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Arata Azuma
Michael S. Schechter *Editors*

Treatment of Cystic Fibrosis and Other Rare Lung Diseases

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Arata Azuma • Michael S. Schechter
Editors

Treatment of Cystic Fibrosis and Other Rare Lung Diseases

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Editors
Arata Azuma
Div of Pulmonary Medicine
Nippon Medical School
Tokyo, Japan

Michael S. Schechter
Children's Hospital of Richmond
Virginia Commonwealth University
Richmond, Virginia
USA

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Preface

Some of the most exciting discoveries in pulmonary medicine have come from studying rare diseases. Insights gained from uncommon lung diseases often shed light on normal physiology as well as the mechanism of more common lung diseases. For example, investigations into cystic fibrosis (CF) have clarified the role of innate defense and mechanism of mucociliary clearance in the airway, as well as the function of the cystic fibrosis transmembrane conductance regulator protein in maintenance of airway surface liquid. The study of lymphangioleiomyomatosis (LAM) has led to an understanding of genes that control cell energy utilization, growth, and movement, potentially lending insights into the cellular and molecular basis of cancers. An understanding of the role of granulocyte macrophage colony-stimulating factor (GM-CSF) in the regulation of surfactant and other components of the complex biological systems in lung host defense as seen in pulmonary alveolar proteinosis (PAP) has led to GM-CSF being developed as an immunity-enhancing treatment for several other diseases.

Drug development for these conditions has been limited by a lack of understanding of the underlying mechanisms of disease and the relative unavailability of subjects for clinical trials, as well as the prohibitive cost of investing in novel pharmaceutical agents with poor market potential. Over the last several decades, however, legislation has been created in the USA (1983), Japan (1993), Australia (1998), and Europe (2000) to provide incentives for the commercial development of new "orphan drugs" to treat rare diseases, including those of the lung. In the USA, the National Institutes of Health has established the Rare Lung Diseases Consortium within its Office of Rare Diseases, and patient advocacy organizations such as EURORDIS are valuable allies in the fight against rare lung disease, by educating, supporting, and organizing patients and families in a manner that facilitates research. Central databases, registries, and research networks such as the Rare Lung Diseases Consortium in the USA and Orphanet in Europe are also useful adjuncts. With such rare disorders, international cooperation is critical for accumulating sufficient numbers of patients for research.

This volume of Milestones in Drug Therapy is dedicated to a discussion of a somewhat arbitrarily chosen group of rare lung disease—we must point out that many others, in which exciting new research is currently being performed, could have been chosen, but were not, in the interest of keeping the volume from being too overwhelming. We have included introductory chapters on the pathogenesis and current standard treatment of the diseases of interest, followed by chapters discussing the biologic basis of current and new investigational treatments for those conditions. We pay special attention to CF, which incidentally serves as a special example of successful alignment of governmental, academic, foundational, and pharma resources; research into this condition has led to extraordinary advances in our understanding of the underlying genetic and molecular basis of this disease and to dramatic improvements in survival and quality of life for affected individuals. Chapters in Part II focus on treatments directed toward the sequential well-defined steps in the pathogenic pathway of the disease. Additional chapters in Parts I and III discuss diffuse panbronchiolitis, for which the salutary effect of macrolides has been brought to attention due to their anti-inflammatory effect and potential benefit in a host of other inflammatory conditions; idiopathic pulmonary fibrosis, a previously untreatable condition that is now becoming better characterized and for which several effective drugs have become available, with others on the horizon; PAP, a disease for which the discovery of the underlying abnormality related to GM-CSF offers promise of effective treatment; and LAM, for which identification of the key role of dysregulation of the mTOR pathway has identified multiple novel therapeutic targets.

We wish to thank Jutta Lindenborn, our editorial contact from Springer, for keeping us on track and assisting in the organization of contributions from our valued chapter contributors, who of course did most of the “heavy lifting” for this volume. We hope this contribution will be of benefit to clinicians, students, and researchers looking for an introduction into the current investigations that are taking place in regard to the diseases discussed.

Michael S. Schechter, MD, MPH
Professor and Chief
Division of Pulmonary Medicine
Department of Pediatrics
Virginia Commonwealth University
Children’s Hospital of Richmond at VCU

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Part I
Etiopathology and Genetics of Rare Lung Diseases

Chapter 1
An Introduction to Clinical Aspects of Cystic Fibrosis

Nauman Chaudary and Michael S. Schechter

Abstract Cystic fibrosis (CF) is the most common life shortening inherited disease in people of Northern European background. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. CFTR is a 1480 base protein belonging to the ABC transporter family. It acts as a cAMP-activated chloride and bicarbonate channel and also regulates Na reabsorption through its effect on the epithelial sodium channel (ENaC). The loss of CFTR-mediated inhibition of ENaC leads to excess sodium and water reabsorption, resulting in dehydration of airway surface materials, and dehydration of airway surface materials. Concomitant loss of chloride efflux prevents the epithelium from correcting the low airway surface water volume. The subsequent decrease in periciliary water volume results in a reduction in the lubricating layer between the epithelium and mucus, causing inhibition of normal ciliary clearance of mucus. In addition to abnormalities in ion transport, dysregulation of the host inflammatory response appears to play an important role in cystic fibrosis. It is characteristic of CF that the airways become infected with pathogenic bacteria. Compounding the inability to clear infection, patients with CF also exhibit abnormal inflammatory signaling and an excessive inflammatory response. With persistent infection and periodic exacerbations of the chronic infection, progressive lung disease develops. Thus, in spite of the progress in treatment that has been made, the overwhelming majority of patients still die from respiratory failure. Treatment has traditionally focused on the downstream effects of CFTR dysfunction, and includes therapies that correct altered airway secretions (physical airway clearance therapy, dornase alfa, hypertonic saline, and the recently approved inhaled mannitol); anti-inflammatory

N. Chaudary, M.D. (✉)
Division of Pulmonary Diseases and Critical Care Medicine, Virginia Commonwealth University, Richmond, VA, USA

Department of Medicine, Virginia Commonwealth University, Richmond, VA, USA
e-mail: nauman.chaudary@vcuhealth.org

M.S. Schechter, M.D., M.P.H.
Division of Pediatric Pulmonology, Children's Hospital of Richmond, Virginia Commonwealth University, Richmond, VA, USA

Department of Pediatrics, Children's Hospital of Richmond, Virginia Commonwealth University, Richmond, VA, USA

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therapies (high-dose ibuprofen and alternate-day azithromycin); anti-infective therapies (inhaled anti-pseudomonal antibiotics such as tobramycin and aztreonam, along with intermittent treatment with systemic antibiotics of episodic pulmonary exacerbations), and when lung damage is severe, lung transplantation. The recent development of first generation CFTR modulating agents to treat CFTR dysfunction (ivacaftor, a potentiator that activates defective CFTR at the cell surface, and lumacaftor, a CFTR corrector that facilitates transport of class II mutations to the apical cell surface) marks the beginning of a new era of mutation- specific therapies to improve the function of defective CFTR protein. A number of next-generation modulators and other agents are currently moving through the drug development pipeline, offering hope for increased optimism regarding continuing improvements in the long-term outlook of this difficult disease.

Keywords Cystic fibrosis • Genetics • Pathophysiology • Diagnosis • Gene therapy • Ion transport

1.1 Introduction

Cystic fibrosis (CF) has been previously described as the most common *lethal* inherited disease in people of Northern European background, but in view of how much the outlook has changed over the last half-century, we now describe it as *life shortening*. The incidence in affected populations is about 1/3000 births overall, but there is significant variation by region, ethnicity, and race. The incidence is 1/1400 in Ireland. In the USA, CF is reported to occur in 1/3200 Caucasian births, compared with 1/15,000 African American births and 1 in 10,000 Latin American and Native American births. The prevalence in East Asians is considerably lower but hard to ascertain due to likely underdiagnosis (Bobadilla et al. 2002; O'Sullivan and Freedman 2009).

This chapter will serve as an overall introduction to the disease, its pathogenesis, pathophysiology, and treatment in order to provide a background for the chapters to follow.

1.2 Genetics

CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Of the >1900 CFTR mutations that have been identified, the functional abnormality of a relatively small number is known (Table 1.1) (O'Sullivan and Freedman 2009; Gallati 2014; Mickle and Cutting 1998). Class I mutations lead to the complete absence of any functional CFTR, either due to the presence of a premature stop codon resulting in the production of a truncated protein (so-called nonsense mutations) or the presence of splicing defects with no

Table 1.1 Classification of CFTR mutations

	Effect on CFTR	Functional CFTR present	Example mutations
Class I	Lack of protein production	No	G542X, 711 +1G→T
Class II	Protein trafficking defect	No	F508del
Class III	Defective regulation	No	G551D
Class IV	Reduced chloride transport	Yes	A455E, R117H
Class V	Splicing defect with reduced production	Yes	IVS8-5T
Class VI	Accelerated turnover	Yes	4326delTC

protein production. Class II mutations are associated with the production of a CFTR molecule that may retain some chloride channel function but is misfolded and degraded shortly after synthesis, before it can reach its site of action at the cell surface. The most important example of this mutation class is Fdel508 (in which the protein lacks a phenylalanine residue at position 508), as it is present in approximately 60–70 % of defective CFTR alleles and 80–90 % of all patients with CF. CFTR produced by class III mutations assumes the correct position at the cell surface but fails to be appropriately activated by ATP or cAMP to regulate ion transport. Class IV mutations are associated with reduced (but not absent) chloride transport through the normally positioned CFTR molecule; as a result of the small amount of residual CFTR function, these patients will usually have normal pancreatic function, at least initially. Class V mutations lead to a splicing defect that causes decreased production of CFTR protein; these mutations are especially important if associated with another mutation that causes the production of a CFTR molecule with decreased function. Class VI mutations produce a molecule that reaches the cell surface but is unstable and short lived.

The different mutation classes compromise CFTR function to varying degrees, with class I–III mutations typically associated with virtually no CFTR function and class IV–V mutations allowing some residual CFTR function (Mickle and Cutting 1998). Class VI mutations are variable in their expression. The organ that appears to be most sensitive to CFTR dysfunction is the vas deferens; men with mutations that retain residual function (most notably R117H) may exhibit congenital bilateral absence of the vas deferens (CBAVD) as their primary or only clinical manifestation of CF, depending upon the nature of the coexisting IVS8 polythymidine tract allele (Mickle and Cutting 1998; Gilljam et al. 2004). Pancreatic function is typically well predicted by genotype; patients with class I–III mutations are reliably pancreatic insufficient, while those with class IV–V mutations are pancreatic sufficient. In contrast, a looser genotype–phenotype correlation is seen for lung disease. While lung function, on average, is less severe in patients with class IV–V mutations, this is not uniformly the case due to the important role that both modifier genes and environmental factors play in determining the extent and severity of airway dysfunction (Collaco and Cutting 2008; Cutting 2010; Schechter 2011; Vanscoy et al. 2007).

1.3 Pathophysiology of CF

CFTR is a 1480 base protein belonging to the ABC transporter family (Fig. 1.1). It acts as a cAMP-activated chloride and bicarbonate channel and also regulates Na reabsorption through its effect on the epithelial sodium channel (ENaC) (Anderson et al. 1991; Quinton 2008; Collawn et al. 2012; Collawn and Matalon 2014). In addition, CFTR impacts transport of adenosine triphosphate (ATP) out of the cell activating outwardly rectifying chloride channel (ORCC) and regulation of chloride/bicarbonate transport, and it may inhibit calcium-activated chloride channels (CaCCs) due to lack of ATP at P2Y2 required to activate CaCC (Collaco and Cutting 2008; Cutting 2010; Schechter 2011; Vanscoy et al. 2007; Anderson et al. 1991).

