytes
Smooth muscle and fibroblasts	Healing and repair (hemostasis)	Contraction of smooth muscle, proliferation of fibroblasts

cell necrosis [38]. Because cell stabilization required the O-linked glycan, this phenomenon was not observed with Bik-lacking glycans [94].

Anti-inflammatory activity of Bik is highly correlated to glomerulonephritis [4, 95, 96]. Bik provides protection to renal cells from ischemia/reperfusion injury by reducing immune-mediated apoptotic signals that typically lead to cell death [4, 30, 81]. Bik also has a protective affect on proximal tubule epithelial cells under stress [97]. Bik levels increase with α-1-microglobulin during renal tubule damage [4].

Glomerular lesions, such as those found in diabetes and glomerular nephritis, are characterized by basement membrane thickening and an increase in collagen-like substances within the mesangial regions that ultimately lead to proteinuria. Protease inhibitors prevent thickening of the basement membrane and reduce proteinuria.

5.7. VASCULAR DISEASE AND COAGULATION

Inflammation leads to vasodilation that damages the endothelial and epithelial layers, thus promoting vascular disease [4]. Kallikrein, neutrophil elastase, and mast cell tryptase release kinins from kininogens. Kinins are

vascular dilators that regulate blood pressure, affect sodium homeostasis, and alter renal and cardiac function. Increased concentration of kinins leads to increased dilation and decreased blood pressure [98]. Vascular damage and ischemia/reperfusion injury increase with dilation due to neutrophil chemotaxis and adherence to the endothelium and basement membrane.

Bik decreases ischemia/reperfusion injury by inhibiting proteases that cause kinin release [4, 99]. Reversion to a normal blood pressure occurs in two ways: through inhibition of kallikrein with protease inhibitors and by destruction of kinins by kinase. Bik decreases kinin formation through their effect on kallikrein. The duration of kinin formation and destruction ranges from 2 to 30 min [100, 101]. After 30 min, little kinin activity is detectable. As inflammation abates, so does neutrophil chemotaxis and endothelial adherence to the basement membrane. PAR also regulates vascular tone and participates in response to vascular injury. Bik inhibits PAR activation [79, 80].

Multiple factors are involved in the coagulation cascade with Factors VII and X playing critical roles [102]. Factor X cleaves prothrombin into thrombin that in turn activates conversion of fibrinogen into fibrin. Bik has a protective effect against disseminated intravascular coagulation (DIC) during coronary artery bypass grafting surgery (CABG) [4]. Fibrin degradation products, fibrinogen concentrations, prothrombin time, partial thromboplastin time, platelet counts, and the number of renal glomeruli with fibrin–thrombin move toward normal values as Bik causes inhibition of coagulation factors, fibrinolysis, and platelet aggregation.

5.8. DIABETES

Chronic inflammation is often associated with diabetes mellitus and autoimmune disorders such as rheumatoid arthritis and organ failure. Hyperinsulinemia increases WBC and elastase [103, 104]. Excess heavy chains can result due to uncoupling of Bik from the cell matrix during chronic inflammation. PAR-triggered cells appear to be a primary cause of gene expression polymorphism and likely precede detectable abnormalities within damaged cells.

The trypsin family of proteases plays a role in acute and chronic pancreatitis, as well as leads to its ultimate destruction [4, 105]. In pancreatitis, active exocrine enzymes are prematurely released inside the pancreatic duct. Various factors can contribute to the development of acute pancreatitis. Trypsinogen, chymotrypsinogen, procarboxypeptidase, and proelastase are inactive proforms of proteolytic enzymes produced by the pancreatic acinar cells. Following secretion these enzymes are activated in a cascade that converts trypsinogen to trypsin in the duodenum and/or small intestine.

Early activation of the enzyme in the pancreas leads to autodigestion, acute hemorrhage, and necrosis [4]. Trypsin in the small bowel converts all proforms (including trypsinogen) to their active forms. Bik protects acinar and endocrine pancreatic cells from self-digestion. Factors that prevent premature trypsin release and injury to the pancreas include intracellular localization of zymogens, sustained rise in extracellular calcium, breakdown of F-actin, and activation of the transcription factor NF-κB. Pancreatitis may lead to a hyperstimulation of the immune system resulting in distant organ damage, especially the lungs. In addition, Bik inhibition of enteropeptidase release disrupts the digestive hydrolase cascade [33].

6. Summary

uTis are a distinct group of Bik protease inhibitors that are central to the body's innate anti-inflammatory response. Bik provides a measure of acute and chronic inflammatory conditions and allows insight to the cellular response to inflammation. It is therefore plausible that screening for Bik especially in the urine may provide a diagnostic tool for assessing inflammation.

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GENE EXPRESSION ASSAYS

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1. Abstract

The study of the profile of gene expression in a cell or tissue at a particular moment gives an insight into the plans of the cell for protein synthesis. Recent technological advances make it possible to analyze the expression of the entire genome in a single experiment. These ''gene expression assays'' complement or replace previous assays which measured the gene expression of only one gene, or a select group of genes. Within this chapter we outline the development of the gene expression assay and provide examples of the wide range of disciplines in which it is used. An overview of the current technologies is given, and includes an introduction to laser capture microdissection and linear amplification of RNA, both of which have extended the application of gene expression assays. Illustrative examples in the field of cancer and neuroscience highlight the scientific achievements. This technology has made in understanding the pathogenesis of diseases, including breast cancer, Huntington's disease, and schizophrenia. With recent advances including exon arrays to investigate alternative splicing, tiling arrays to investigate novel transcription start sites, and on-chip chromatin immunoprecipitation to investigate DNA–protein interactions, the future of gene expression assays is set to further our understanding of the complexities of gene expression.

2. Introduction

Transcriptomics, or the study of the complete set of RNA transcripts present at a specified moment in a cell or tissue, provides an insight into the cell's plans for protein synthesis in response to the current cellular environment. Following recent technological advances, it is now possible to analyze the gene expression of the whole genome in a single hybridization experiment. These assays provide far more information, far more quickly, than the conventional gene expression assay—the Northern blot—where expression levels of a single RNA are determined by hybridization of a labeled complementary DNA sequence, and the results obtained following 2–5 days of autoradiography.

Evolution of the gene expression assay has produced the cDNA and oligonucleotide microarray. Originally described in *Science* in 1991 [1], the first microarray study monitored the differential expression of 45 *Arabidopsis* genes [2]. By labeling two separate RNA samples with different color fluorophores, following hybridization to cDNA sequences spotted onto a glass substrate, the relative fluorescence represented the level of gene expression in the two samples. The following year saw the use of microarrays to monitor gene expression of 1046 human genes [3] and 864 yeast genes [4],

in addition to the first application of microarrays to cancer, with the analysis of the expression pattern of 1161 genes in a human cancer cell line [5]. Following the commercial release of microarrays in 1996, the number of microarray publications on PubMed has grown from 21 in 1998 to 834 in 2001 and 4453 in 2005.

The applications of this technology span all fields of biology. Within the medical sciences, studies compare disease with normal samples to elucidate the mechanisms of disease pathogenesis [6–8], and comparison of heterogeneous clinical phenotypes identifies subtypes of disease, which may have implications for prognosis [9, 10] and treatment [11, 12]. Following treatment with a drug, the response, in terms of differential gene expression, can be determined, allowing understanding of the molecular mechanisms and the specificity of action of the drug [13]. More basic science applications include treatment of cells with an environmental stimulus, such as a heat shock, to determine pathways involved in the response [14], or microarray analysis following gene knock out, to determine the functions of the gene [15]. In addition, comparing samples at different time points to identify temporal changes, such as in development [16] or ageing [17] or comparing two different cell (or tissue) types to identify specific markers for that cell type [18], can further our understanding of basic biological mechanisms.

Other examples where microarray analyses have been used in a wider range of disciplines include: (1) in nutritional and food sciences, the effect of soy protein in the diet [19] and the detection of microbial pathogens in food [20]; (2) in microbiology, the mechanisms of host–pathogen interaction [21] and antibiotic action and resistance [22]; and (3) in plant sciences, the mechanisms of steroid hormone signaling [23] and elucidation of the response to low temperatures [24]. In this last example, in addition to understanding the basic biology of how plants survive at freezing temperatures, the results also have practical applications when designing strategies to improve cold tolerance in crop varieties.

A complete review of all the current gene expression assays is beyond the scope of this chapter, given the range of disciplines and variety of experiments. Therefore, this chapter will begin by giving an overview of the current technologies available, and will also comment on the development of laser capture microdissection (LCM) and linear amplification of RNA, which has extended the use of microarrays to specific cell types and smaller sample sizes. It will then focus on the applications and achievements made in cancer biology, and in neurological and neuropsychiatric diseases, using illustrative examples. Finally, with the advent of the exon arrays, and the potential for predictive diagnostics using characteristic expression patterns, termed ''gene signatures,'' the future for microarrays and their potential clinical application will be discussed.

3. The Technology

There are a number of methods available for detecting and quantifying gene expression levels such as Northern blots, S1 nuclease protection assays, differential display, and serial analysis of gene expression (SAGE) [25]. However, such methods are often labor intensive, rather complex and designed to analyze only a small number of genes at a time. They have been augmented with the development of array-based technologies which expand the number of genes. It is possible to analyze at one time. Each of these technologies enables the analysis of two aspects of gene expression; both the static measure within the cell or tissue in which a particular gene is expressed and the dynamic measure of the relative expression of a particular gene in different cells or tissues [25]. Such analyses have developed from the early spotted arrays of the late 1980s and early 1990s [26, 27]. They have been described as multiple Northern or Southern blots, but the technology is now far more advanced than the early filter-based assays, to a level which was unimaginable when the first filters were produced. The DNA expression chip is a gridded array of nucleic acid species bonded to a flat, solid support, typically glass, generally not larger than a microscope slide. The ordered arrangement of the nucleic acid sequences on the chip surface, termed "probes," allows for parallel analysis of a multitude of hybridization events occurring between the various probes and the sample, or "target" material [28] which allows for the profiling of expression of tens of thousands of genes in a single experiment [25]. Essentially, DNA expression arrays continue to exploit the sequence complementarity of the two DNA strands and the fidelity of the hybridization of nucleotides to their base-pairing complementary nucleotides [29]. An important factor in the development of the microarray technology has been the move to a solid support for the probe sequences. This provides several advantages; the target nucleic acid can make immediate contact with the probe (without any potential for diffusing into the substrate), and the solid support improves the efficiency of the post-hybridization washing steps due to the elimination of the potential for diffusion. Interestingly, these factors simplify the calculation of the hybridization kinetics which need to be considered when designing the array [29]. For the remainder of this chapter, we shall focus the discussion on the solid substrate-based technologies.