There are four hypotheses regarding how CFTR dysfunction leads to the clinical manifestations of cystic fibrosis, and it may be possible that aspects of all four contribute to the pathogenesis of the disease.

The low-volume hypothesis postulates that the loss of CFTR-mediated inhibition of ENaC leads to excess sodium and water reabsorption, resulting in dehydration of airway surface materials (Matsui et al. 1998, 2006; Boucher 2007). Concomitant loss of chloride efflux prevents the epithelium from correcting the low airway surface water volume. The subsequent decrease in periciliary water volume results in a reduction in the lubricating layer between the epithelium and mucus, causing inhibition of normal ciliary clearance of mucus (Fig. 1.2). According to this hypothesis, mucus on the epithelium forms plaques with hypoxic niches that can harbor bacteria, particularly *Pseudomonas aeruginosa (PA)* (Boucher 2007; Worlitzsch et al. 2002).

A less well-accepted alternative "high-salt hypothesis" argues that in the absence of functional CFTR, excess sodium and chloride are retained in airway surface liquid (Smith et al. 1996; Zabner et al. 1998). The increased concentration of chloride in the periciliary layer disrupts the function of important innate antibiotic molecules (e.g., human β-defensin 1), allowing bacteria that are cleared by

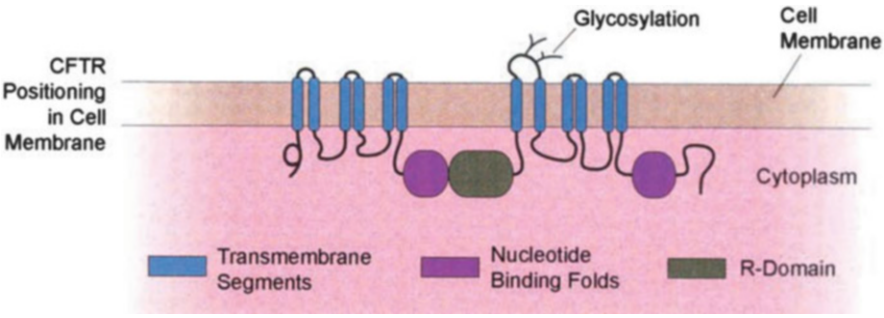

Fig. 1.1 Structure of the cystic fibrosis transmembrane conductance regulator (CFTR) molecule, consisting of transmembrane segments, nucleotide-binding folds, and a regulatory (R) domain. Adapted from Gibson et al. (2003)

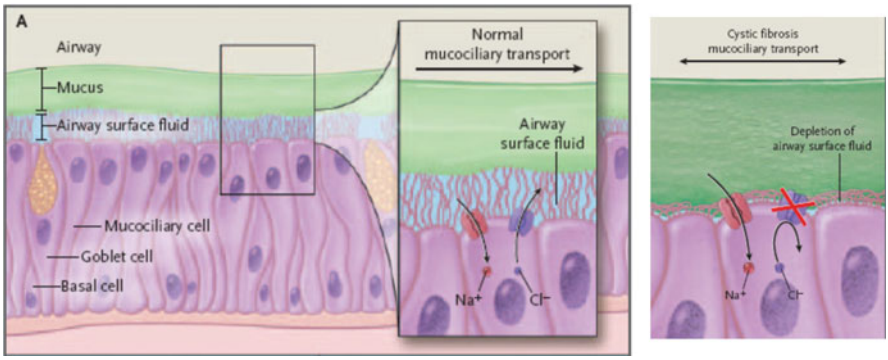

Fig. 1.2 Normally functioning CFTR determines airway surface fluid depth by regulating Cl^- (and bicarbonate) secretion and Na^+ reabsorption [the latter indirectly through its influence on the epithelial Na channel (ENaC)]. CFTR dysfunction and the resulting abnormalities in ion transport lead to reduced airway surface fluid and pH, inhibiting mucociliary clearance and innate defenses to lead to chronic infection and concentrating inflammatory mediators at the epithelial surface. Adapted from the *New England Journal of Medicine* 2006

normal airways to persist in lungs (Goldman et al. 1997). Studies in the CF knockout pig have indicated that depletion of airway surface liquid is not present in CF pig airways (Chen et al. 2010), lending some support to the "high-salt" hypothesis. The CF pig also has reduced CFTR-dependent bicarbonate secretion in the airways (Chen et al. 2010), and it has been suggested that reduced bicarbonate secretion leads to reduced airway surface pH which impairs innate bacterial defense mechanisms (Pezzulo et al. 2012).

In addition to abnormalities in ion transport, dysregulation of the host inflammatory response appears to play an important role in cystic fibrosis. Abnormally high concentrations of inflammatory mediators are seen in CF cell cultures and uninfected ex vivo tissue samples (Carlstedt-Duke et al. 1986; Tirouvanziam et al. 2000; Karp et al. 2004; Machen 2006). Furthermore, findings from lung lavage studies show that inflammation is present in children as young as 4 weeks of age who are apparently free of infection (Khan et al. 1995). An increase in proinflammatory molecules such as interleukin-8, interleukin-6, tumor necrosis factor-α, and arachidonic acid metabolites has been found in patients with CF (Freedman et al. 2004; Zaman et al. 2004; Colombo et al. 2005). Stimulation of the nuclear factor-kB pathway, platelet hyperreactivity, and abnormalities in neutrophil apoptosis has also been reported (Carrabino et al. 2006; O'Sullivan and Michelson 2006; Rottner et al. 2007). At the same time, low concentrations of native anti-inflammatory substances such as interleukin-10 and lipoxin favor unabated inflammation.

In addition, there may be a primary predisposition to infection due to CFTR dysfunction. In normal hosts, *PA* binds to functional CFTR and initiates an innate immune response, which is rapid and self-limiting. In patients with CF, an increase in asialo-GM1 in apical cell membranes allows increased binding of PA and

Staphylococcus aureus to the airway epithelium, without initiation of the CFTR-mediated immune response (Rottner et al. 2007; Campodonico et al. 2008). As a result, there may be a combination of compromised elimination of PA from the airways along with enhanced attachment of bacteria to the epithelial surface.

Whatever the explanation, it is characteristic of CF that the airways become infected with pathogenic bacteria. PA and *S. aureus* are typically thought of as the two most common infecting pathogens, but recent research regarding the airway microbiome suggests a much more complex microbiological picture than what had previously been theorized (Caverly et al. 2015). Compounding the inability to clear infection, patients with CF also exhibit abnormal inflammatory signaling and an excessive inflammatory response (Muhlebach et al. 1999, 2004; Chen et al. 2008). Paradoxically, the abundant neutrophil infiltration in the CF airway fails to clear the bacterial infection and instead contributes to airway injury, impaired innate immune function, and ineffective mucus clearance through the release of destructive neutrophil proteases and reactive oxygen species and of neutrophil DNA, which markedly increases mucus viscosity. With persistent infection and periodic exacerbations of the chronic infection, progressive lung disease develops. Thus, in spite of the progress in treatment that has been made, the overwhelming majority of patients still die from respiratory failure (Gibson et al. 2003). Pulmonary complications such as massive hemoptysis and pneumothorax occur in patients with end-stage disease.

While the lungs are the primary organ affected in most CF patients, CF is a multisystem disease, the manifestations of which can be best understood by considering it a disease that can affect most tubular epithelia. Abnormal secretions lead to obstruction of the pancreatic ducts, leading to pancreatic insufficiency in a majority of patients, with malabsorption of calories and fat soluble vitamins (A, D, E, and K); those with milder compromise of CFTR activity will have normal pancreatic function but may develop pancreatitis. Abnormal intestinal secretions lead to meconium ileus in 15–20 % of newborns and a propensity toward constipation and distal intestinal obstructive syndrome past the newborn period. Biliary obstruction may cause cholestasis and even cirrhosis. Nearly half of all adult patients also have CF-related diabetes. Other complications include sinusitis, osteopenia, osteoporosis, arthritis, and male (but not female) infertility due to CBAVD.

1.4 Diagnosis

The vast majority of patients with CF are currently diagnosed by newborn screening in the USA, Australia, and most of Western Europe. Newborn screening is most commonly accomplished in a multistep approach that begins with measurement of immunoreactive trypsinogen (IRT), followed by a screening of CFTR mutation

panel in infants with elevated IRT and followed by a sweat test in children who are found to carry at least one CFTR mutation. This process has a sensitivity of >95 % (depending upon IRT cutoff values) but has only been introduced in the last 10–15 years so that many older children and adults may still remain undiagnosed even in those countries that employ newborn screening. In the absence of newborn screening, CF is most commonly recognized at birth or in early infancy (classic form) because of bowel obstruction (meconium ileus) or low weight gain due to digestive problems. Presenting symptoms in newly diagnosed adults may include infertility in males due to CBAVD, recurrent pancreatitis, or respiratory manifestations such as recurrent bouts of sinusitis, bronchitis, and bronchiectasis. Patients diagnosed in adulthood are more likely to have class IV–V mutations and have milder manifestations; some may be classified as having "CFTR-related disorder" rather than classical CF if they have minimal disease and CFTR mutations with residual function (Boyle 2003). The term "CF-related metabolic syndrome" has been suggested for infants with mild CFTR dysfunction but no clear clinical manifestations who are found through the newborn screening process (Borowitz et al. 2009). These patients should be evaluated at CF centers with expertise and experience with unusual clinical presentations.

The sweat test remains the most readily available and clinically useful test for making the diagnosis of CF, provided it is performed at a laboratory that adheres to strict guidelines using pilocarpine iontophoresis and a quantitative determination of chloride concentration (LeGrys et al. 2007). A sweat chloride greater than 60 mmol/L is almost always diagnostic of CF. Patients with a sweat chloride in the so-called intermediate range (30–59) will often be found to have abnormalities in CFTR function. Sweat testing is feasible and reliable by 3 days of age, although it might be more difficult to obtain adequate sweat in young infants, especially those with low birth weight (Eng et al. 2005).

CF may also be initially diagnosed by identifying two disease-causing mutations, and a number of analytical methods for CFTR mutation detection are commercially available. In general, the use of a discrete panel of mutations is faster and less costly than expanded mutation analysis, and incorporation of approximately 40 of the most frequent disease-associated mutations will detect over 90 % of affected individuals in most populations. However, the diagnosis will be missed if the subject is affected by a mutation that is not included on the panel in use. Full sequence analysis will detect virtually all CFTR mutations, but its interpretation is sometimes difficult because it often uncovers polymorphisms and novel mutations whose significance may not be known (Farrell et al. 2008).

Nasal transepithelial potential difference (NPD) measurement is sometimes used to assess patients who may have CF but do not meet classical diagnostic criteria. However, NPD is labor intensive and technically difficult and is only available at a relatively small number of CF research centers. It is primarily a research tool for the evaluation of the ability of pharmaceutical agents to alter chloride channel function, and it has had limited validation as a diagnostic tool (Farrell et al. 2008).

1.5 Management

When the CF was first described in 1938, most children died in early childhood, primarily due to malnutrition (Davis 2006). The most important breakthrough that prolonged survival in those early years was the development of pancreatic enzyme replacement therapy (PERT). While attention over the last several decades has primarily focused on the treatment of lung disease, the benefits of maintaining good nutrition in regard to long-term survival and lung health are well established (Peterson et al. 2003; Konstan et al. 2003), making nutritional support an integral component of disease management from early infancy. Treatment with pancreatic enzymes should be used in those patients demonstrating pancreatic insufficiency as documented by low human fecal elastase-1 levels (Borowitz et al. 2002). Dosing is titrated to minimize fat malabsorption and is standardized according to the lipase activity of the enzyme preparation. Patients with CF can and should be expected to maintain BMI at or near the 50th percentile for age.

Over the last half-century, survival has increased substantially such that in 2014, the median expected age at death for patients with CF had improved to about 40 years in most affluent nations (Patient Registry 2007 Annual Report 2007). Increased survival of people with CF may be attributed not only to the availability of new therapies but also to increased expertise and more proactive use of both older and newly available therapies. Our discussion of treatment follows the pathogenetic steps shown in Fig. 1.3 and primarily focuses on those treatments that are currently available and used by clinicians; a description of the development of novel treatments may be found in the following chapters.

1.5.1 Gene Therapy for the Treatment of the CFTR Gene Mutation

The identification of the CFTR gene in 1989 immediately led to high hopes for gene therapy as a treatment and cure for CF. However, the early optimism that resulted from initial reports of apparent transfection of virus-delivered genetic material was dashed by subsequent reports of toxicity and failure of delivery. Continued efforts are underway using both viral and non-viral vectors; the reader is referred to several recent overviews of the current status of gene therapy (Oakland et al. 2012; Griesenbach and Alton 2013).

1.5.2 CFTR Modulating Agents to Treat CFTR Dysfunction

The recent development of ivacaftor marks the beginning of new era of mutation-specific therapies to improve the function of defective CFTR protein. Ivacaftor is a

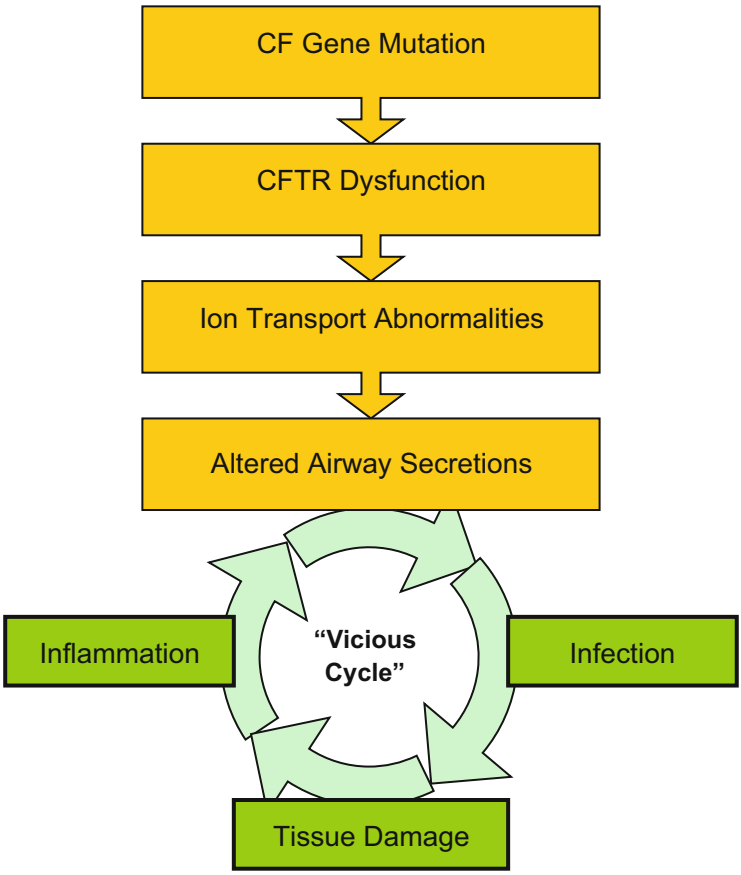

Fig. 1.3 Pathogenesis of cystic fibrosis. According to the commonly accepted low-volume theory, the gene mutation on chromosome 7 leads to an abnormal CFTR molecule whose dysfunction results in abnormalities in cation and anion conduction, leading to a dehydration of periciliary fluid that interferes with normal mucociliary clearance and failure of innate immunity. As a result, the airway is unable to clear bacteria; the ensuing inflammatory response, augmented by an increase in concentration of mediators in the pericellular fluid and other related abnormalities associated with oxidative stress, as well as extracellular release of neutrophil DNA, leads to increased obstruction that in turn further compromises airway clearance, adding to the difficulty in clearing infection. The ongoing unabated infection and inflammation lead to bronchiectasis, further abetting the compromise in airway clearance and attenuating the ability to clear the infection

small-molecule *potentiator*, delivered orally, that activates defective CFTR at the cell surface. The initial primary target for this therapy was a class III gating mutation mutated CFTR in which glycine has been replaced by aspartic acid at position 551 (G551D), but subsequent to the demonstration of successful augmentation of CFTR function in individuals with that mutation, it has been shown to be effective in patients with other mutations associated with normal trafficking of

CFTR to the cell surface but abnormal chloride transport (De Boeck et al. 2014). More recently, a combination of lumacaftor (a CFTR *corrector* which facilitates transport of class II mutations to the apical cell surface) and ivacaftor has been shown to cause modest but significant improvements in lung function (FEV1%), BMI, and decrease likelihood of pulmonary exacerbations at 24 weeks versus placebo in patients homozygous for F508Del (Wainwright et al. 2015). As described in detail in the chapter by Siracusa and Clancy in this volume, a number of additional correctors are currently under development, as well as drugs addressing read through of class I mutations associated with premature termination codons (Sermet-Gaudelus et al. 2010). Molecules that improve the splicing of CFTR in class V mutations and that stabilize protein at the cell surface in class VI mutations are currently undergoing preclinical evaluation as well, as are agents that nonspecifically amplify CFTR function.

1.5.3 Therapies to Correct Ion Transport Abnormalities

As discussed in the chapter in this volume by Daviskas, early studies indicated that amiloride, which blocks sodium reabsorption, corrected the overactive epithelial sodium channel (ENaC) abnormality, but clinical trials have failed to confirm a clinical effect (Burrows et al. 2012). Similarly, P2Y2 receptor agonists such as denufosol failed to live up to initial promise (Ratjen et al. 2012). Investigations of a number of new agents that inhibit ENaC are ongoing, but at the moment there are no available therapies that correct ion transport abnormalities.

1.5.4 Therapies that Correct Altered Airway Secretions

The "low-volume" hypothesis of the pathophysiology of CF, described earlier in this chapter, naturally led to investigations of the benefit of inhaling osmotic agents to increase the hydration of the airway surface liquid, and indeed this approach has been shown to be clinically successful. Inhalation of nebulized hypertonic saline (usually 7 %) is a mainstay of current CF therapy, used by about 2/3 of patients in the USA over the age of 6 years (Cystic Fibrosis Foundation 2013). Hypertonic saline has been shown in clinical trials to lead to an improvement in lung function and a decrease in the number of pulmonary exacerbations (Elkins et al. 2006). Its benefit in infants is currently undergoing evaluation, as initial negative trials have been attributed by some investigators to the need for an appropriate outcome measure in young children with normal lung function (Rosenfeld et al. 2012; Subbarao et al. 2013; Dasenbrook and Konstan 2012). Dry powder mannitol has also recently been shown to convey benefit in phase III clinical trials (Bilton et al. 2013), and it is being used in Europe but not currently in the USA. These therapies are described in more detail in the chapter by Daviskas.

As mentioned earlier, in addition to abnormal ion transport, CF is also characterized by immune dysregulation with an intense influx of neutrophils into the airway secretions. When neutrophils participating in the inflammatory response undergo necrosis, they release DNA that increases viscoelasticity and adhesiveness of CF sputum. Dornase alfa (recombinant human DNase) breaks down extracellular DNA, improving mucus clearability. It was the first drug specifically developed for use in CF and to show efficacy in clinical trials (Fuchs et al. 1994). It is commonly used in patients over the age of 6 years (86 % of patients in the USA) (Cystic Fibrosis Foundation 2013; Flume et al. 2007) and increasingly in younger patients although there is less evidence of efficacy in this group (Quan et al. 2001).

The aerosolized pharmacologic therapies directed toward correcting abnormalities in airway secretions are used in conjunction with physical airway clearance therapies such as percussion and postural drainage, positive expiratory pressure, active-cycle-of-breathing technique, autogenic drainage, oscillatory PEP, and high-frequency chest compression to help loosen thick mucus that is then removed with huffing or coughing. These techniques are generally considered to be a vital cornerstone to CF therapy and are begun at diagnosis (Flume et al. 2009).

1.5.5 Anti-Inflammatory Therapies

A number of anti-inflammatory agents have been shown to be beneficial in cystic fibrosis, but there is currently ongoing controversy and inconsistency regarding their use. Systemic steroids have long been known to improve airway function when used chronically, but are associated with the expected significant side effects such as glucose intolerance and cataracts (Eigen et al. 1995). There are no studies of their acute episodic use, but they are commonly employed in some settings during acute pulmonary exacerbations. Inhaled corticosteroids are used commonly, particularly in patients with apparent airway reactivity, but their benefit has not been clearly demonstrated (Ren et al. 2008). Ibuprofen, when given in high doses, provides long-term benefits in lung function, particularly in children. Concerns regarding gastrointestinal and renal toxicity have inhibited their adoption by most clinicians, even though experience has shown these adverse effects to be unusual (Konstan 2008).

The chronic use of macrolide antibiotics have been shown to improve lung function in CF patients, especially those infected with *P. aeruginosa* (PA) (Saiman et al. 2003). The mechanism of their immunomodulatory effect is unclear, and there may be an influence on PA virulence factors as well (Schultz 2004). Guideline recommendations have led to its use in about 2/3 of PA+ patients in the USA, but there are recent concerns raised about an antagonistic effect on inhaled tobramycin that still needs to be further elucidated (Nick et al. 2014).

A number of new anti-inflammatory agents are currently being evaluated for use in CF, and these are discussed in the chapter by Chmiel and Dasenbrook.

1.5.6 Anti-Infective Therapies

1.5.6.1 Chronic

The CF airway is characterized by chronic infection that begins in infancy (Sly et al. 2009) and may be controlled but never eradicated (O'Sullivan and Flume 2009). The microbiology of infection in CF patients follows a fairly typical course with most infants initially harboring *H. influenzae* and/or *S. aureus*; without specific intervention, *P. aeruginosa* eventually becomes the predominant organism found in the airways (Cystic Fibrosis Foundation 2008). The liberal use of antibiotics is another cornerstone of therapy, and they are given by the oral, intravenous, or inhaled route. The benefit of using chronic prophylactic antibiotics directed against *S. aureus* is controversial due to concerns that it may increase the likelihood of acquiring PA (Flume et al. 2007), but this approach has been commonly used in the UK (Smyth 2005). Patients who have chronic persistent airway infection with PA benefit from the regular use of inhaled antibiotics, such as tobramycin, aztreonam, or colistin (Flume et al. 2007); other antibiotics are currently being developed for inhalational use. There is a pronounced survival benefit for those patients who remain free of *Pseudomonas* infection (Konstan et al. 2007). For this reason, heightened surveillance for *P. aeruginosa* has become a common practice, combined with strategies to eradicate early *Pseudomonas* infection (Treggiari et al. 2007). These are discussed in more detail in the chapter by Flume and VanDevanter.