3.1. Manufacturing Arrays

There are a number of alternative methodologies available to the array manufacturer. In the first instance, the array can hold cDNA or oligonucleotide probes. Second, the arrays can be produced by spotting the probe

directly onto the substrate or using a process to build the probes *in situ*. A convention has arisen wherein the arrayed material is described as the ''probe'' and the RNA species which is hybridized to the array is known as the ''target.'' This nomenclature is derived from the terminology used in Northern blots where the sequence applied to the blot was known as the probe and the probes are now represented many times on the array [30].

The probes for cDNA arrays are normally polymerase chain reaction (PCR) products of 0.6–2.4 kb in length [25], generated using vector- or gene-specific primers from cDNA libraries or clone collections. Once prepared and appropriately cleaned up, the cDNA probes are printed onto the substrate at defined locations. cDNA probes generated by PCR need partial purification to remove unwanted contaminants such as salts, detergents, PCR primers, and proteins which are present in the PCR reaction mixture. The cDNA spots typically contain a few nanoliters of a product at 100- to 500-ng/μl spaced 100- to 300-μm apart and equidistantly. Using this technology, arrays can be constructed with more than 30,000 cDNAs on a single microscope slide [30]. The spotting is, wherever possible, carried out by a robot and the technology applied relies on a nib, piezo-, or ink-jet spotter. The cDNA-spotted arrays suffer slightly from at least two drawbacks: first, the relatively large size of the probe sequence can create a stearic hindrance and reduce accessibility of the probe to the target [28] and second, when measuring expression of members of gene families, the cDNA approach can lack the resolution necessary to distinguish between family members with high sequence similarity [28].

The use of short oligonucleotides as the probe sequence has some advantages. Bioinformatic sources are crucial to the probe design and they can be highly specific. The development of the various genome projects to define the genome of different organisms has provided a wealth of information that is used to inform the probe design process [25]. The length of the oligonucleotide used as a probe sequence depends on the technique used to derive the oligo. It was found that the photolithographic process as used by Affymetrix [31] becomes less efficient when long oligonucleotides are being prepared. For this and other reasons, the Affymetrix probes are 25-mers [26] and the company has attempted to overcome the potential lack of specificity of the shorter probes by including multiple probes for each gene and a perfect match/mismatch strategy. This feature provides a probe which is designed to match a target sequence exactly and a partner, mismatch, probe which is manufactured with a nonmatching base at the middle (position 13) of the oligonucleotide. An important feature of the use of short oligos as probes is that these molecules enable the discrimination of single mismatched base pairs [29]. This is advantageous both in the subsequent analysis of the Affymetrix GeneChip arrays where the relative levels of hybridization

between the perfect match and the mismatch are used to generate a "Present call" but also when applied to the novel resequencing and single nucleotide polymorphism arrays that are available from Affymetrix. The manufacturers such as Agilent, who have adopted an ink-jet spotting process for their oligonucleotide synthesis, are able to build longer molecules efficiently and have settled on a 60-mer length oligo for their probes [26]. These provide greater sensitivity than the shorter probes but the increased length can be a hindrance in the design process. Other technologies are available where presynthesized oligonucleotides can be spotted onto the arrays, micromirrors can be used to create the oligos *in situ* (as used by Nimblegen and Febit) or oligonucleotides can be built onto beads (as used by Illumina). These different strategies have been well described by Stoughton [26] and Knudsen [32]. The process of *in situ* synthesis has certain advantages over the spotting of sequences onto an array. The yields of probe are high and consistent between fabrications. However, it can be more difficult to carry out a full quality control on the products produced this way compared to synthesized oligos [29].

3.2. Laser Capture Microdisection and Linear Amplification of RNA

Whatever design method is used, the DNA microarrays can be used to examine global expression in tissues or cells comparing, for example, affected vs unaffected or treated vs untreated samples. The cDNA arrays can be used in circumstances where no sequence information is available by using anonymous clones from cDNA libraries [28]. These assays are most applicable to simple cells, particularly from cultured material because they rely on good quality RNA being available from the material under investigation. There is, however, increasing use of material from tissue collected from tumors or postmortem. This brings with it the added problem of tissue heterogeneity. A method for unambiguous analysis of individual cells from within the tissue is to utilize the process of LCM [18, 33], to isolate the cells of interest, and to extract the RNA from these alone. However, this process reveals an additional problem—that of RNA quantity. In the normal process of preparing the labeled target for application to the DNA chips, there is a necessity for a modest amplification as well as incorporation of label such as Cy5, Cy3, or biotin (Fig. 1) [26]. When RNA quantity is not limiting, then fluorescent dyes can be incorporated in the first-strand synthesis of cDNA [30]. However, the use of primary cells and LCM material means the quantity of RNA is more limited and procedures have been developed to increase sensitivity and reduce the quantity of RNA required. In particular, amplification of the target RNA by one or two rounds of *in vitro* transcription has resulted in a situation where it is possible to produce sufficient labeled antisense RNA

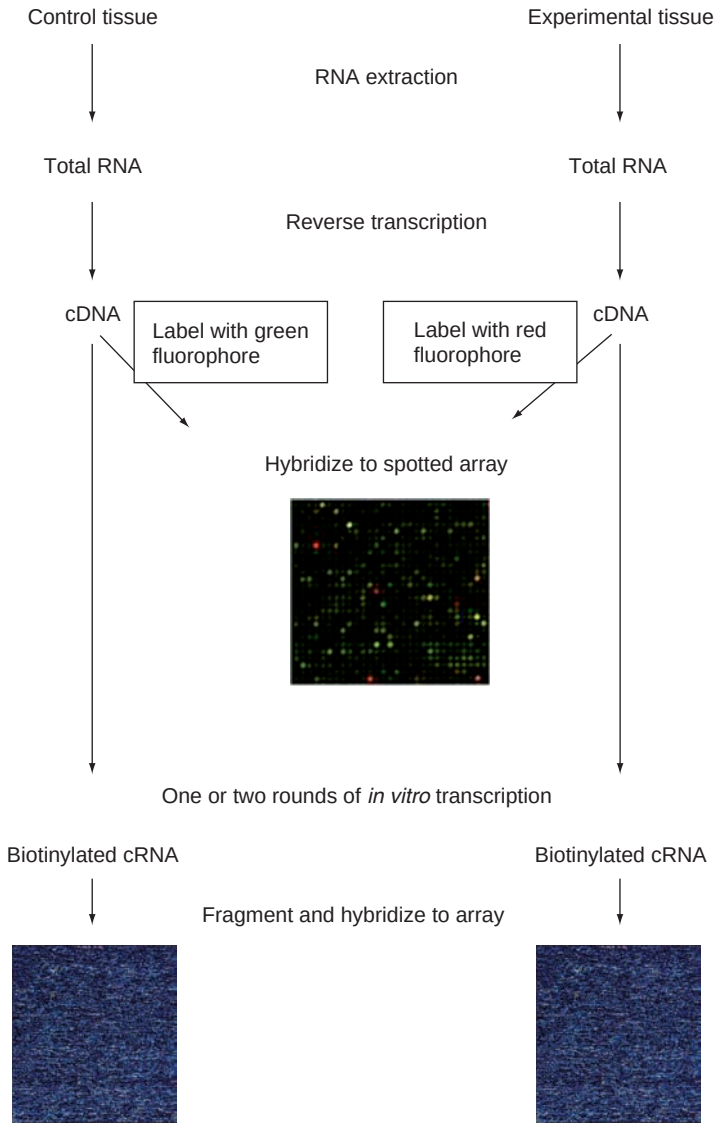

FIG. 1. Preparation of the microarray target.

from 10 ng of input RNA to apply to a microarray [30, 34, 35]. There are also other processes of amplification where PCR-based strategies are used to amplify the target or post-hybridization amplification is achieved using labeled antibodies or multiple-labeled fluorophores [26]. Whichever method

is employed, it is important to monitor the linearity of the amplification process because any nonlinear amplification could introduce a marked bias in the results.

3.3. Hybridization and Data Analysis

During the process of hybridization, the complementary target sequences will find their probe preferentially over noncomplementary or mismatched sequences. These hybridization events can be affected by altering the fundamental parameters of time, stringency, concentration, and complexity of sample, in addition to density of available probes [26]. These processes are also influenced by the secondary effects of probe length, dye chemistry, and surface chemistry. The target sequence will attach and dissociate several times during the hybridization reaction. The kinetics of the hybridization reaction are modified to increase the time the target remains bound in well-matched duplexes. As with any nucleic acid hybridization reaction, the most specific hybridization is produced with long incubations at high stringency. These processes can be speeded up with agitation, buffers, surfactants, and acoustic sonication [26]. Following high stringency washes, the level of hybridization is quantitated by measuring the fluorescent signal and the data are then adjusted to remove the background level of non-specific hybridization. Before comparing different arrays, the signals are normalized, most commonly using a global normalization strategy. However, it is possible within most software programs to specify particular invariant calibrator genes, although these do not always correspond to the classical ''housekeeping genes'' of actin, glyceraldehyde-3-phosphate dehydrogenase, and clathrin.

The ultimate aim of the experiment is to quantify large numbers of mRNA transcripts, in order to provide a detailed insight into the cellular processes being investigated [30]. This has necessitated a development of sophisticated methods of data analysis. Numerous different algorithms are available to obtain the list of differentially expressed genes, including robust microarray analysis (RMA), GC-RMA (which is based on RMA but takes into account the GC content of the probe), probe logarithmic intensity error (PLIER), DNA-Chip analyzer (dCHIP), and statistical analysis of microarrays (SAM). Some of these are free software downloads (dCHIP, SAM), while others are options in commercially available software programs such as ArrayAssist (Iobion) and GeneSpring (Silicon Genetics) (Table 1). The expression profile of sample A1 can either be compared in a pairwise fashion with B1 or replicate data can be analyzed by a multiple comparison, either using the average of replicates from sample A and comparing them with the average of replicates from sample B, that is, average (A1, A2, A3) vs average (B1, B2, B3) or by using a nine-way analysis, where each replicate of sample A is

TABLE 1
GENE EXPRESSION ASSAYS ANALYSIS TOOLS

Commercial tools	Supplier
Software	
Array Assist	Stratagene
Gene Spring	Agilent
Gene Sifter	Gene Sifter
Gene Maths XT	Applied Maths
Gene Sight	BioDiscovery
Expressionist	Gene Data
Chip Inspector	Genomatix
Teragenomic	IMC
JMP microarray	SAS
Genowiz	Ocimum Biosolutions
Genomics Suite	Partek
Resolver	Rosetta
Decision Site	Spotfire
Avadis	Strand Genomics
Freeware	
http://genome-www5.stanford.edu/resources/restech.shtml	A thorough website outlining many of the freely available analytical tools

compared with each replicate of sample B (A1 vs B1, A1 vs B2, A1 vs B3, and so on). Each of these strategies will provide the researcher with a list of genes differentially expressed between the two samples, following the setting of criteria within the software as to what is considered "differentially expressed," for example, a twofold change and significance level $p < 0.05$.