1.5.6.2 Episodic

Patients experience intermittent episodes of worsening signs and symptoms of airway disease which are associated with increased inflammation and airway obstruction that are labeled as "pulmonary exacerbations" and treated with discrete courses of systemic antibiotic therapy. There is currently no uniformly accepted defining criteria of what constitutes a pulmonary exacerbation of CF (Goss and Burns 2007), in part because of the evolving perception that a liberal approach to systemic antibiotic treatment of pulmonary exacerbations appears to lead to better long-term pulmonary outcomes in CF patients (Johnson et al. 2003; Szaff et al. 1983; Regelmann et al. 1990). Typically, treatment with oral antibiotics is initially attempted, especially in milder and younger patients, and if this does not successfully return the patient to baseline status, intravenous antibiotics are used. In addition to systemic antibiotics, therapy for a pulmonary exacerbation of CF generally includes increased use of airway clearance techniques and improved nutrition. The optimal treatment of pulmonary exacerbations has not been well studied; combination antibiotic therapy with agents displaying different modes of action is conventional, and the duration of therapy usually approximates 14 days (Doring et al. 2000). Home treatment with IV antibiotics is feasible, but may not be

as efficacious as hospital-based therapy (Nazer et al. 2006). Controversies in the treatment of pulmonary exacerbations are also discussed further in the chapter by Flume and VanDevanter.

1.5.7 Tissue Damage

The end result of chronic, lifelong infection and inflammation is irreversible bronchiectasis and eventual respiratory failure. At this point, the only remaining option is bilateral lung transplant, which is generally offered when death is likely to be imminent. The 5-year survival following lung transplantation is approximately 50 %, with bronchiolitis obliterans due to chronic rejection an ongoing problem. How best to select patients, especially children, for this high-risk procedure is being vigorously debated (Aurora et al. 2008).

1.6 Future Developments: CF Clinical Research

The development of new therapies for CF has been significantly accelerated by the development of clinical research consortia that have enabled collaborative efforts to enroll patients into clinical trials in a way that would not be possible without this kind of infrastructure. The US CF Foundation Therapeutic Development Network, founded in 1998, consists of 82 accredited CF care centers with experience and staffing to conduct clinical research, supported by a central coordinating center and a group of laboratories and interpretation centers, called National Resource Centers, with specialized expertise in developing and measuring CF clinical trial outcomes (https://www.cff.org/Our-Research/Therapeutics-Development-Network/). The European CF Society Clinical Trial Network has been active since 2008 and brings together 43 large and experienced CF centers located in 15 different countries throughout Europe (https://www.ecfs.eu/ctn). The capabilities provided by these two research consortia facilitate recruitment of patients into trials to support efficiencies in drug development that would otherwise be impossible given the relatively small number of patients with the disease cared for at any given center. With this infrastructure in place, there are high expectations for programs to discover and test new drugs in all the categories outlined above.

The improving clinical outcomes seen in patients with CF, especially children, had been presenting an increasing challenge to therapeutic development. The efficacy of most therapies in current use was shown by way of their impact on lung function and the occurrence of pulmonary exacerbations. However, these outcomes are inadequately sensitive in patients with mild disease; children, for example, can be shown to have considerable lung disease well before they are symptomatic or demonstrate either abnormal or deteriorating lung function (Sly et al. 2009; Hall et al. 2011). Newer techniques to detect and follow lung disease,

including chest CT and MRI (Tepper et al. 2016), infant pulmonary function testing (Rosenfeld et al. 2013) and multiple breath washout/lung clearance index (Owens et al. 2011), and measurement of inflammatory mediators in lung fluid, sputum (Sagel et al. 2012), and blood (Nick et al. 2013), are all currently undergoing evaluation to validate their use in research as well as clinical care (Tiddens et al. 2015).

Additional details regarding research directions are provided in the chapters that follow.

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Chapter 2
Diffuse Panbronchiolitis

Naoto Keicho and Minako Hijikata

Abstract Diffuse panbronchiolitis is included in the category of chronic inflammatory lung diseases and characterized by accumulation of lymphocytes and foamy macrophages in respiratory bronchioles and by mucus hypersecretion and neutrophilic inflammation in both upper and lower airways. By the absolutely beneficial effect of long-term macrolide therapy, we can say the prognosis of this disease was definitely improved. Patients with diffuse panbronchiolitis were mainly seen in East Asian countries, and genetic predisposition to the disease has been investigated. A strong association with human leukocyte antigen (HLA)-B54 has been reported historically among Japanese patients, whereas HLA-A11 was reportedly associated with Korean patients. Considering these findings, we have recently cloned novel mucin-like genes including *MUC22* in the candidate region between HLA-A and HLA-B loci in the short arm of human chromosome 6. Because of rarity of the disease, it is currently challenging to calculate exact odds ratio of the candidate genes for disease development.

Keywords Neutrophilic inflammation • Macrolide therapy • Human leukocyte antigens • Mucin genes • Genetic predisposition

2.1 Background

Diffuse panbronchiolitis is included in the category of chronic inflammatory lung diseases and characterized by accumulation of lymphocytes and foamy macrophages in respiratory bronchioles as well as mucus hypersecretion and neutrophilic inflammation in both upper and lower airways (Homma et al. 1983; Homma 1986; Keicho and Kudoh 2002). The term “diffuse” refers to the scattered distribution of the lesions in both lungs, while “pan-” refers to the inflammatory involvement of all layers of the respiratory bronchioles. Because of the above features, diffuse panbronchiolitis is distinct from commonly seen chronic obstructive pulmonary

N. Keicho (✉) • M. Hijikata
Department of Pathophysiology and Host Defense, The Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, 3-1-24 Matsuyama, Kiyose, Tokyo 204-8533, Japan
e-mail: nkeicho@jata.or.jp

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diseases and was proposed as a unique disease entity by a Japanese clinician and researcher, Homma and Yamanaka, in the 1960s. Unfortunately this proposal was not easily accepted internationally, because this disease was rarely seen in Western countries (Homma et al. 1983; Homma 1986).

2.2 Epidemiology

In Japan, for undefined reasons including improvement of socioeconomical status or hygiene, the incidence of this disease appears decreased recently (Kono et al. 2012). The prevalence was 11.1 in 100,000 among 70,000 employees in the Japanese National Railway corporation in 1980, but according to a recent unofficial survey in Japan, it appears to be decreased to 3.4 in 100,000 (unpublished observations). In a previous domestic survey among Japanese, age at onset is distributed with a peak on 40–60. Male to female ratio among clinically defined cases in the Japanese nationwide survey in 1980 was 1.4–1. Two thirds of the patients were not related to smoking. Predominance of non-smokers is also distinct from the features of chronic obstructive pulmonary disease (Keicho and Kudoh 2002).

In other Asian countries, the number of case reports gradually increased as the concept of this disease is recognized clearly and widely. Outside Asia, the number of cases is still limited, and typical cases are often from Asian immigrants even in Western countries (Keicho and Kudoh 2002; Urbano Aranda 2012).

2.3 Clinical Signs and Symptoms

Major symptoms of this disease are chronic cough with a large amount of purulent sputum and exertional dyspnea. Most of the patients have present or past history of chronic rhinosinusitis. Chest computer tomography reveals numerous small centrilobular nodules in the periphery of both lung fields. The appearance is called “tree in buds.” Because small nodules showing similar appearance are also observed in the limited area of the lung of other diseases such as pulmonary mycobacterial infection, scattered distribution of the nodules in the both lungs is an important feature of “diffuse” panbronchiolitis. These nodules consist of lymphocytes and foamy macrophages around respiratory bronchioles pathologically. When the disease advances, secondary dilatation of bronchioles and bronchi presents appearance of diffuse bronchiectasis of unknown cause on radiography and pathological sections. Typical findings of pulmonary function test are reduced FEV1.0 showing an obstructive pattern. Lymphoid-tissue hyperplasia around the bronchioles may cause airflow limitation in part. Hyperinflation is also observed. Blood gas analysis reveals hypoxemia particularly in the progressed disease.

2.4 The Diagnostic Criteria

The diagnostic criteria proposed in 1998 by a working group of the Ministry of Health, Labour, and Welfare of Japan are still useful for epidemiological survey of this disease (Nakata 1999):

1. Persistent cough, sputum, and exertional dyspnea
2. History of or current chronic sinusitis
3. Bilateral diffuse small nodular shadows on a plain chest X-ray film or centrilobular micronodules on chest CT images
4. Coarse crackles
5. FEV1/FVC <70 % and PaO_2 <80 mmHg
6. Titer of cold hemagglutinin equal to or higher than 64

Definite cases should fulfill criteria 1, 2, and 3 and at least two of criteria 4, 5, and 6.

High titer of cold hemagglutinin is often observed in Japanese patients, but this finding is not commonly described in the reports in other Asian countries. As a result of chronic bacterial infection, *Haemophilus influenzae* and other species are frequently detected from sputum. Bacteria in the airway tend to be replaced by *Pseudomonas aeruginosa* when the disease is advanced. The natural history of the disease is progressive and reaches chronic respiratory failure and fatal outcome with 60 % of the 5-year survival rate in those days (unpublished observation).

2.5 Starting Macrolide Therapy to the Disease

In 1982, Dr. Shoji Kudoh in Tokyo Metropolitan Komagome Hospital had a chance to examine a patient with the disease; his condition was remarkably improved after medication for 2 years received by a clinic in his hometown. From his detailed prescription records, he speculated that 600 mg of erythromycin every day for 2 years was potentially effective. Thus an open trial for low-dose, long-term erythromycin therapy was successfully done in his hospital (Kudoh et al. 1987). Since then, without conducting large-scale randomized controlled trials, Japanese physicians clearly noticed that erythromycin had a really strong therapeutic effect (Sawaki et al. 1986; Yamamoto et al. 1990; Nagai et al. 1991; Fujii et al. 1995). Prognosis of this disease has been dramatically improved, and now diffuse panbronchiolitis is regarded as a curable disease by the treatment (Kudoh et al. 1998). Although we have not yet proposed a single definitive mechanism by which macrolide antibiotic is so effective and specific to the treatment of this disease, macrolide therapy has been further advanced and used for various inflammatory diseases, such as COPD (Albert et al. 2011) and non-CF bronchiectasis (Wong et al. 2012; Altenburg et al. 2013). This would be discussed in another chapter of this book. In this chapter, we thus focus on the recent advances of the

genetic approach to the cause of this disease with much higher prevalence in Asians than non-Asians.

2.6 Pathogenesis

Airway defense is managed by three systems: physical barrier including mucociliary transport, innate immunity regulated by epithelial cells or phagocytes on the mucosal surface, and acquired immunity mediated by immunoglobulin and T-cell receptors. In chronic airway infection, one can assume at least one of these systems are defective (Pasteur et al. 2000; Bals and Hiemstra 2004; Zhang et al. 2000). When bacteria are inhaled into the airway with such a defect, they can be easily fixed on the surface of airway and then replicated and injure the surrounding tissues, by inducing inflammatory response. Neutrophils are mainly recruited to the site by the substances derived from the bacteria themselves or neutrophil chemotactic factors produced by the mucosal cells. Activated neutrophils in the airway further release proteolytic enzymes and reactive oxygen species. Copious sputum production with accumulation of neutrophils in the proximal airway is one of the important features of diffuse panbronchiolitis (Ichikawa et al. 1990). Hyperplasia of goblet cells and submucosal glands with abundant mucins is observed in the pathological section (Kamio et al. 2005). Although concrete mechanism for mucus hypersecretion is not clear, the volume of purulent sputum is remarkably reduced, and mucus rheology is normalized after the macrolide therapy, facilitating mucociliary clearance (Tamaoki et al. 1995; Rubin et al. 1997). Neutrophil numbers and elastase activity were significantly high in the bronchial fluid from the patients (Kadota et al. 1993), and excessive neutrophil chemotactic factors such as IL-8 and LTB4 and upregulation of adhesion molecules such as CD11b are presumably triggers of neutrophils that are recruited to the proximal airways (Oishi et al. 1994). It will further promote fixation of bacteria on the injured airway. Although epithelial cells have a potential to produce a variety of antimicrobial peptides, these molecules are not enough to kill pathogens at disease sites in such a case: Mucus is often excessively produced and mucociliary clearance is seriously disturbed in the disease. Vicious cycles of chronic airway infection are thus proposed. Upper and lower airways can be damaged by a possibly common mechanism. Many researchers believe the above pathogenesis is applied to that of diffuse panbronchiolitis (Homma et al. 1983; Homma 1986; Keicho and Kudoh 2002). However, another unique feature of this disease, lymphocytes and foamy macrophages around the small airway, is not simply explained by the possible defect of airway defense system. It remains unknown that the bronchiolar inflammatory reaction is caused by neutrophilic inflammation accompanied by bacterial infection or in the large airway.