Most microarray experiments generate a large amount of data which needs to be fully investigated to uncover the meaningful results. The biological significance of the changes needs to be investigated and the results verified using an alternative technique such as real-time PCR, *in situ* hybridization, or analysis of protein expression. However, the sheer volume of data means that it is important to recognize at a very early stage in an array experiment the importance of a stringent experimental design [32]. The technology remains expensive and the scientist needs to consider carefully the question(s) being asked before embarking on an experiment, in order to be confident that the analysis will produce a meaningful result.

Microarrays provide a global view of gene expression which has proved useful in identifying genes that share a common expression pattern in response to a particular stimulus. This is made more apparent when clustering algorithms are used to interrogate the data such as hierachical clustering, principal component analysis (PCA), and self-organizing maps (SOM). A particular

value of this type of analysis has been the ability to identify genes which share a common expression pattern in the experimental paradigm being investigated [30]. This identification of apparently coregulated genes may also lead to assignation of new functions to previously known gene products or describe a function for previously undescribed gene products.

3.4. Limitations

There are some important limitations to the expression array approach. Since this analysis examines only the transcriptome, it fails to identify the important regulatory points at the level of translation and enzyme activity [29], in addition to the processes of protein–protein interaction. Currently, the expression arrays do not address the issue of alternative splicing, but there are new generations of arrays being produced to examine these events.

An important point to remember is that mRNA is a labile molecule with some molecules having a very short half-life and others being transcribed very rapidly in response to a given stimulus. Unless the material from which the RNA is being extracted is dealt with rapidly, it is possible that some molecules will be lost or some new unexpected molecules will be identified. This could lead to an altered expression level for some mRNAs. Hence, the analysis of gene expression changes might represent effects of the collection process and are not truly representative of the transcriptome of the cell or tissue being investigated [29]. One can consider this issue with respect to the concept of the steady state levels of mRNA. If the molecule is at a steady state, then the level of synthesis is matched by the level of degradation; hence, $d(\text{mRNA})/dt = 0$. However, an increase in the level of expression of a particular mRNA may not occur purely through an increase in transcription but may reflect a decrease in degradation or a release of mRNA from a protected untranslatable form. Whatever be the reason, it is likely to result in increased translation and an increase in the level of the gene product.

4. Gene Expression Assays in Cancer

The technology and expertise required for analysis of gene expression were adopted at a very early stage in its development by the field of cancer biology. This was because it was recognized that, while there were available a number of useful clinical, morphological, and molecular parameters for diagnosis and/or prognosis of human malignancies [36], there remained a substantial margin of error. It can be the case that, even with these tools, patients receiving the same diagnosis can have markedly different treatment outcomes. Cancer diagnosis is often subject to subtyping of diagnostic categories

as new diagnostic tools are developed. There was a pressing need for a diagnostic tool which would complement existing histopathological evaluation and enable the profiling of cancer cells in tissues [37]. In addition to providing accurate diagnosis, there was a further need to provide biomarkers which would be useful in (1) detecting cancerous cells early in the disease, (2) providing accurate pretreatment staging, (3) determining the biological aggressiveness of disease in order to tailor appropriate therapy, (4) predicting treatment outcome, and (5) monitoring disease progression [37, 38].

4.1. Gene Expression Assays in Breast Cancer

Some early studies demonstrated the potential of the new technology [39–41] to examine the differences between normal and malignant colonic tissue [39] and identifying potential subclassifications of breast cancer [41]. An interesting aside to these studies was the different analytical methods used by the authors to analyze and interpret the data. This variability of approach remains a feature of microarray studies and can hinder the comparison of data from different studies. However, there are now processes in place which attempt to standardize at least some of the analyses [42].

It is not possible to review all applications of gene expression arrays in the field of cancer and a number of excellent reviews are available to examine different aspects of the field, for example [43–48]. However, as an introduction to how the methodology has been applied, we will discuss breast cancer as an illustrative example.

4.1.1. *Early Studies in Breast Cancer*

Following the early pioneering work, a series of studies have been carried out to examine breast cancer malignancies more thoroughly. As stated previously, a number of tools are available for the classification of tumors. These include histopathology, immunocytochemistry, cytogenetics, fluorescent *in situ* hybridization, comparative genome hybridization, and whole genome allelotyping [49]. However, these can be limited in their diagnostic capability with subclasses of disease being indistinguishable and are of little prognostic value. The study of breast cancer by gene expression arrays, taken as an exemplar, can be used to demonstrate how the field has developed both technically and analytically. It is a feature of breast cancer, unlike pathologies such as leukemia, that there is no clear cytogenetic marker associated with the disease. As will be discussed later (Section 4.1.4) it has been clearly demonstrated that some breast cancers have a familial aspect [50, 51], but early studies of gene expression in breast cancer examined nonfamilial cases. A study by Perou *et al.* [52], using cDNA arrays featuring 8102 human genes, profiled 42 breast tissues from both tumor (39) and normal

(3) samples. Using a hierarchical clustering algorithm, the authors were able to distinguish a number of important differences between the samples. Twenty tumor samples were examined twice, once before treatment and again after treatment with doxorubicin for an average of 16 weeks. Pre- and posttreatment samples from the same individual were more similar to each other than samples from different individuals. The tumor samples could be separated according to whether they were estrogen receptor positive or negative. In addition, the estrogen receptor positive group were characterized by the expression of many genes expressed by breast luminal cells and were found, with one exception, to express low levels of the Erb-B2 gene. (Erb-B2, also known as HER-2 or neu, is a transmembrane tyrosine kinase receptor whose expression status has been further shown to be correlated with poor prognosis [49] in this and other cancers). The study identified four classifications of tissue: estrogen receptor positive/luminal cell-like, basal cell-like, Erb-B2 expressing, and normal breast tissue. An important feature of the data was the finding that the gene expression profile of a primary tumor is similar to that of a metastasis from the tumor. This would suggest a clonal development of the tumors and indicate a basis for individually defined treatment.

Bertucci *et al.* [49] carried out a similar study and reached a broadly similar result. Also, using a hierarchical clustering approach, 34 primary breast cancer samples were compared using cDNA arrays on a nylon membrane format. An important feature of this study was that the cDNAs spotted on the arrays were a select group predetermined to be important in breast cancer. Two distinct groups of tumor were identified, characterized by the outcome after therapy. As found in the earlier study [52], expression of Erb-B2 was associated with a poor prognosis, and this is suggested to reflect the behavior of the protein as an enhancer of cellular motility and hence possibly predisposing to a greater risk of metastasis.

4.1.2. *Identification of Gene Signatures*

Although early studies were designed to distinguish between normal and tumor material, a later study [53] set out to identify a gene signature that was associated with a short-time interval before metastasis and hence a poor prognosis. The current predictors of metastasis, lymph node status, and the histological grade of the tumor were not adequate predictive tools and it was recognized that many patients were receiving chemotherapy and/or hormone therapy when it was not needed. The study examined tumors from young patients, under the age of 55 years, who had received surgical treatment alone. An analysis which examined clinical outcome identified a poor prognosis signature comprising 70 genes that were associated with a short interval to metastasis from lymph node negative tumors. This offered a predictive model for patients who would benefit from adjuvant therapy and also would

allow identification of those patients for whom adjuvant therapy was less likely to be needed. Further studies on a larger cohort of patients have indicated that the 70 gene signature is a powerful predictor of disease outcome [54]. A feature of these studies is that no attempt was made to examine different cell types from within the tumor, which limits the specificity of the analysis. The predictive power of the gene expression assay would be furthered if the precise cells that were likely to develop into tumors could be identified and analyzed.

4.1.3. *Dissecting Out Tumor Types Using Gene Expression Analysis*

As has been discussed (Section 3.2), the techniques of LCM enable the researcher to separate individual cells from within a heterogeneous tissue. This should increase the specificity of the gene expression assay and increase its predictive power. Sgroi *et al.* [55] were the first to apply LCM to the isolation of different cell types from breast cancer tumors. They examined normal, invasive, and metastatic cell populations and described gene expression differences between each of the groups of isolated cells, demonstrating the potential use of the combined approach. Ma *et al.* [56] used LCM and arrays to determine whether tumorigenesis was a multistep process with a defined series of stages occurring in response to the acquisition of advantageous gene changes. If this were the case, these changes might be similar across different individuals. In breast cancer, the designated stages of disease progression are: premalignant atypical ductal hyperplasia (ADH), followed by ductal carcinoma *in situ* (DCIS), and finally invasive ductal carcinoma (IDC). Unfortunately, these stages are poorly defined histologically in breast cancer, with an alternative grading system being used in which the tumors are described as well, moderately or poorly differentiated and given the numerical identifier I, II, or III, respectively. A number of different parameters were examined and a few features identified. It was not possible to define stage-specific gene expression profiles by hierarchical clustering but some distinction could be made between the tumor grades. In particular, grade I was quite different from grade III and grade II appeared to be a hybrid signature of the two extremes. A subset of genes were identified that could be associated with grade III (poorly differentiated) tumors and the DCIS stage. This subgroup was more likely to be associated with an occult invasive phenotype. A particular gene, *RMM2*, was identified which may play a part in disease progression. RMM2 is the rate-limiting component for the conversion of ribonucleotides to deoxyribonucleotides required for DNA synthesis. It is thus a point of control for the rate of cell division. Increased RMM2 production would support rapid cell division and proliferation. As has been noted before, the profile of expression of different stages of tumor from the same individual were quite similar, again indicating that the

tumors are clonally derived and do not necessarily represent changes in gene expression that are common to the disease process.