Persistent bacterial infection may dysregulate system of acquired immunity, affecting mucosal defense mechanisms through the local production of IgG and IgA (Sato et al. 1992). Increased number of dendritic cells in the bronchiolar tissues

of the disease has also been reported, which may imply an enhanced mucosal immune response around the bronchioles through promotion of antigen presentation (Todate et al. 2000). However these defense mechanisms do not appear to clear bacteria from the airway effectively. Another group of researchers analyzed T lymphocytes collected from bronchoalveolar lavage fluid and found that the absolute number of T lymphocytes, especially activated CD8-positive cells, is increased together with a number of neutrophils (Mukae et al. 1995). These findings suggest the relationship between innate immunity and acquired immunity in this disease.

2.7 Findings that Show Immunological Abnormalities

Laboratory findings also suggest immunological abnormalities, which may be secondarily associated with chronic infection (Sugiyama 1993). The titer of cold hemagglutinin, autoantibodies against surface antigens of red blood cells, is continuously raised in many patients with no evidence of *Mycoplasma* infection (Takizawa et al. 1986). Increased serum IgA and positive rheumatoid factor are also observed.

2.8 Genetic Predisposition of Chronic Airway Diseases

Historically, rare genetic diseases characterized by chronic airway infection are well known: cystic fibrosis, primary ciliary dyskinesia, and IgG subclass deficiency are typical examples showing a role of genetic factors in the development and progress of chronic airway infection (Hoiby 1994; Corne 1996). It implies that genetic predisposition is definitely involved in functional impairment of upper and lower airways. In these genetic disorders, it is likely that airway mucosal defense systems are more or less damaged. Diffuse panbronchiolitis is also strongly influenced by host genetic factors, although it is not a simple Mendelian genetic disease.

2.9 Genetic Predisposition to Diffuse Panbronchiolitis

Although it is reasonable to assume that interaction between environmental and genetic factors is important to the development of diffuse panbronchiolitis, it is reported mainly in East Asian countries including Japan, Korea (Kim et al. 1992), and China (Chu et al. 1992; Tsang et al. 1998). In Western countries, the disease is quite rare and the cases are often atypical. In addition, half of the reports from those countries are from Asian immigrants (Hoiby 1994; Corne 1996). Thus researchers hypothesized that genetic variations in Asian populations might be involved in

development of the disease. Familial cases have also been described in domestic records in Japan, though seen only in domestic non-English reports.

2.10 Human Leukocyte Antigen and the Disease

Human beings recognize "self" through HLA class I molecules on the surface of their own cells. As mentioned later, defective expression of HLA molecules designated as bare lymphocyte syndrome causes serious signs and symptoms (Klein and Sato 2000a, b). Because of vital roles of these molecules, HLA has been investigated extensively in the variety of medical fields including infection, inflammation, autoimmunity, and transplantation medicine (Cooke and Hill 2001).

Human major histocompatibility complex (MHC) spans 3600 kb on the short arm of the sixth chromosome (6p21.3) and contains genetic loci encoding classical class I (HLA-A, HLA-B, and HLA-C) molecules, class II (HLA-DR, HLA-DQ, and HLA-DP) molecules, and many other genes contributing to immune and inflammatory reaction. In the past decade, entire nucleotide sequences of MHC region were determined (The MHC sequencing consortium 1999), and their variations carrying different HLA haplotypes were also reported (Horton et al. 2008). These studies have demonstrated that a lot of immune-related genes are densely packed in the MHC region, which appears to be associated with many disease processes (Shiina et al. 2009). From genetical standpoint, MHC regions are characterized by strong linkage disequilibrium, which means a set of genetic variations is preserved in the wide region, mostly unchanged through generations without events of chromosomal recombination. This characteristic of MHC regions is considered to be a main cause of difficulties in identifying a single gene responsible for a disease of interest by the ways of conventional genetic analysis including genome-wide association studies (Bakker et al. 2006).

The association of HLA with diffuse panbronchiolitis was initially reported by Sugiyama et al. (1990). They analyzed 38 patients and demonstrated that HLA-B54 (former HLA-Bw54) antigens are much more frequently observed in the disease group than in control group. Interestingly individuals carrying HLA-B54 antigens inhabit East Asian countries such as Japan, Korea, and China (Tokunaga et al. 1996). This finding provided the first approach to our question why this disease is predominantly seen in East Asians (http://pypop.org/popdata/2008/maps/B-5401.gif). Later, our group also confirmed this HLA association in 76 patients at DNA levels; 36.8 % of the Japanese patients had HLA-B54 by serological typing and HLA-B*54:01 by DNA typing, whereas 14.6 % of the control had the antigen (Keicho et al. 1998). Another rare allele in Asia, HLA-B*55:04 was also found in the process of HLA genotyping. Diffuse panbronchiolitis is thus a chronic lung disease strongly associated with an HLA class I gene in Asians, and it is likely that HLA-B*54:01 and B*55:04 are both susceptible to diffuse panbronchiolitis at least among Japanese (Fig. 2.1).

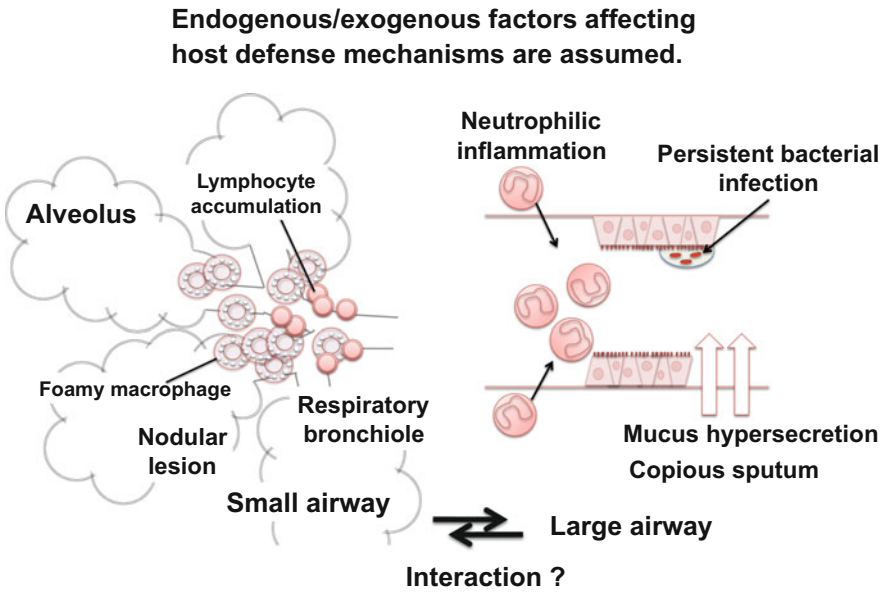

Fig. 2.1 Schematic representation of pathology and proposed pathogenesis of diffuse panbronchiolitis. Lymphocytes and foamy macrophages around the small airway and mucus hypersecretion with huge number of neutrophils in the large airway are characteristic to the disease. Unknown defect in host defense mechanisms is assumed

2.11 Hypothesis of an HLA-Related Disease Susceptibility Gene

This finding raised two possibilities about pathogenesis of the disease: the first one is the distinctive molecular structure of HLA class I alleles (HLA-B*54:01 in Japanese) themselves which are crucially important. In this case, CD8+ T cells that recognized pathogen-derived peptides through the particular HLA-B molecules may cross-react with tissue-specific peptides with a sequence motif similar to the exogenous peptides and induce chronic inflammation at the sites of disease, although we have not had any definite evidence to support this hypothesis. The second one is that another gene located near the HLA-B genetic locus on the same chromosome contributes to genetic predisposition. In fact, clinical characteristics and treatment response of the disease were not distinguished by the presence or absence of HLA-B54 (Keicho et al. 1998; Kadota et al. 2004). It may support possible influence of another gene rather than direct effect of HLA-B54. Actually reports from non-Japanese did not demonstrate clear disease association with HLA-B54: Tsang et al. reported no HLA-B54 observed in seven patients in Hong Kong (Tsang et al. 1998). Park et al. reported only 13.3 % of 30 Korean patients had HLA-B54 antigens, indicating no difference from the antigen frequency in their

control population (12.5 %) (Park et al. 1999). She et al. in Shanghai, mainland China, showed no association with HLA-B antigens either, by the analysis of 24 patients (She et al. 2007). Because of rarity of this disease, sample size of each study was rather small, and it is difficult to draw a definite conclusion. Nevertheless, it was notable that association with HLA-B antigens has not been reproduced in non-Japanese populations even in Asia. Interestingly Park et al. revealed a strong disease association not with HLA-B54 but with HLA-A11 (Park et al. 1999). Shanghai's report was also consistent with the association with HLA-A11 (She et al. 2007).

These findings raised an attractive hypothesis that one of the disease susceptibility genes is located between HLA-A and HLA-B loci. A presumably disease-bearing HLA haplotype (a set of alleles on a single chromosome), HLA-A11-B54 is typically observed in East Asians. According to the hypothesis, mutation that determines disease susceptibility might have occurred on a common ancestral chromosome carrying HLA-B54 and HLA-A11 (Keicho et al. 1998). After many generations, in Japanese, recombination events presumably happened frequently between HLA-A and the disease loci in the class I region, and subsequently the HLA-B54 might have been kept with the mutation. While in Koreans, recombination might have separated the relationship between HLA-B and the disease loci in the class I region, and probably the HLA-A11 has been maintained together with the mutation, because genetic evolution histories of Japanese and Koreans are closely related. This hypothesis is schematized in Fig. 2.2.

2.12 The Candidate Region of the Disease Susceptibility Gene in the MHC Region

Based on the hypothesis, we narrowed down the candidate region of the disease susceptibility gene in the targeted HLA class I region (Keicho et al. 2000). Between HLA-A and HLA-B loci spanning around 1400 kb, 14 genetic markers with short tandem repeats were investigated. Then haplotypes associated with the disease were analyzed. A segment commonly shared by disease haplotypes was identified, and a marker showing the strongest association was found inside the segment, which was a 200 kb candidate region for disease susceptibility gene and 300 kb apart from the HLA-B locus, in the direction of HLA-A locus on the short arm of human sixth chromosome.

A series of these studies were now summarized and registered as MIM604809 in a public database, Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/omim/604809).