A study [57] examined the gene expression profile of 286 patients with lymph node negative breast cancer. These authors were able to describe a 76 gene signature list: 60 for estrogen receptor positive and 16 for estrogen receptor negative, which could be used to recommend systemic adjuvant chemotherapy where it would be most appropriate. Sotiriou *et al.* [53] examined the gene expression profile of 99 breast cancer patients, both lymph node positive and negative. As has been seen previously, the major division was between the estrogen receptor positive, luminal cell-derived tumors, and estrogen receptor negative, basal cell-derived tumors, with some smaller subgroups being identified. It was suggested that a minimum classifying set of genes might become available when further studies had been carried out. A similar study [58] also set out to identify the genes that were associated with the transition from DCIS to IDC and used LCM to isolate the cells. The study also identified a series of genes differentially regulated in IDC compared to DCIS, with more upregulated than downregulated (445:101). However, the list of genes shared only four with the Ma study [56]. This reflects the differences in approach with different array platforms being used and, to some extent, the analytical methods applied. There were similarities in the outcome of both studies. In both instances, the progression stages from the same patient are more closely clustered than the corresponding stages from different patients. Both groups also consider that the approach provides a molecular insight into the transition from noninvasive to invasive tumor and begins to characterize a set of differentially expressed genes that might underpin the process.

4.1.4. *Gene Expression Profiling of Familial Breast Cancer*

It appears that the relative risk of breast cancer increases with the number of female relatives one has who suffer from the disease, particularly if the age of onset in the relatives is under 40 years [50]. The two major known inherited forms of breast cancer, which are caused by mutations in the genes *BRCA1* or *BRCA2*, account for ~15% of the excess familial risk of the disorder, which suggests there are other potential inherited causes [50]. A few genes have been implicated: *TP53, PTEN, CHK2*, and *ATM*, but the numbers of affected individuals are very small. Several large-scale studies have examined the influence of the *BRCA1* and *BRCA2* gene mutations on familial breast cancer [51]. The study by Malone *et al.* reviewed some of these and demonstrates that, while the overall numbers of sufferers can differ between different populations, the population frequency of *BRCA1* mutations in breast cancer patients was about 2.4% compared to only 0.04% of controls. The figure for *BRCA2* mutations was 2.3% in patients and 0.4% in controls.

These data are similar to previous studies, although the frequency of *BRCA1* mutations was slightly lower and *BRCA2* slightly higher [51]. However, it is apparent that the level of gene mutation leading to breast cancer is low and other mechanisms leading to disease need to be investigated.

Hedenfalk *et al.* [8] examined the differential expression profiles of the inherited forms of breast cancer. They examined seven each of *BRCA1*, *BRCA2*, and sporadic breast cancer samples and demonstrated that there were different profiles for the groups. *BRCA1*-derived tumors differed from *BRCA2* in the expression of 176 genes. In addition the *BRCA1* mutations were more likely to be associated with estrogen receptor negative cells while *BRCA2* tumors were more likely to be estrogen receptor positive. Therefore, the study demonstrated significant differences between the germ line mutations *BRCA1* and *BRCA2* at the level of their global gene expression profiles. The familial forms of breast cancer were also different from the sporadic form. The study also identified a sporadic case with altered expression of *BRCA1* through a promoter defect, which gave confidence in relation to the power of the study.

4.1.5. *Profiling the Effects of Drug Treatments*

One area of research that has progressed in the field of breast cancer analysis has been the use of gene expression arrays to examine drug effects on tumor progression in order to improve treatment strategies [59–61]. The rationale behind such studies is that, while adjuvant systemic therapy after surgery for breast cancer is widely used and represents a crucial intervention in reducing mortality, it is difficult to predict which patients are likely to benefit from particular treatment regimes. Chang *et al.* [59] were interested in the prediction of response to taxane drug treatment using the drug docexatol. They were able to examine the effect of neoadjuvant therapy on tumor development and by association determine a gene expression profile that lent itself to a positive outcome to drug treatment. Using the Affymetrix HgU95-Av2 GeneChip, they focused on genes which gave a consistent response would enable the identification of gene expression patterns that could be used as a predictive test for the effect of the taxane treatment. The 92 genes that were identified consisted of 14 genes overexpressed in treatment-resistant tumors and 78 genes overexpressed in treatment-sensitive tumors. The genes associated with docetaxel treatment resistance fell into categories including protein translation, cell cycle, and RNA transcription suggesting an involvement in cell growth and division, while the categories represented in the treatment groups included stress or apoptosis, cell adhesion, cytoskeleton, protein transport, transduction, and RNA splicing which is in keeping with cell maintenance or cell death roles. These results were consistent with a role for docetaxel in apoptosis induction and suggested

that a profile of genes associated with potential treatment strategies had been determined. The 92 genes identified in this experiment were tested in a leave-one out cross-validation study which showed them to be 92% successful for predicting a positive response to the drug and 83% successful in predicting a negative outcome. A further test of this profile in an independent cohort of samples was successful in describing six patients with drug-sensitive tumors. This level of predictive value compares favorably with other clinically validated markers.

A study by Ayers *et al.* [60] set out to describe a pathological complete response (pCR) to a combined neoadjuvant chemotherapy approach consisting of paclitaxel and fluorouracil + doxorubicin + cyclophosphamide. Forty-two patients were examined by gene expression array prior to drug treatment and the response monitored. Thirteen of the patients achieved complete tumor loss and these were split into two groups: six were examined to define a gene expression profile associated with sensitivity to the drug treatment and the remainder used in a validation set. A complex analytical methodology gave rise to a 74 gene predictive group which were tested on a validation set and were found to be 78% accurate in predicting the response of a tumor to the drug treatment (three patients predicted to demonstrate pCR showed a complete tumor loss). Further predictive models using smaller numbers of genes were less efficient. While the list of genes important in the predictive process included few previously considered important to the tumorigenic process, this experimental protocol indicates the value of the methodology and may be developed into a diagnostic tool with a larger number of patients.

It is not possible in a short review to examine fully how gene expression arrays have been used to examine breast cancer. However, the above discussion illustrates the principles of the approaches used and the potential clinical value of the data emerging. Brenton *et al.* [62] and Sorlie *et al.* [63] have attempted to mine the published data in order to synthesize an overview of the research in this field. It is apparent that breast cancer is a heterogeneous disorder with marked differences between familial disease related to expression of BRCA1 or BRCA2 and the sporadic form of the disease. The location of the initial tumor in a basal or luminal cell type is also significant in terms of prognosis. The gene expression studies have demonstrated that the profile of expression can be used as an adjunct to the histopathological and histochemical approaches, and provide additional information concerning the potential effects of adjuvant therapy. Hence, different subtypes can be identified using gene expression data: basal or luminal cell derived, Erb-B2, (HER-2), positive or negative, BRCA1 or BRCA2 positive, and estrogen receptor positive or negative. There remains a lack of consensus as to the most appropriate set of genes to examine, but it is noted that basal cell-derived

tumors have the worst prognosis and this is exacerbated by the presence of Erb-B2 expression and estrogen receptor status. The potential of the approach has been demonstrated and what is now needed is a large-scale study, or reanalysis of the current datasets, to enhance the information that can be used in patient diagnosis.

4.2. Gene Expression Assays in Other Cancers

Although this chapter has focused on breast cancer, it is also interesting to briefly describe how the technology has impacted on the field of hematological cancers. This has been thoroughly discussed by Dunphy [64] and demonstrates how new stratifications of disease are being identified by their gene expression signature and hence new markers being made available for more cost-effective diagnostic methods such as IHC and flow cytometry. It is clear that gene expression profiling is useful as an adjunct approach with other methodologies.

Valk *et al.* [9] investigated genes differentially expressed in acute myeloid leukemia (AML). Current methodologies offer only about 50% of the information, predicting patient therapy and prognosis. In this study, 285 AML patients and 8 healthy controls had their gene expression profile of blood or bone marrow determined, and 16 clusters were identified. Some of these clusters were associated with known karyotypic abnormalities or particular gene mutations, but several of the clusters had a normal karyotype. There was a unique cluster which was associated with AML cases with a poor prognostic outcome. With new clusters of AML having their own characteristic gene signatures, this demonstrated how the technology could aid diagnosis and provided a step toward the identification of a set of genes that could be ordered on a diagnostic array. Another study of AML examined 116 AML cases of which 45 had normal karyotypes [65]. Gene expression signatures were demonstrated for different subtypes. Progress of the disease was followed up in an attempt to construct a gene expression-based outcome predictor. A group of 133 genes could be used to predict outcome for karyotypically abnormal cases. In patients with a normal karyotype, there were two subgroups based on their gene expression profile. These studies demonstrate that there is a role for gene expression profiling in both improving classification and predicting the prognosis of AML.

4.3. Considerations When Comparing Array Studies

It is appropriate to consider some of the general themes that have already become apparent in the short time that microarray technology has been available. Cancer biology is complicated by the mechanisms that can

lead to a tumor developing and progressing. As has been discussed, tumor development can be associated with chromosomal rearrangements or gene mutations which are subject to selection pressure during cancer progression leading to a cancer cell that has a selective advantage [66]. This explains the clonal development of tumors which has been demonstrated by gene expression profiling. Previously, many studies in cancer examined single markers and correlated them with a diagnosis and clinical outcome [67]. This process is a simplification of the etiology and progression of cancer.

Many pathways interact in the development of a tumor and the advantage that gene expression profiling brings is the potential, particularly through clustering analyses, to identify groups of genes that are differentially regulated together. This grouping process can further identify regulatory processes that are a part of the functional relationships modified in the tumor [67]. There are three potential approaches to tumor analysis: whole tumor examination, tumor-derived cell lines, and microdissection profiling [67]. Each approach has its benefits and drawbacks. The whole tumor will provide a gross picture of the expression profile but the cancerous cells may be a small fraction of the tumor mass. Cultured cells provide an idea of the expression profile of the cancerous cell but in an isolated environment. Microdissected cells are able to examine the gene expression changes in what may be a small population of cells within the tumor, but it may not be easy to identify the appropriate cell and the small numbers of cells will provide a limited quantity of RNA, which means additional processing steps are required to produce the gene expression profile. However, the field has progressed rapidly and microarray technologies have been used to profile the gene expression profile of tumors, leading to the discovery of particular disease susceptibility genes, therapeutic targets, and profiles related to disease outcome and drug sensitivity and resistance [66]. What has become apparent is that from the many studies that have been carried out in a variety of tumor types, a consensus has not yet been reached which can be applied in all studies. Different groups have used different array types and different analytical tools. This can lead to apparently discrepant results. There is now acute awareness of this problem in the field of microarray research and mechanisms are in place to try and address the difficulties. The adoption of the MIAME standards [42] for publication of material has focused attention on the parameters required to validate a study. The next step is a further examination of strategies for data analysis that will strengthen the data examination and allow studies to be more easily compared and interpreted. The challenge will be to correlate these findings with high-throughput proteomics analyses, and finally to the development of novel diagnostic, predictive, and prognostic biomarkers, as well as therapeutic targets.

5. Applications of Arrays to Neurological Disorders

Initial gene expression studies in neurology focused on using tissue homogenates from affected regions of the brain to gain further insights into the pathogenic mechanisms responsible for neuronal cell death. More recently, the application of LCM and linear amplification has interrogated the gene expression of the vulnerable neuronal cell type, without the dilution effect from supporting cells of the central nervous system (CNS). Illustrative examples from Huntington's disease (HD), Alzheimer's disease (AD), multiple sclerosis (MS), and motor neuron disease (MND) demonstrate how microarray technology has been used to advance the knowledge of these diseases.

5.1. Huntington's Disease

HD is a midlife onset, progressive neurodegenerative disease characterized by motor impairment, cognitive decline, and psychiatric symptoms. Histopathologically, the earliest and most severe neurodegeneration occurs in the GABAergic cells, or medium spiny neurons of the caudate nucleus, although atrophy is also seen in the putamen, globus pallidus, and cerebral cortex [68]. For this reason, gene expression studies have focused on these regions of the brain. In 1993, the genetic cause of the disease was identified as an expanded repeat of the CAG codon encoding the polyglutamine tract, in exon 1 of the *IT15*, or *HD* gene [69]. The encoded protein, huntingtin (HTT), is ubiquitiously expressed throughout the brain and body, suggesting a selective vulnerability in the neurons affected.

Many studies have been carried out on HD mouse models and human material to investigate the basic mechanisms of neurodegeneration of mutant HTT protein [70–72], the effects of mutant HTT protein length [73], the variability in expression changes in different regions of the brain [74], the effects of potential HD drug treatments [13, 75, 76], and to identify potential biomarkers which correlate with the progression of the disease [77]. The next sections will discuss each of these applications in turn.

5.1.1. *Gene Expression Studies in HD Mouse Models*

To provide an insight into the pathways involved in HD neurodegeneration, gene expression studies of the striatum were performed on the mouse model of HD, R6/2, which expresses exon 1 of the *HD* gene with 140–150 CAG repeats under control of the *HD* promoter [70]. Eighty percent of cells in the mouse striatum were estimated to consist of medium spiny GABAergic neurons. Decreased neurotransmitter receptor gene expression was identified

in the striata of transgenic mice. In addition, decreases were seen in other components of neuronal signaling pathways (specifically those of dopamine and glutamate), and in genes involved in calcium signaling pathways, ion channels, transcription, metabolism, and cell structure, while increases were seen in inflammation and cell cycle genes. Interestingly, differential gene expression was seen early in the disease, at 6 weeks, when only subtle motor defects were evident, suggesting an accumulation of neuronal damage underlies the progressive nature of the disease.

To determine the extent of gene expression changes responding specifically to mutant HTT, rather than a polyglutamine repeat, further gene expression studies were conducted using a mouse model of dentatorubral–pallidoluysian atrophy (DRPLA) [71]. This is also an autosomal dominant neurodegenerative disease, caused by expansion of 49–88 CAG repeats in the atrophin-1 gene (*Atn1*). The At-65Q mice contained an expansion of 65 repeats in *Atn1* under control of the same promoter as the *Htt* gene in the N171-82Q mice. These mice express a peptide consisting of 171 amino acids of the N-terminus of HTT, with 82 CAG repeats. Analysis of cerebellar gene expression profiles in the two models compared to wild-type litter mates identified 184 genes in common, suggesting these are altered in response to an expanded polyglutamine, rather than representing gene expression changes specific to the mutant protein. In addition, 74 of these changes were also seen in the R6/2 HD mice [72], and four genes, including enkephalin, were also confirmed as altered in the cerebellum from mouse models of two other CAG repeat disorders, spinal cerebellar ataxia 7 and spinal bulbar muscular atrophy [71]. However, these changes were not seen in transgenic mice expressing a full-length HTT protein with 72 CAG repeats.

To determine the extent of the effect of HTT protein length on gene expression, studies have also been conducted comparing mouse models expressing the short truncated HTT protein with expanded repeats (R6/2 and N171-82Q mice) with a larger truncated HTT protein (HD46 and HD100 containing 46 and 100 CAG repeats along with the first 964 amino acids) and full-length mutant HTT protein (YAC72 containing 72 CAG repeats) [73]. These results indicated that striata expressing the longer HTT proteins showed fewer expression changes compared to those with the shorter truncated protein at pathologically equivalent ages. In addition, previously identified gene expression changes in the R6/2 and N171-82Q mice were not among those genes differentially expressed in mice transgenic for the longer mutant HTT proteins. However, it should be noted that shorter, proteolytically cleaved HTT is present in both HD and control brain tissue [78], and with changes in the R6/2 mice also present in human HD brain [70], it has been proposed that mutant HTT has an effect following proteolysis of the complete protein into the small N-terminal fragments.

5.1.2. *Gene Expression Studies in HD Human HD Cases*

The most comprehensive study to date of human postmortem HD tissue analyzed gene expression from four brain regions in 44 HD cases and 36 controls [74]. Gene expression profiles were obtained from the caudate nucleus, which shows the most severe and earliest pathology, cerebellum which shows little pathology, motor cortex which controls the motor function that is altered early in the disease, and prefrontal association cortex, involved in cognitive processing which deteriorates later in the disease. Twenty-one percent of the genes interrogated were differentially expressed in the HD caudate samples, compared to 3% in the HD motor cortex and 1% in the HD cerebellum, while prefrontal cortex showed no changes other than those expected by chance. The HD caudate samples also showed the largest fold changes in expression. Interestingly, of the genes in the motor cortex also altered in the caudate, the majority were altered in the same direction, suggesting similar changes occur in different brain regions as well as changes specific to that cell type. To address the issue of whether the large number of gene changes in the caudate was due to cell loss of the medium spiny neurons, LCM was used to isolate the same number of neurons from both HD cases and controls. Subsequent microarray analysis confirmed 77% of the decreased genes and 65% of the increased genes were concordant with those genes differentially expressed in the whole caudate samples. As expected, the largest number of gene expression changes was in the category of neuronal signaling and homeostasis, specifically in the neurotransporter receptors. In addition, genes involved in intracellular signaling, and proton and metal ion transport were also implicated. Thus, this global gene expression study confirms and extends the genes differentially expressed that occur in the presence of the mutant HTT protein, while demonstrating the both similarities and differences in regional expression changes that occur in the HD brain.

Mutant HTT has been found to bind to other polyglutamine containing proteins, including transcription factors [79], and forms aggregates, with sequestration of other cellular proteins. Thus, the proposed mechanism of action for mutant HTT is through aberrant protein–protein interactions, particularly involving transcriptional dysregulation, as demonstrated by the human and mouse microarray studies [71, 72, 74]. This subsequent disruption of transcription is thought to underlie the apparent involvement of oxidative stress, mitochondrial dysfunction, apoptosis, energy metabolism disturbances, and excitotoxicity in neuronal cell death.

5.1.3. *Transcriptional Effects of Drug Treatments in HD*

The dysregulation of transcription has provided a target for therapeutic intervention, and microarray analysis has been used to follow the effect of the drug in the treated samples. For example, acetylation of histones regulates

the gene expression of between 2 and 5% of genes [80], allowing transcription factors access to regions of DNA which are tightly packed in the chromatin. While histone acetyltransferases add the acetyl group onto the lysine residues found in the histones N-terminal tails, the histone deacetylases (HDAC) remove them, in a highly dynamic process. Thus, gene transcription occurs when the rate of acetylation exceeds that of deacetylation. Mutant HTT was found to bind and sequester p53- and CREB-binding protein (CBP) into aggregates of HTT, and was associated with gene repression of two p53 promoters [81]. CBP is a transcriptional activator and functions as a histone acetyltransferase [82]. Following the demonstration that cognitive defects observed in CBP+/− transgenic mice were ameliorated following treatment with HDAC inhibitors, HDAC inhibitiors have been trialed in mouse models of HD and the gene expression response analyzed using microarrays.

In R6/2 mice injected with an HDAC inhibitor, sodium butyrate, from 3 weeks of age, survival was extended in a dose-dependent manner by up to 20%, motor performance was enhanced and pathological improvements showed reduced atrophy of the striatum, with a 73% reduction in lesion volume [75]. Subsequent microarray studies, in mice treated for 2 weeks from 6 weeks of age, showed selective changes in the sodium butyrate-treated mice, rather than a global reversal of gene expression. It was suggested that this may be due to prior sequestration of key transcription factors into the mutant HTT aggregates. A further study looked at the treatment of a different HD mouse model, N171-82Q, with an alternative HDAC inhibitor—phenylbutyrate [13]. The aim of this study was to investigate the effects of drug treatment following symptom onset. Intraperitoneal injections from 75 days extended survival of the HD mice by 23%, and this was associated with reduced gross brain atrophy, specifically striatal neuron atrophy. Analysis of striatal gene expression on Affymetrix U74Av2 GeneChips reported 11 genes significantly increased, including glutathione *S*-transferase (GSTm3), proteasomal subunits (Psma3 and ATPase3), and ubiquitin-specific protease 29, and 6 genes significantly decreased by phenylbutyrate treatment, including apoptotic genes (Casp9 and Cflar). Both apoptosis and aberrant protein degradation have been demonstrated in HD, and therefore, reduction of caspase-9 expression, which was associated with a decrease in active caspase-3, and increases in the ubiquitin–proteasome subunits may contribute to the beneficial effects of phenylbutyrate treatment.

An alternative therapeutic strategy has focused on improving cognitive features of HD, and the effect of this treatment on gene expression has been studied [76]. R6/2 mice were treated from 5 weeks old, when they exhibit spatial learning difficulties, with a cocktail of tacrine (an acetylcholine esterase inhibitor, which results in a global increase of brain acetylcholine levels), moclobemide (an antidepressant that inhibits monoamine oxidase A and

thereby prevents noradrenaline and 5-hydroxytryptamine breakdown), and creatine (a supplement which has shown to increase muscle mass, improve muscle strength in a wide range of neuromuscular disorders, as well as having a beneficial effect on mitochondrial function [83, 84]). Treated mice showed improved results in two cognitive tasks, compared to untreated mice, although they were not restored to the same level as WT mice, suggesting that the drugs act by preventing further deterioration. Gene expression analysis using U74Av2 GeneChips (Affymetrix) identified 640 differentially expressed genes of which 333 were reversed toward normal levels by the cocktail of drugs. As there were no significant improvements in motor function following treatment, these results highlight those genes specifically involved in the cognitive portion of the disease pathogenesis. The authors suggest, therefore, that combinatorial drug therapies targeting both cognitive and motor defects should be considered.