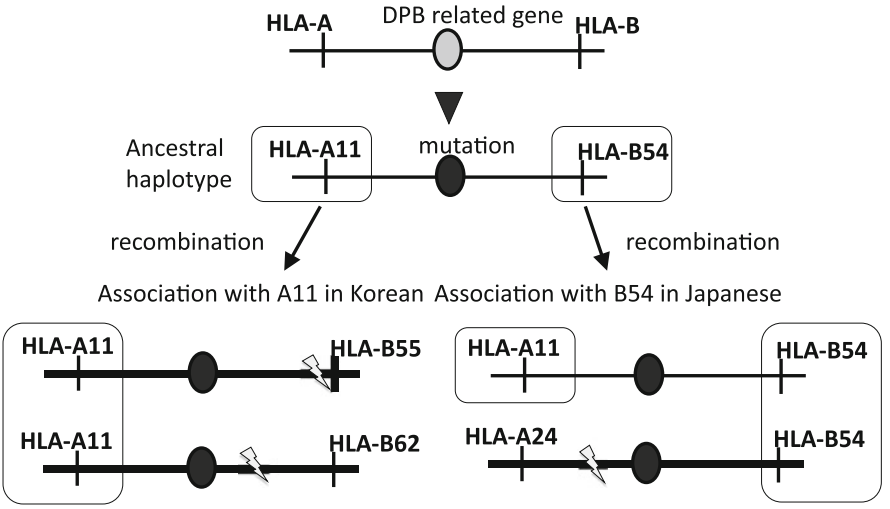

Fig. 2.2 A hypothesis to interpret the findings that diffuse panbronchiolitis is associated with different HLA types in the Japanese and Koreans. A disease susceptibility gene might be located between HLA-A locus and HLA-B locus (see the text for details)

2.13 Mucin-Like Candidate Genes

Fine mapping of the above 200 kb region was performed in our group. We first predicted exon-like structures by using a gene prediction software and then identified more than 100 single nucleotide polymorphisms within predicted exons and exon-intron boundaries. Using these genetic markers, structure of linkage disequilibrium was further analyzed. As a result, a region showing strong linkage disequilibrium was demonstrated within 80 kb of the 200 kb region (Fig. 2.3). Markers showing a strong association with the disease were also located in the 80 kb region. In this region, we cloned new genes designated panbronchiolitis-related mucin-like 1 and 2 (*PBMUCL1* and *PBMUCL2*) (Hijikata et al. 2011), which consist of a mucin-like gene cluster together with two adjacent genes, *MUC21* and *DPCR1* (Itoh et al. 2008; Matsuzaka et al. 2002).

2.14 Mucin Clusters

In human genome, two mucin gene clusters are already known (Rose and Voynow 2006). As far as we know, this is the third mucin or mucin-like gene cluster identified on human chromosomes (Fig. 2.4). PBMUCL1 was designated as *MUC22* later. We found genetic polymorphisms in *PBMUCL1*/*MUC22* that were associated with diffuse panbronchiolitis: in addition to a strong association with HLA-B in the Japanese, it is conceivable that the mucin-like gene *PBMUCL1*/

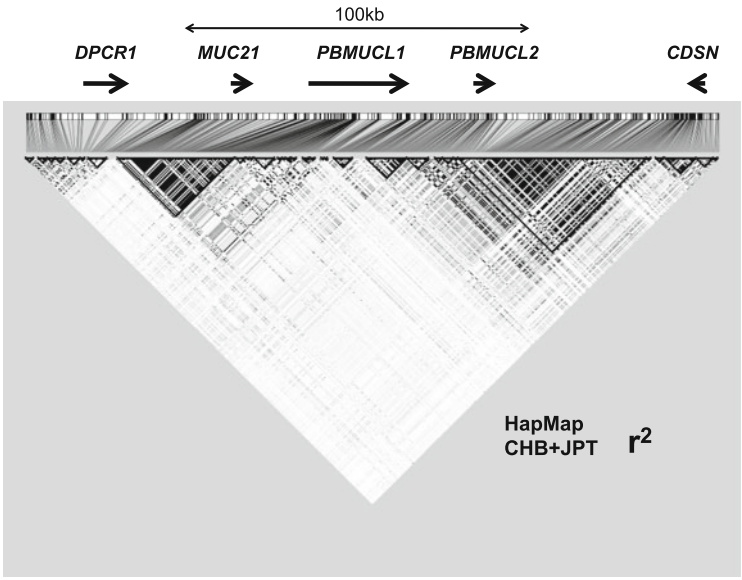

Fig. 2.3 Linkage disequilibrium (LD) pattern of the HLA class I candidate region for the disease susceptibility in the Japanese population. D prime, one of the LD parameters, was calculated and visualized by the Haploview program (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview). Single nucleotide polymorphisms with minor allele frequency >0.05 are shown. Strong linkage disequilibrium structure (a *black inverted triangle* on the *right-hand side*) is observed throughout 80 kb of the 200 kb region. Genetic markers showing a strong association with the disease were also located in the region

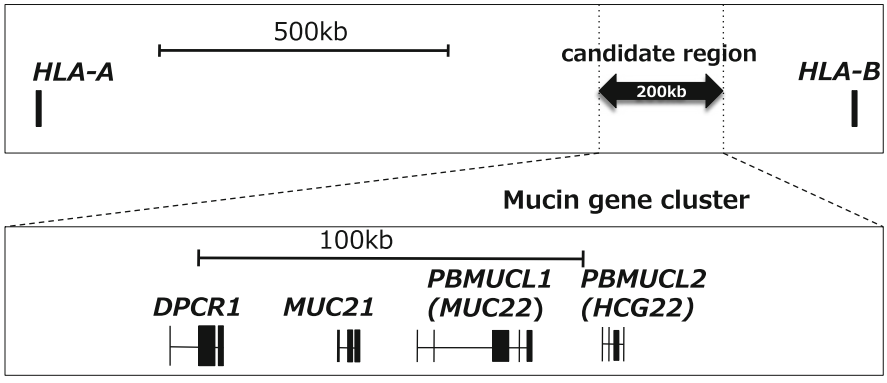

Fig. 2.4 A novel mucin or mucin-like gene cluster of which members showed association with diffuse panbronchiolitis in the HLA class I region on the short arm of the sixth chromosome (6p21.3). *DPCR1*, *MUC21*, *PBMUCL1*/MUC22, and *PBMUCL2* are all mucin or mucin-like genes

MUC22 is also one of the candidate genes for disease susceptibility from genetical standpoint (Hijikata et al. 2011). Functional significance of these genes is thus still unclear, and more investigation is necessary to link disease and the genes.

2.15 Classical Mucin Genes and Diffuse Panbronchiolitis

Identification of mucin-like genes in the disease candidate region raises a possibility that classical mucin genes might also be involved in the pathogenesis of diffuse panbronchiolitis. Excessive airway mucus secretion of the disease is also a reason why mucin or mucin-like genes are important candidates for disease-related genes.

Mucins are high molecular glycoprotein and secreted or membrane bound, depending on the mucin gene structures. Core proteins of the mucins consist of repetitive sequences rich in proline, threonine, and serine residues, and O-linked sugar chains are characteristically bound there (Lang et al. 2007). To date, more than 20 mucin genes have been identified. In the lung and bronchus, at least, expression of nine mucin genes is confirmed (MUC1, 2, 3, 4, 5AC, 5B, 7, 8, and 13) (Copin et al. 2000). MUC1 and MUC4 are expressed in ciliated epithelial cells as membrane-associated mucins; MUC5AC in goblet cells, MUC5B in mucous cells, and MUC7 in serous cells in the bronchial glands are secreted as gel-forming mucins.

Amount of mucus secretion should be optimal to protect airway mechanically and biochemically. Because mucins are rich in sugar chains, they trap some viruses inside the gel before it reaches the surface epithelial cells to be infected (Olofsson et al. 2005). When the secretion is too much, however, it may clog the airway, and ventilation is impaired. Dysregulation of mucus production also interrupts mucociliary transport, which causes frequent infection on mucosal surface of the airway. We once analyzed genetic variations in the regulatory regions in six airway mucin genes in diffuse panbronchiolitis (Kamio et al. 2005). In the regulatory region of the MUC5B gene, a functionally significant insertion/deletion polymorphism was identified: in fact, the deletion allele showed a negative association with the disease (Fig. 2.5). The regulatory region including the deletion allele showed lower transcriptional activity than others by the luciferase assay. These findings suggest that the presence of the deletion allele might protect disease airways from mucus hypersecretion through downregulation of MUC5B mRNA expression.

2.16 Aberrant Expression of MUC5B in Upper and Lower Airways

Expression of the gene is normally limited to submucosal glands. However, in the disease state of chronic airway inflammation, we demonstrated that MUC5B is aberrantly expressed on the surface of the airway mucosa. MUC5AC was expressed

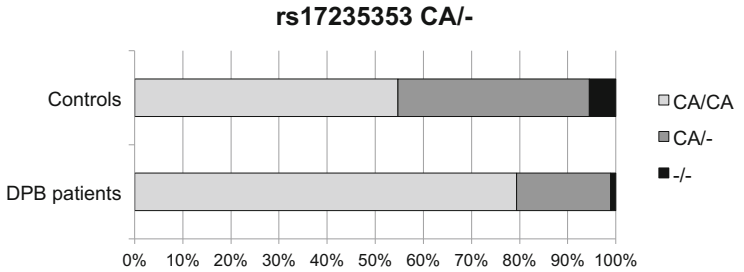

Fig. 2.5 Distribution of functional insertion/deletion polymorphism of the *MUC5B* gene is statistically different between control subjects ($N = 128$) and patients with diffuse panbronchiolitis ($N = 92$). The deletion allele was less frequently observed in the patients (Kamio et al. 2005)

together in airway epithelial cells with goblet cell hyperplasia. This aberrant MUC5B expression has also been demonstrated in the upper airway (Kim et al. 2004a). In healthy individuals, MUC5AC is expressed on the surface of sinus epithelial cells and MUC5B is localized in submucosal glands. By contrast, in inflammatory tissues, MUC5B was expressed in sinus epithelial cells as well as submucosal glandular cells.

It is thus likely that regulatory mechanism of mucin expression is rather different between normal and chronic inflammatory conditions throughout upper and lower airways. Although MUC5AC is known to be predominant as airway mucin and extensively investigated (Kaneko et al. 2003; Kim et al. 2004b), a role of MUC5B may also be crucial in inflammatory state. *MUC5B* regulatory polymorphisms we identified might thus modify the state of excessive mucus secretion characteristic to diffuse panbronchiolitis.

2.17 HLA Class I Deficiency

HLA class I deficiency is a very rare Mendelian genetic disease and also known as bare lymphocyte syndrome type I. We had a chance to see a 51-year-old female patient with this rare autosomal recessive inherited disorder caused by consanguineous marriage. Her clinical profile was previously reported by others (Maeda et al. 1985).

Since her childhood, she has presented symptoms of chronic airway infection. *Haemophilus influenzae* had been detected in her sputum. Chronic cough, purulent sputum, complication of chronic sinusitis, diffuse small nodular shadows on chest X-ray film, abnormal lung sound, impaired lung function, and elevated titer of cold hemagglutinin were her signs and symptoms and typically observed in diffuse panbronchiolitis as well. Therefore, she was treated by 600 mg of erythromycin, and within a few months of the treatment, her symptoms, cough and sputum, were remarkably decreased, signs of hypoxemia were alleviated, and pulmonary function measurements were also improved (Azuma et al. 2001). Surprisingly, all class I

molecules were hardly detected on the blood mononuclear cells of this patient by the conventional serological method, and the diagnosis of bare lymphocyte syndrome type I was made.