5.1.4. *Identification of Biomarkers in HD*

Although HD carrier status can be definitively diagnosed through genetic testing, numerous neurological disorders can only have a confirmed diagnosis at postmortem. However, HD has provided a genetically defined population in which biomarkers for the disease, and indeed biomarker changes that correlate with the progression of the disease, have been discovered in blood [77]. Previous array work demonstrated that mutant HTT, as it is ubiquitiously expressed, caused similar gene expression changes in nonneuronal tissue such as muscle [72], and normal and mutant HTT have been found in HD blood samples [85]. Gene profiles of peripheral blood from symptomatic patients (average 49.6 years) and late presymptomatic individuals (average 39 years) were significantly different to controls, with a large number of genes showing an increase in expression [77]. Following selection of 12 validated genes to use as biomarkers, PCA was able to distinguish the late presymptomatic cases from both controls and symptomatic patients, although the expression levels of these genes were more similar to those of symptomatic cases. Further studies in early presymptomatic individuals (average 22.5 years) demonstrated that expression of these genes increased over time, prior to symptom onset. Most importantly, analysis of blood from HD cases involved in a dose-finding study for the HDAC inhibitor sodium phenylbutyrate showed a significant decrease in the gene expression of those 12 genes following treatment. Thus, following the generation of biomarkers from microarray data, not only can the progression of the disease be monitored, but drug specificity and efficacy can be assessed, just by taking a blood sample. As such, this is an important research advance to be applied to not only other neurological disorders, but many other genetic and sporadic diseases.

5.2. Multiple Sclerosis

MS is an autoimmune disease that attacks the myelin sheath of oligodendrocytes around the neuronal axons. This allows the axonal cytoskeleton to be damaged, bringing about secondary axonal loss and persisting neurological dysfunction. The characteristic pathology is of a lesion or plaque in the CNS white matter, formed by inflammation and demyelination and these can be classified into active, chronic active, or chronic silent plaques [86].

The first report applying microarrays to MS sampled gene expression in normal white matter, and compared this to acute lesions in white matter of the same patient [87]. The changes reflected altered cell metabolism and increased expression of cytokines and cell adhesion molecules characteristic of an immune response. A second study by the same group analyzed changes in gene expression from two patients, the first having both acute and chronically active lesions, while the second had chronic lesions. Comparisons were made with both control human white matter and two animal models of experimental autoimmune encephalomelitis (EAE) [88]. EAE can be induced by immunization of mice with myelin oligodendrocyte protein (MOG), proteolipid protein (PLP), or myelin basic protein (MBP). The resulting symptoms, produced by a targeted attack on the oligodendrocytes, and subsequent recovery following elimination of the immune cells from the CNS, are reminiscent of those in MS, complete with recurrent relapses. Only four genes were consistently upregulated in the MS lesions and both MOG and MBP induced EAE. One of the genes, arachidonate 5-lipoxygenase (5-LOX), was upregulated 83-fold in the acute/chronic active lesion patient and 22-fold in the chronic lesion patient, and is of particular interest as it is an important enzyme in the synthesis of the potent immune mediators, leukotrienes. Follow-up studies showed that 5-LOX knockout mice did exhibit less anxious behavior than controls [89], but contrary to expectations, given that there is an increase in 5-LOX when EAE is induced in normal mice, the 5-LOX knockout mice were more susceptible to EAE than controls [90].

Further investigation of differential gene expression in EAE induced by MOG demonstrated increases in immune response genes such as antigen receptors, MHC molecules, chemokines, and cytokines [91]. In addition, changes in genes specifically expressed in the spinal cord were investigated, and the study found those genes involved with neurogenesis and neuronal repair genes were increased, while genes encoding ion channels, neurotransmitters, and growth factors were decreased. Differential gene expression of this "neuronal response," in contrast with immune response, continues in recovering animals even after the immune response has returned to normal.

Treatment of EAE with metalloproteinase (MMP) inhibitors can prevent or reverse the progression of EAE, and microarray analysis demonstrated

that MMP inhibitors reduced the expression of several genes, including osteopontin (OPN) following treatment of EAE induced in rats [92]. Subsequent studies investigated the relevance of OPN in MS, and several polymorphisms within the gene have been associated with disease susceptibility, disease progression, and age of onset, although not in all studies (reviewed by Steinman and Zamvil [93]). However, expression levels are clearly linked with the disease, with differential increases of OPN in both relapsing and remitting MS cases, and chronic active and acute plaques [94].

The three types of MS lesions have characteristic gene expression changes [95, 96]. Profiling of one acute, two chronic active, and one chronic silent lesion not only identified the involvement of increased proinflammatory cytokine activity and decreased expression of genes encoding myelin-associated proteins but also showed the acute lesion to cluster separately from the chronic silent lesion, which was more similar to the white matter profile of controls [95]. Further work identified there were more differentially expressed genes in the chronic active lesions compared to the chronic silent lesions, and also the margins of the chronic active lesions showed increased transcriptional activity, particularly in genes encoding immune mediators [96].

Thus, microarrays have shown differential gene expression of markers such as OPN and 5-LOX correlate with disease state, while lesion profiling has identified a transcriptional distinction between the types of the lesions found in MS cases.

5.3. Alzheimer's Disease

AD is a neurodegenerative disease characterized by progressive cognitive decline, and is the most common cause of dementia in the elderly. First described by Alzheimer in 1907, a definite diagnosis of AD can only be made postmortem by neuropathological examination, through the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles (NFT), neuronal loss, and synaptic dysfunction in the hippocampus and cerebral cortex. While 95% of AD is late onset (>65 years), genetic factors have been implicated in early onset AD, with mutations identified in the presenilin 1 (PSEN1), presenilin 2 (PSEN2), or amyloid precursor protein (APP) genes. Studies on these genetically defined individuals and transgenic mice for the genes are increasing the understanding of the molecular mechanisms underlying AD, with the aim to improve the diagnosis and treatment of the disease for all cases.

5.3.1. *Gene Expression Studies in AD Mouse Models*

Transgenic mice have been generated carrying missense mutations in the PSEN1 gene, deletion of exon 9 (caused by a G to T substitution in the splice site acceptor site), as well as a PSEN1 knockout mouse. A study by Mirnics

et al. [97] compared the gene expression profiles from hippocampus of the exon 9 deleted mouse (ΔE9) with those from the PSEN1 knockout mice. Interestingly, the majority of the genes in common showed contradictory directions of change, and it was suggested that this represented the effect of the "gain of function" of the PSEN1 mutations, while the similarly changed genes may represent the loss of function effects that have also been reported [98, 99].

Amyloid precursor protein (APP) is alternatively spliced to express several isoforms [100], of which APP695 is preferentially expressed in neuronal tissue [101]. APP is cleaved first by β-secretase, and then by γ-secretase to form β-amyloid-40 and β-amyloid-42. It is these β-amyloid isoforms that aggregate in AD. PSEN1 has been demonstrated to be involved in the γ-secretase proteolytic cleavage of APP, with neuronal cultures from PSEN1-deficient mice showing reduced production of β-amyloid-42 [102]. Microarray analysis of the Tg2576 mouse model (carrying the K670N/M671L mutations) examined the changes in gene expression that occur during the progression of the disease [103]. Transcription profiles of the cerebral cortex of mice at 2, 5, and 18 months, corresponding to long before, immediately before, and after the appearance of β-amyloid plaques, respectively, identified mitochondrial energy metabolism genes (particularly those related to oxidative phosphorylation) and apoptosis genes to be upregulated at all three time points, compared to controls. Additional gene expression changes were identified in the 18-month mice, in functional pathways, including transcription, cell cycle development, and signal transduction. The finding of early mitochondrial involvement correlates with findings in the human disease from Hirai *et al.* [104], and the presence of this dysfunction in the mouse model now allows the exact nature of mitochondrial involvement to be determined, and also whether mitochondrial-enhancing drug treatments will be beneficial [103].

Double transgenic mice, carrying the APP (K670N/M671L) and the PSEN1 (A246E) mutations, show cognitive impairment which is consistently present by 15 months, and show this to be progressive as β-amyloid deposition accumulates [105]. Gene expression profiling showed a decrease in genes required for normal memory function (Arc, Nur77, Zif268) and neuronal/synaptic activity (NaKATPaseαIII), and an increase in inflammatory-related and the acute-phase response genes (including GFAP and ApoE), reflecting glial activation, in the double-mutant mice [106]. Interestingly, most of these changes were restricted to regions of the brain showing amyloid deposition. Q-PCR of human AD brain also showed reduced expression of the memory-related genes specifically in regions affected by β-amyloid deposits. Thus, the authors proposed that memory dysfunction occurs early, before synaptic degeneration and neuronal cell death, which is seen in human postmortem material.

5.3.2. *Gene Expression Studies in Human AD Cases*

One of the first studies to apply microarray technology to human postmortem AD cases used linear amplification of RNA methodology to examine the expression profile of tangle bearing CA1 neurons from AD patients compared to normal CA1 neurons in controls [107]. This demonstrated decreases in genes whose proteins had previously been implicated in AD such as those involved in the cytoskeleton, the synapse, and both glutamate and dopamine receptors. In addition, genes such as utrophin and glutaredoxin were found upregulated in the tangles and were thought to represent novel mediators of NFT formation, or neurodegeneration. Microarray analysis of AD brains has identified gene expression changes that correlate with AD markers such as Mini Mental Status Examination (MMSE) and NFT scores [108]. For example, the microfibrillary-associated protein 1 was upregulated in AD cases as NFT score increased, while the G–protein-coupled receptor 22 was decreased as the MMSE score decreased in AD cases. A further study has shown that analysis of genes expressed in fibroblasts from carriers of APP (K670N/M671L and E693G) and PSEN1 (H163Y) mutations allows them to be clustered together separated from siblings free of the mutation, independently of the mutant gene. This suggests a similar molecular mechanism underlies the effect of these mutant genes on the cell, which correlates with PSEN1 being involved in β-amyloid production. The expression profiles of senile plaques in the hippocampus have also been investigated, and cholinergic basal forebrain neurons, which supply the cholinergic fibers to the hippocampus and cerebral cortex (as reviewed by Ginsberg *et al.* [109]). These studies have identified that the senile plaques contain predominantly neuronal mRNAs, suggesting that they are formed through the accumulation of degenerating neurons [110], and that expression levels of high-affinity nerve growth factor receptors (trk) are significantly downregulated during the progression of the disease, expression correlates with declining MMSE scores, and therefore may represent an early biomarker at disease onset [111].