Bare lymphocyte syndrome type I is caused by a defect in antigen presenting system. The class I molecules themselves are synthesized normally, while their expression on the cell surface is markedly downregulated through the defect: during the course of antigen presentation, fragmented antigens enter endoplasmic reticulum through antigenic peptide transporter TAP1/TAP2 and encounter class I molecules and beta-2 microglobulin therein. Then the molecular complex generated there is further transported and presented on the cell surface. If either TAP1 or TAP2 is defective, heterodimer of the TAP molecules does not function as a transporter any longer, and antigenic peptides are not provided in the endoplasmic reticulum properly. As a result, most of the class I molecules without binding to antigenic peptides are unstably located on the surface and hardly detected by the routine serological method. In 1994, de la Salle reported a homozygous defect of the TAP2 gene in patients with HLA class I deficiency (de la Salle et al. 1994; Donato et al. 1995). Interestingly these patients also had chronic sinusitis and bronchial inflammation, similar to our case (http://omim.org/entry/604571). Our case was homozygous for a single nucleotide substitution in the splice acceptor site of the first intron of TAP1 gene. A frameshift due to this mutation was found to be a cause of functionally deficient TAP1 molecule (Furukawa et al. 1999). Although a causative mutation of this disease was thus clearly demonstrated, it remains unknown why it resembles diffuse panbronchiolitis in disease phenotype and why long-term macrolide treatment was effective. Further investigation would be necessary to identify common mechanism by which phenotype of chronic upper and lower airway infection is presented in diffuse panbronchiolitis and HLA class I deficiency.

2.18 Cystic Fibrosis and Diffuse Panbronchiolitis

Cystic fibrosis is mainly observed in European descendants and shows marked contrast from diffuse panbronchiolitis in geographical distribution (Hoiby 1994). Cystic fibrosis is caused by mutation of cystic fibrosis transmembrane conductance regulator (CFTR) gene located on the long arm of the seventh chromosome (7q31). CFTR ΔF508 is a predominant mutation observed in European descendants. This founder mutation presumably occurred after Europeans had been separated from Asians (The HUGO Pan-Asian SNP Consortium 2009; Normile 2009). This founder effect clearly explains high prevalence of European cystic fibrosis and its rarity in Asians. The CFTR deletion impairs mucociliary clearance and airway microenvironment and leads to a progressive cycle of infection and inflammation and possibly in chronic airway diseases (Lee et al. 2003; Casals et al. 2004).

In the case of diffuse panbronchiolitis, another founder effect of a different disease susceptibility gene has been proposed in Asians, although it is not following

Mendelian mode of inheritance, but multifactorial genetic disease, suggesting contribution of each responsible gene to diffuse panbronchiolitis is not large. In fact, CFTR ΔF508 mutation has not been observed in diffuse panbronchiolitis (Akai et al. 1992).

2.19 Functional CFTR Polymorphisms

The poly-T and TG repeats in intron 8 (IVS8) are CFTR polymorphisms commonly found in Asians and cause abnormal RNA splicing in the CFTR gene itself (Chu et al. 1993; Cuppens et al. 1998). It is conceivable that such variations may have relevance to Asian lung diseases (Noone et al. 2000; Pignatti et al. 1996), even though incidence of classical CF is very low in many Asian populations. We also tested this hypothesis and found significantly higher frequency of the T5 (five thymine residues containing) allele of the poly-T in intron 8 in patients with another type of chronic airway infection, pulmonary *Mycobacterium avium* complex infection, than in healthy controls (Mai et al. 2007). All T5 alleles were associated with long TG repeats; the TG12 or TG13 allele. Thus, Asian CFTR alleles might be involved in susceptibility to at least a part of chronic airway infection, and we cannot exclude the possibility that accumulation of other minor mutations of the CFTR gene might account for a small proportion of the category of diffuse panbronchiolitis or modify its phenotype or severity.

2.20 Conclusion

Four decades have passed since diffuse panbronchiolitis was first described in Japanese clinicians and researchers. Although neither environmental factors nor infectious agents specific to the disease have been demonstrated, studies on the etiology of the disease have progressed in the context of a genetic predisposition unique to Asians. Diffuse panbronchiolitis is regarded as a complex genetic disease affecting East Asians and is strongly associated with the class I HLA-B54 in Japan and HLA-A11 in Korea. Based on a hypothesis that one of the major susceptibility genes for diffuse panbronchiolitis is located within the 200 kb in the class I region 300 kb telomeric of the HLA-B locus on the chromosome 6p21.3, we have cloned novel mucin-like genes including *MUC22* in the candidate region. Together with newly found genes, HLA class I genes themselves and other mucin genes may also influence development of the disease in part (Keicho and Hijikata 2011). Functional significance of candidate genes would be further elucidated by in vitro and in vivo studies. Different genetic or environmental background may cause a similar phenotype and treatment response of the disease that are hardly distinguished from the original type of diffuse panbronchiolitis. This idea is important especially when non-Asian patients with diffuse panbronchiolitis are reported.

Although the advent of macrolide therapy has strikingly improved the prognosis of this disease, we still do not know whether the beneficial effect of macrolides in vivo can be completely separated from the original antimicrobial effect. We do hope that genetic approach will be helpful in identifying a target molecule and design of novel types of anti-inflammatory drugs for chronic lung diseases.

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Chapter 3
Idiopathic Pulmonary Fibrosis

Chiko Shimbori, Pierre-Simon Bellaye, Philipp Kolb, and Martin Kolb

Abstract Idiopathic pulmonary fibrosis (IPF) is a chronic and fatal disease of unknown cause representing the most common form of idiopathic interstitial pneumonias. It is believed that the development of IPF is influenced by both genetic and environmental factors. The pathogenesis of IPF is complex, and many contributing factors to fibrogenesis are known to date. In recent years there have been important advances in the understanding of the pathogenesis of IPF. A large number of experimental studies have highlighted the importance of epithelial cell injury, fibroblast differentiation and myofibroblast activation, the involvement of inflammatory and progenitor cells, and the effect of genetic and epigenetic factors. IPF is characterized by progressive fibroblast proliferation and differentiation followed by excessive deposition of extracellular matrix (ECM). This results in the damage to the structure of the lung, which eventually causes dyspnea and respiratory failure in patients with IPF. This fibrotic ECM microenvironment is characterized by altered biochemical and biomechanical properties and stores abundant amounts of growth factors, all of which can affect the behavior of structural lung cells and also inflammatory cells. This chapter summarizes the newest insights into the pathogenesis of IPF and tries to describe how this knowledge helps to find new therapies for the patients who are suffering from this devastating disease.

Keywords Idiopathic pulmonary fibrosis • Pulmonary fibrosis • Interstitial lung diseases • Fibrosis • Rare lung diseases

3.1 Introduction and Clinical Features

Idiopathic pulmonary fibrosis (IPF) is characterized by relentless progression of interstitial fibrosis with a progressive decline in gas exchange, which often results in respiratory failure and death within 4–5 years after diagnosis. IPF is characterized by dyspnea, cough, and a restrictive defect in lung function. Identifiable causes

C. Shimbori • P.-S. Bellaye • P. Kolb • M. Kolb (✉)
Department of Medicine, Firestone Institute for Respiratory Health, McMaster University, Hamilton, ON, Canada
e-mail: kolbm@mcmaster.ca

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of lung fibrosis have to be excluded. Both radiology and histopathology have to show a distinct pattern of usual interstitial pneumonia (UIP). Until recently there were no therapies available, but two just published phase III clinical studies have shown that two novel antifibrotic drugs, pirfenidone and nintedanib, can slow the progressive decline in lung function in patients with IPF (King et al. 2014; Richeldi et al. 2014) and were recently approved for clinical use in IPF by the FDA. Despite this, therapeutic options are still limited, and the development of novel drugs targeting different fibrotic mechanisms within IPF is imperative (Hambly et al. 2015).

3.1.1 Epidemiology

IPF is the most common form of idiopathic interstitial pneumonias (IIP). The annual incidence of IPF is 5–16 per 100,000, and the prevalence is 13–20 per 100,000 individuals (Raghu et al. 2006, 2011; King et al. 2011). The morbidity associated with IPF is substantial, with 50 % of patients dying within 3–4 years. This equates to a mortality higher than for most common cancers (Vancheri et al. 2010; Ley et al. 2011; Raghu et al. 2011). IPF is more frequent in men (approx. 2:1) compared to women (Raghu et al. 2006) and typically occurs in middle-aged and elderly adults (median age 66 years). It is very uncommon to see IPF under the age of 50 (Raghu et al. 2011; King et al. 2011).

3.1.2 Pathology

The main pathological features of UIP are heterogeneous degrees of fibrosis throughout the lungs with subpleural and paraseptal fibrosis, excessive and disorganized deposition of ECM (mainly collagen), honeycomb cysts, and destruction of lung architecture (Raghu et al. 2011). In small areas of active fibrosis, clusters of fibroblasts and myofibroblasts accumulate (fibroblastic foci). There is spatial and temporal heterogeneity of the process and indicates an ongoing disease. Smooth muscle metaplasia in the interstitium is commonly seen in areas of fibrosis. Inflammation is usually mild and consists of a patchy interstitial infiltrate of macrophages, lymphocytes, and plasma cells associated with the hyperplasia of type II pneumocytes and bronchiolar epithelial cells (Todd et al. 2012).

3.1.3 Diagnosis

The diagnosis of IPF includes a thorough history taking to exclude known and treatable causes for lung fibrosis. Patients with IPF typically present a gradual onset of symptoms among which the most disabling are dyspnea and a nonproductive

cough. Physical examination reveals digital clubbing in 25–50 % (Turner-Warwick et al. 1980; Johnston et al. 1997), and coarse end-inspiratory crackles are present in more than 80 % of patients. Impairment of gas transfer within the lung leads to decreased PaO_2 at rest or during exercise (Mahendran and Sethi 2012). Associated systemic symptoms are rare but can occur and include weight loss, fatigue, arthralgias, and myalgias, but presence of these should always trigger a more thorough investigation for connective tissue diseases. The consensus statement from the American Thoracic Society, European Respiratory Society, Japanese Respiratory Society, and Latin American Thoracic Association recognizes IPF as a type of chronic, progressive interstitial lung disease with specific findings on high-resolution computed tomography (HRCT) scan and surgical lung biopsy (Raghu et al. 2011). The characteristic HRCT finding for IPF is a "UIP pattern," consisting of reticular abnormalities in a subpleural and basal distribution, with rows of subpleural cysts (honeycombing).

The clinical management of IPF remains a major challenge not only due to limited availability of effective drugs, but also a lack of good indicators for disease progression. Currently, there are no validated biomarkers for use in the clinical care of patients with IPF (Vij and Noth 2012). There are some promising candidates, including KL-6, surfactant proteins A and D, Mucin 5B (MUC5B), matrix metalloproteases (MMP) 1 and 7, CCL18, VEGF, YKL-40, osteopontin, fibrocytes, and T-cells (Vij and Noth 2012; Hambly et al. 2015). Although none of these have been established as a specific biomarker for IPF, this is an active field of investigation, and the use of multiple biomarkers in combination may eventually become useful for diagnosis and monitoring disease progression and will eventually contribute to develop personalized medicine.