In a recent publication, the largest number of AD brains to date were analyzed by microarray. Unlike the previous studies, the RNA from 61 AD frontal cortex samples and 53 controls samples were pooled, and multiple hybridization experiments took place on two-array platforms. Only 3 of the top 30 differentially expressed genes from each platform were found to be in common: RGS4 and RAB3A were decreased and ITPKB was increased. However, real-time PCR with the individual samples confirmed these changes were specific to the AD patients, and these data support the evidence of disrupted intracellular calcium signaling in AD [112].

Finally, a review has compared the expression data obtained from both transgenic AD models and human postmortem AD material [113]. The human

postmortem studies show more overlap in genes that are downregulated than upregulated, while the mouse studies, which include mice carrying single and double AD mutant genes, show a greater overlap with the study by Blalock *et al.* [108] than the other human studies. This may be explained by the inclusion of cases in the early stages of AD, rather than just advanced AD cases, and the mice models, such as the APP mutant mice, mimicking earlier gene expression changes such as changes in energy transduction, before loss of neurons. On the basis of these microarray findings, the authors proposed a model for AD progression, beginning with localized oligodendrocytes causing remyelination responses in the neuronal axons [113]. This causes secretion of growth factors from the oligodendrocytes, and compensatory tumor suppressor responses in neurons and astrocytes. This then leads to protein aggregation, abnormal axonal–myelin interactions, and NFT formation, and may also explain the observation that pathological changes in AD progress along myelinated axons.

5.4. Motor Neuron Disease

MND is a group of progressive neurodegenerative disorders encompassing the clinical subtypes of amyotrophic lateral sclerosis (ALS), primary lateral sclerosis, primary bulbar palsy, and progressive muscular atrophy. Characterized by the cell death of upper and/or lower motor neurons in the motor cortex, brainstem, and spinal cord, the exact mechanisms of motor neuron injury are unknown, but are thought to involve oxidative stress [114], excitotoxicity [115], protein aggregation [116], mitochondrial dysfunction [117], and genetic factors [118]. While the majority of cases are sporadic, 5–10% of cases are familial, usually with an autosomal dominant inheritance. The first gene to be identified as causative, and to date the most common, is the Cu/Zn superoxide dismutase (SOD1) gene [119]. Encoding a ubiquitously expressed free radical scavenging enzyme, mutations lead to the mutant protein possessing a toxic gain of function, thought to result from aberrant handling of free radical species and/or protein aggregation.

5.4.1. *Profiling Spinal Cord Homogenates in MND*

Microarray technology has been applied to determine the exact mechanisms by which mutant SOD1 causes motor neuron cell death. Due to clinical similarity of the SOD1-related MND cases to other familial and sporadic cases, further understanding of the pathogenesis in the SOD1 cases may well be applicable to MND as a whole. The first studies used lumbar spinal cord homogenates from a transgenic mouse cell line generated to express the human mutant G93A SOD1 protein [120–122]. These mice develop symptoms around 90 days, with hind limb weakness progressing to paralysis

at 140–150 days. However, pathological changes occur in the motor axons as early as 30 days, with the vacuolar changes becoming evident in the motor neuron cell body by 60 days, along with Golgi fragmentation and mitochondrial swelling. By 90 days, glial activation is present as are inclusion bodies and SOD1 protein aggregates.

These gene expression studies using the G93A SOD1 transgenic mice have also allowed the progressive nature of the disease to be studied. Fewer differentially expressed genes have been identified in the presymptomatic mice (60 days) compared to early symptomatic mice (90 days), while the majority of gene expression changes are seen at end stage of the disease (120 days) [121, 122]. In symptomatic mice, there was strong evidence of an inflammatory response from reactive astrocytes and activated microglial, as well as a potential adaptive response to metal ion dysfunction, postulated to be the result of increased intracellular iron caused by mitochondrial dysfunction. Other pathways affected included lipid metabolism [121], cytoskeletal architecture [122], and proteins involved in the differentiation/maturation of the spinal cord population [122]. The relevance of these pathways to the human disease is supported by gene expression profiling studies of postmortem tissue from sporadic ALS cases [123–125]. Interestingly, analysis of the human postmortem material also identified significant alterations in genes encoding ubiquitin/proteasome pathway components and other protein degradation mechanisms, though whether dysregulation of protein degradation is the cause of the protein aggregations observed, or whether these are a protective measure, sequestering faulty cytosolic proteins, remains to be determined. Dangond *et al.* [123] also analyzed the gene expression of two familial ALS cases, one of which carried a mutation in the SOD1 gene, and found a significant number of genes which were altered specifically in these two cases, including increased expression of genes expressed in response to high calcium levels and genes involved in the cell cycle.

5.4.2. *Profiling Cellular Models of MND*

To determine the specific response of motor neurons to the presence of mutant SOD1, without contamination of other cell types and the effects of their interactions with motor neurons, gene expression profiling has been undertaken on the motor neuronal cell line, NSC34, transfected with mutant SOD1 [126]. This study demonstrated, in contrast to the whole tissue homogenate studies, that there was a marked degree of transcriptional repression in the presence of mutant SOD1, with reduced gene expression demonstrated in the antioxidant response pathway, and particularly the "programmed cell life" genes under the transcriptional control of Nrf2. Additional pathways affected included genes involved in protein degradation, cell death/survival, the immune response, and the heat-shock response.

5.4.3. *Profiling Motor Neurons in MND*

Gene profiles from motor neurons in the spinal cord can now be determined through the combined technologies of LCM and linear amplification of RNA. A comparative study of ventral horn and isolated motor neurons in ALS cases and controls demonstrated how few motor neuron-specific gene expression changes were represented in the whole tissue homogenates from the ventral horn [127]. Consistent with the cell model findings, ALS motor neurons showed more genes were decreased (3%) than increased (1%), whereas in the ALS ventral horn samples, more genes were increased (0.7% vs 0.2% of 4845 transcripts interrogated). This can be explained by the influence of reactive gliosis and other cellular reactions in the nonneuronal cells, and through reduced motor neuron number in the ventral horn of ALS cases. Major functional groups that were found to have genes downregulated included cell receptors and intracellular signaling, transcription, metabolism, and cytoskeleton architecture, while genes encoding cell death associated proteins, secreted and extracellular communication proteins, and cell cycle regulators were all upregulated.

LCM has also been applied to isolate MN from the transgenic G93A SOD1 mice [128]. The authors demonstrate the increasing changes in gene expression that occur as the disease progresses, although, in contrast to the human and cell model studies, they show a larger number of genes increased than decreased. The majority of increased genes were involved in cell growth and/or maintenance. Only 12 genes were differentially expressed at all three time points though the progression of the disease, and one of the genes increased encodes vimentin. This is an intermediate filament involved in retrograde transport and may also play a role in neurite extension. Vimentin inclusions were seen in presymptomatic mice, and became more abundant as the disease progressed [128]. Interestingly, this increased gene expression is not specific to the SOD1 mice but also occurs in two other neurodegenerative mouse models, pmn and wobbler [129].

Gene expression studies on presymptomatic mice demonstrated vimentin to be the only gene differentially regulated in all three models, although 11 genes were differentially expressed in the pmn and wobbler mice [130], which are both autosomal recessive models of neurodegeneration arising from a spontaneous mutation event [131, 132]. These studies aimed to identify early events and responses in the neurodegenerative process, and have determined both common and gene-specific induced changes in gene expression.

In summary, gene expression studies have demonstrated the involvement of the ubiquitin/proteasome pathways, and dysregulation of mitochondria and cytoskeletal structures, supporting previous hypotheses. One of the novel findings that MN are trying to reenter cell division is surprising as in

these postmitotic cells this will lead to cell death. Most importantly, these studies have demonstrated that gene expression changes of whole tissue homogenates do not portray all the differentially expressed genes in the individual cell types present.

5.5. Expression Profiling Other Neurological Disorders

In addition to the examples listed above, gene expression arrays have also been applied to Parkinson's disease (PD), identifying abnormal iron metabolism [133], oxidative stress, and protein aggregation occurring in both familial [134] and sporadic PD [135], as well as identifying genes differentially expressed in a cellular model of DJ-1-associated familial PD [136]. Further insights into Creutzfeldt–Jakob disease (CJD) have been discovered by gene expression profiling the frontal cortex, demonstrating increases in genes encoding immune and stress response, cell death, and cell cycle proteins and decreases in genes encoding synaptic proteins [137]. Importantly, although there were common pathways affected in the human CJD and the prion disease mouse models, there was a larger immune response present in the mouse, while the human CJD cases showed more apoptotic-related genes to be differentially expressed. It is crucial to be aware of such differences, when focusing on therapeutic targets identified in mouse models to slow down the progression of the human disease.

The application of microarrays to these neurological disorders, both genetic and sporadic in origin, has generated a significant amount of information regarding the molecular events occurring in both the affected regions and vulnerable cell populations. Disease progression, drug treatment, and nonneuronal samples, such as blood, can all be scrutinized at the level of transcriptional responses, generating further therapeutic targets, and identifying candidate susceptibility genes and biomarkers.

6. Application of Microarrays to Neuropsychiatric Disorders

Neuropsychiatric disorders such as schizophrenia (SZ), bipolar disorder (BD), and major depressive disorder (MDD) are complex diseases, whose exact etiology is unknown. Evidence suggests genetic, developmental, and environmental factors combine to create each disorder, with susceptibility loci for SZ, BD, and MDD identified throughout the genome. With an absence of clearly defined genetic factors on which cellular and animal models can be generated, to further the understanding of this spectrum of disorders, gene expression analysis of postmortem material has been utilized to distinguish

particular clinical phenotypes, to determine specific gene signatures [138], and to identify novel genes implicated in the disorder [139].

6.1. Schizophrenia and Bipolar Disorder

SZ is a common psychiatric disorder affecting 1% of the population. Onset usually occurs in late adolescence or early adulthood, coinciding with a developmental reduction in brain synapse density, and symptoms include delusions, hallucinations, impaired motivation, and changes in cognitive responses such as attention span and working memory. Pathologically, the hippocampus, superior temporal gyrus, and thalamus are affected, with predominant pathology occurring in the prefrontal cortex, which is thought to underlie the cognitive impairment symptoms. Gene expression studies have identified the involvement of presynaptic secretion, mitochondrial dysfunction, and oligodendrocyte impairment in the molecular pathogeneis of SZ.

BD occurs in 1% of the population and affects an individual's emotional response, with symptoms cycling between depression and mania. It is a complex genetic disease thought to require additional environmental factors for onset. Gene expression studies in BD have identified many changes in common with SZ, including oligodendrocyte abnormalities [140] and mitochondrial dysfunction [141].

6.1.1. *Expression Profiling in SZ and BD*

In the first microarray study to sample gene expression of the prefrontal cortex in 10 SZ patients compared to matched controls, the major group of transcripts differentially expressed belonged to those involved in the presynaptic secretory machinery [142]. This was also seen in a further five SZ cases and in subsequent microarray studies conducted by Vawter *et al.* [143] and Hemby *et al.* [144]. Interestingly, two of the SZ cases in the initial study were not taking medication, suggesting that these changes were specific to SZ, and were not produced in response to drug treatment. Additional array and *in situ* hybridization studies were performed on prefrontal cortex from monkeys treated with haloperidol, an antipsychotic drug [142]. These studies failed to detect any decreases in the presynaptic genes that were altered in the SZ cases, supporting the hypothesis that these changes are SZ specific.

Subsequent studies demonstrated the involvement of dysfunctional mitochondrial energy metabolism in SZ, with decreases in the mitochondrial malate shuttle system and the tricarboxylic acid (TCA) cycle [145]. In this study, comparison of these gene expression changes with those occurring in haloperidol treated monkeys identified malate dehydrogenase as increased following treatment, suggesting a direct therapeutic effect of the drug on mitochondrial metabolism.

Mitochondrial involvement was further analyzed by Iwamoto, whose study included both SZ and BD cases [141]. Focusing on the expression of 676 mitochondrial-related genes, global downregulation was seen in genes, including those involved in mitochondrial cell respiration, the TCA cycle, and mitochondrial transcription and translation. A previous study demonstrated that decreases in energy metabolism genes correlated with brain tissue of low pH (correlating with prolonged agonal state), rather than with the psychiatric disorders BD and MDD [146]. However, the study by Iwamoto reanalyzed the data using only high-pH samples. Although there were some changes to the genes identified as differentially expressed, the global downregulation of the mitochondrial-related genes was still evident. Fifty-seven transcripts were found to be in common between SZ and BD, while SZ exhibited 25 specific changes, and 19 changes were specific for BD. However, rather than using monkeys to assess the effect of the drug treatments, two non-medicated SZ cases and four non-medicated BD cases were analyzed for gene expression changes. Interestingly, these cases did not demonstrate a global downregulation of mitochondrial genes, but a tendency for upregulation, particularly in those genes involved in the respiratory chain components. Thus, the drug treatments in these patients are suggested to have a repressive effect on mitochondrial genes in the brain.

Early studies on prefrontal cortex of SZ cases also identified decreases in myelination-related genes, implicating oligodendrocyte dysfunction in the pathogenesis of SZ [147]. This hypothesis was supported and extended by Tkachev, who demonstrated oligodendrocyte and myelination genes, and the transcription factors controlling the expression of these genes, were downregulated not only in SZ but also in BD [140]. In addition, the changes in myelination-related genes are not specific for the prefrontal cortex, as they have also been identified in the temporal cortex of SZ cases [148]. A further study investigated whether these oligodendrocyte-related gene changes were due to drug treatments in SZ patients [149]. Although a few gene changes were found in common with monkeys treated with haloperidol for 3 months, unlike the mitochondrial gene changes, the majority of the data supported a role for oligodendrocyte impairment in SZ.

The identification of oligodendrocyte abnormalities and mitochondrial dysfunctional in both SZ and BD provide targets for symptomatic relief, and the similarity in pathways affected may reflect those genes involved in psychosis, and may explain why particular drugs developed for SZ are used successfully to treat BD. However, genes specifically altered in BD have been identified such as decreases in distinct mitochondrial genes [141] and increases in procaspase-8 and transforming growth factor β1 (TGFβ1) [150]. Since TGFβ1 is neuroprotective, the decrease in expression may contribute to the neurotoxicity seen in the disorder.

6.1.2. *Identifying Susceptibility Genes in SZ*

While the above studies have demonstrated how the molecular pathogenesis of SZ has been revealed using gene expression assays, they also provide an example of how novel susceptibility genes for SZ are identified. Regulator of G-signaling 4 (RGS4) was originally identified as the only consistently downregulated gene out of 7800 transcripts interrogated in 6 SZ cases, compared to 6 controls [139]. Analysis of 5 further SZ and 10 MDD cases confirmed the decrease as specific to SZ. Furthermore, the decrease was also evident in two SZ cases not on medication. Since RGS4 is also localized close to a region previously identified as an SZ susceptibility locus, chr 1q21–22 [151], sequencing of the coding regions in the SZ cases was carried out to determine if there were any mutations or functional polymorphisms. None were found. However, subsequent studies have focused on four single nucleotide polymorphisms (SNPs) located upstream and in intron 1 of the RGS4 gene.

The first study screened 13 SNPs from the RGS4 region in 3 cohorts of SZ cases and found 4 SNPs which showed distorted transmission [152]. However, the haplotypes differed between two American cohorts from Pittsburgh and the NIMH. Further studies by Morris *et al.* [153], Williams *et al.* [154], and Chen *et al.* [155] confirmed an association of SZ with RGS4, while Sobell *et al.* [156] and Brzustowicz *et al.* [157] have failed to find supporting data. To try to clarify the results, a meta-analysis of 13,807 samples were used from 13 cohorts to evaluate RGS4 as a susceptibility gene [158]. Although no individual risk factor was identified, at least two common haplotypes conferred a risk of SZ, and it is suggested this is due to the presence of an undetected risk factor which is only localized on these two haplotypes. Thus, the location of this susceptibility gene was enhanced by gene expression analysis.

6.1.3. *Identifying Biomarkers in SZ and BD*

As with HD, following gene expression assays to further the basic molecular pathology of the disease, identification of biomarkers in biological samples that can be taken while patients are alive, such as blood, presents the next goal. The first study of this kind in psychiatric cases describes the identification of a unique gene signature in blood samples which allows the SZ, BD, and control samples to be distinguished [138]. Following validation of key markers by RT-PCR, eight potential biomarkers were used to discriminate between SZ, BD, and control samples with an accuracy of 95–97%. Blood-derived biomarkers have potential benefits for diagnosis of these disorders, as early diagnosis and correct drug treatment can provide those affected with the disorders with a better quality of life.

6.2. Major Depressive Disorder

MDD describes individuals who experience a depression event of more than 2 weeks duration, though this may be a single or recurrent event. Unlike BD, there is no mania associated with the depression. Due to the complex interaction of genetic and environmental factors, gene expression arrays have been used to provide an overall view of the disorder and identify disease mechanisms. Although pathological studies implicate prefrontal and temporal cortices, as well as limbic structure, the temporal cortex is thought to be important in regulating emotional states, and functional magnetic resonance imaging (fMRI) and low-resolution electromagnetic tomography demonstrate differential responses in MDD cases compared to controls. Therefore, gene expression profiling of the temporal cortex (Brodmann area 21) in 12 MDD cases, including 7 under treatment, was performed and compared against control samples [159]. The study identified 225 changes in gene expression, including genes involved in neurogenesis, cell communication, chromatin and gene expression, and the cell cycle. To clarify that these changes were specific for the disease, rather than an effect of the drug treatments, the five samples from unmedicated individuals were used for analysis. All 225 changes identified initially were also found in this subsequent analysis. Therefore, these changes were due to MDD. The decreases in neurogenesis genes, specifically the myelin-related changes, correlate with the reduced density of oligodendrocytes seen in Brodmann area 9 [160] and amygdala of MDD cases [161], and may underlie the white matter changes seen by imaging techniques [162].

7. Future Advances

Gene expression arrays have advanced our understanding of many medical conditions, and allowed us to study the progression of the disease, or the effect of drug treatments. While the advent of LCM and linear amplification of RNA have widened the application of arrays to smaller sample sizes, recent advances in methodologies will extend the information available from microarrays yet again. The human genome is far more complex than was originally envisaged. Even the earliest iteration of the human genome indicated this to be the case, since it described only 33,000 genes, while it is known that there are a far greater number of proteins transcribed in humans. Thus, gene expression arrays in their current state only provide us with a partial view of the overall picture of the transcriptome of the cell. In order to investigate further the complexity of this situation, a panoply of new array types are being developed. Of particular interest to those studying gene expression are the alternative splicing, or exon arrays. These enable the

examination of alternative splicing events, which are important for identifying the range of proteins being produced by a cell, and are also able to uncover information about alternative promoter usage and alternative termination events [163] (www.affymetrix.com: Affymetrix Application Notes; Microarrays in Cancer Research).

In-depth analysis of the genome by projects such as ENCODE [164] has identified a large number of novel transcriptional start sites throughout the genome that were not recognized by the original algorithms [165,166], which suggest a greater degree of complexity in transcriptional control than was originally envisaged [167]. The new tiling arrays can be used for whole genome transcript mapping and will enable further investigation of these phenomena (www.affymetrix.com: Affymetrix Data Sheet Tiling Arrays).

In addition, the application of the "GeneChip" is reaching beyond measuring gene expression, and arrays are being used to investigate interactions between proteins and DNA, using on-chip chromatin immunoprecipitation (ChIP on-chip) [168, 169]. These have developed from earlier experiments [170, 171] and allow the investigation of multiple interactions between genes and a particular promoter protein. With the entire human genome now tiled out as 25-mer oligos, regions of protein–DNA interaction can be mapped, using methods already reported using a tiled array set containing chromosomes 21 and 22 [166, 172]. These assays should enable a more thorough examination of the processes that control gene transcription.

The single nucleotide polymorphism arrays are also useful for scientists investigating gene expression because they are able to demonstrate regions of genome amplification and/or deletion. While such rearrangements have long been known to be important in the field of cancer biology, this phenomenon has also been found to play a large part in normal human diversity [173].

What is apparent is that the gene expression array technology provides a useful introduction to the transcriptome of a group of cells. Some important and interesting genes involved in pathogenic processes have been identified using these tools. However, it is also evident that the processes that lead to disease require more investigation than simply compiling a list of genes which are differentially expressed in a particular situation. The more advanced methodologies becoming available will add to the armamentarium of the scientist trying to unravel the complexities of the changes that occur in diseased cells.

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