3.2 Risk Factors

The etiology of IPF is complex, likely involving a mix of genetic and environmental influences, which interact over extended periods of time. Some known risk factors for IPF include exposure to cigarette smoke, metal and wood dust, drugs, contact with infectious agents, age, and gender (Raghu et al. 2011; King et al. 2011; Selman and Pardo 2014). Genetic factors are also relevant, highlighted by the significant presence of familial IPF (Hodgson et al. 2002; Hambly et al. 2015). Interestingly, all these risk factors that predispose to IPF—age, sex, cigarette smoke, and genetic variants—are able to influence epigenetic marks within the genome.

3.2.1 Genetic Predisposition

Recently, major advances have been made to understand the genetic predisposition to develop IPF by analyzing genome-wide association study (GWAS) data, which may eventually help early detection in genetically susceptible individuals. Several genes are associated with IPF, and with every year of research, more are being discovered. The late presentation of patients renders the precise contribution of genetic transmission to IPF which is difficult and uncertain, but it appears to be responsible for 5 % of IPF cases (Noble et al. 2012). Mutations in SP-C and SPA2 encoding genes (SFTPC and SFTPA2, respectively) have been associated with IPF (Thomas et al. 2002; Wang et al. 2009; Maitra et al. 2012). These proteins are exclusively expressed and secreted by type II alveolar epithelial cells. Although these studies provide strong evidence for linkage of surfactant proteins mutations to specific familial cases of pulmonary fibrosis, these mutations occur in only 1 % of sporadic cases of IPF. Interestingly, mutations in the aging-related genes such as the TERT and TERC, two components forming telomerase, are associated with the development of IPF (Armanios et al. 2007; Tsakiri et al. 2007). These polymorphisms may contribute to telomere shortening, a hallmark of aging. Mutations in TERT and TERC may be present in 8–15 % of patients with familial and 1–3 % of patients with sporadic IPF. Leukocyte telomere lengths of these patients are generally shortened as compared to age-matched controls. Although persistent inflammation, senescence, and depletion of alveolar epithelial progenitor cells have been observed, the precise mechanisms by which telomerase deficiency contributes to IPF remain elusive.

In the past years, a common polymorphism in the promoter region of the MUC5B has repeatedly been associated with IPF (Seibold et al. 2011; Zhang et al. 2011; Stock et al. 2013). MUC5B is expressed in the bronchial epithelium and not in type II alveolar epithelial cells under physiological conditions. However, most individuals in the general population with this polymorphism (present in 19–20 % of control subjects) do not develop pulmonary fibrosis (Seibold et al. 2011; Zhang et al. 2011). Although the mechanism by which the MUC5B promoter variant leads to IPF is still unknown, a recent animal model suggested that MUC5B may be involved in regulating homeostatic and pathological microbial populations in the lung (Fletcher and Evans 2014). Mutations in toll-interacting protein (TOLLIP), which negatively regulates innate immunity inhibiting toll-like receptor signaling and genes associated with cell–cell adhesion and DNA repair, are linked to IPF. Interestingly, TOLLIP interacts with Smad7 suppressing TGF-β signaling and, among other effects, inhibits epithelial–mesenchymal transition (EMT) (Zhu et al. 2012). Both MUC5B promoter and TOLLIP variants have been linked to the survival in IPF (Noth et al. 2013; Peljto et al. 2013), and the MUC5B promoter variant is also associated with asymptomatic interstitial lung abnormalities in the general population (Hunninghake et al. 2013). A recent case–control GWAS identified several gene variants, critical for the epithelial integrity, as important risk factors in IIPs (Fingerlin et al. 2013). The association between IPF

and TERT, MUC5B, and TERC mutations has been confirmed by this study. Several newly associated loci have been reported, including FAM13A, DSP, OBFC1, ATP11A, and DPP9, which are related to host defense, cell–cell adhesion, DNA repair, epithelial injury, and aberrant repair (Shulenin et al. 2004; Zhang et al. 2005; Acharya et al. 2006; Delva et al. 2009; Cho et al. 2010; Levy et al. 2010; Mangino et al. 2012; Mathai et al. 2016). Interestingly, a recent study reported that certain genotypes can affect on the treatment response to *N*-acetylcysteine in IPF (Lozano-Wilhelmi 2015). Because of the extremely complicated and diverse pathophysiology of IPF, personalization of treatment might be indispensible in the future.

3.2.2 Environmental Factors

Numerous environmental exposures have been associated with the development of IPF. The fact that asbestos can cause lung fibrosis, often indistinguishable from IPF on histology (Rom and Harkin 1991; Brody 1993; Liu et al. 1997), highlights that inhaled factors may contribute to IPF as well. A strong association has been demonstrated between cigarette smoking and IPF (Schwartz et al. 1994; Baumgartner et al. 1997). Cigarette smoke remains a risk factor for the development of IPF even years after smoking cessation (Baumgartner et al. 1997), suggesting that the fibroproliferative process induced by cigarette smoke may at some point become self-sustaining (Spira et al. 2004). While it is well known how cigarette smoke contributes to chronic obstructive pulmonary disease, it is obscure what drives cigarette smoke-induced pulmonary fibrosis (Selman and Pardo 2014). Epidemiological studies have also established associations between metal or wood dust exposure and IPF in the United States (Baumgartner et al. 2000), the United Kingdom (Hubbard et al. 1996, 2000), and Japan (Iwai et al. 1994; Miyake et al. 2005). Viral particles have been detected in the lungs of IPF (Egan et al. 1995; Tang et al. 2003). For instance, Epstein-Barr virus has been found in 40 % of patients with IPF (Stewart et al. 1999). The involvement of influenza, *Cytomegalovirus*, and hepatitis C virus has also been suggested in the pathogenesis of IPF (Raghu et al. 2011). It is very interesting to note that considerable variability exists in the extent of pulmonary fibrosis among workers exposed to similar concentrations of fibrogenic dusts or organic antigens. For instance, after exposure to asbestos, similarly exposed individuals may experience different outcomes (Polakoff et al. 1979; Selikoff et al. 1979). This is likely due to that fact that the development of lung fibrosis involves a complex interaction of multiple genes and environmental factors.

Genetic predisposition plays also a role in animal models of lung fibrosis. Inbred strains of mice differ in their susceptibility to fibrogenic agents. In comparison to BALB/c or Sv129 mice, C57BL/6 mice are more susceptible to lung fibrosis when challenged with either bleomycin (Rossi et al. 1987; Ortiz et al. 1998) or asbestos (Corsini et al. 1994; Warshamana et al. 2002). Genomic analyses of bleomycin- and

radiation-induced mouse models of fibrotic lung disease have revealed distinct susceptibility loci. These studies identify the major histocompatibility complex II antigen H2-Ea as a potential target gene (Haston et al. 2002).

3.2.3 Aging

Many of the hallmarks of aging, e.g., genomic instability, telomere shortening, epigenetic alterations, mitochondrial dysfunction, and cellular senescence, have been proposed as essential mechanisms in the development of IPF (Selman and Pardo 2014). It is important to emphasize that aging impairs the repair capacity of tissue and usually worsens the fibrotic response (Torres-Gonzalez et al. 2012; Fernandez Perez et al. 2013). GWAS studies highlighted age-related genes variants such as TERT, TERC, and OBFC1 as involved in IPF (Fingerlin et al. 2013). For example, strong evidence supports that microsatellite DNA instability occurs in IPF (Demopoulos et al. 2002). Other factors involved in aging such as abnormal telomere shortening, impaired autophagy, dysfunctional mitochondria, excessive reactive oxygen species (ROS) production, as well as mitochondrial-mediated AEC apoptosis have been described in IPF (Kuwano et al. 2002; Alder et al. 2008; Araya et al. 2013). Furthermore, the susceptibility to endoplasmic reticulum (ER) stress increases with age, and ER stress has an important effect on the physiology of AECII mitochondria and influences susceptibility to lung fibrosis. A recent study showed that deficiencies in expression of PINK1 are related to aging and ER stress (Wei et al. 2013; Bueno et al. 2015).

3.2.4 Related Diseases

Several studies have suggested that abnormal gastroesophageal reflux may be a risk factor for IPF (Tobin et al. 1998; Patti et al. 2005; Raghu et al. 2006, 2011). Other risk factors such as diabetes mellitus have been described (Gribbin et al. 2009). Patients with rheumatoid arthritis (RA) and other autoimmune diseases (e.g., systemic lupus erythematosis (SLE), systemic sclerosis) show NSIP or UIP pattern (Cojocaru et al. 2011; Gifford et al. 2012). Interestingly, several autoantibodies have been reported in IPF cohorts (Dobashi et al. 2000; Ogushi et al. 2001; Feghali-Bostwick et al. 2007; Kahloon et al. 2013). The production of autoantibodies with specificities for varied autoantigens is a common feature of auto-immunological diseases (Solomon et al. 2002). Further research is required to elucidate this mechanism and possible cause–effect relationship.

3.3 Pathogenesis of Pulmonary Fibrosis

Historically, IPF was considered the result of an uncontrolled inflammatory response in the lungs. Due to a lack of correlation between conventional inflammatory markers with IPF progression and the poor response to anti-inflammatory therapy, the prevailing hypothesis has shifted toward repetitive injury of the alveolar epithelial cells and abnormal wound repair (Selman et al. 2001; Sisson et al. 2010). Despite this the role of inflammation in IPF initiation and progression has not been fully abandoned, and low-level inflammation may still be a relevant component of wound repair and fibrosis (Wynn and Ramalingam 2012). In IPF, epithelial injury is thought to induce a local inflammatory response with secretion of cytokines and growth factors that contribute to a profibrotic microenvironment (Wilson and Wynn 2009; Homer et al. 2011). It is therefore crucial to keep an open mind and consider that both inflammation and epithelial injury may participate in the development of lung fibrosis. The subsequent accumulation of ECM causes irreversible destruction of the lung parenchyma. This fibrotic ECM can drive progressive fibrogenesis in the lung.

3.3.1 Epithelial Cell Injury

IPF probably has a long asymptomatic period, during which clinically silent alveolar epithelial cell microinjuries occur. As injuries and repair progress, the abnormalities reach a certain threshold at which symptoms appear. Endoplasmic reticulum stress (ER stress) and the subsequent unfolded protein response may also contribute to epithelial cell vulnerability and myofibroblast differentiation (Wynn 2008; Wei et al. 2013; Korfei et al. 2008; Lawson et al. 2011). Aberrant activation of alveolar epithelial cells (AEC) after injury may provoke the migration, proliferation, and activation of mesenchymal cells leading to the formation of fibroblast foci. There are numerous studies showing that abnormally activated bronchiolar and AECs express most of the growth factors and chemokines responsible for the migration, proliferation, and activation of fibroblasts. Transforming growth factor (TGF)-β1, one of the main inducers of the fibrotic response, and the αvβ6 integrin, one of the activators of TGF-β1, are produced by epithelial cells (Horan et al. 2008; King et al. 2011; Selman and Pardo 2012). The loss of epithelial integrity leads to an aberrant reaction of the epithelium, which is mediated by sustained activation of profibrotic signaling pathways. Several studies in mice have shown that the absence of molecules essential for epithelial integrity can provoke a fibrotic response without inflammation. For example, ablation of the tyrosine phosphatase Shp2, a potent inductor of branching morphogenesis and alveolarization during lung development, causes marked reduction in surfactant proteins, disorganized lamellar bodies, increased epithelial apoptosis, and pulmonary fibrosis without inflammation (Zhang et al. 2012). Likewise, attenuation of TGF-β signaling in the lung

epithelium protects from bleomycin-induced fibrosis despite increased inflammation, indicating a critical role for epithelial TGF-β signaling in regulating the fibrotic process (Degryse et al. 2011). Mice expressing the mutant L188Q of the SP-C protein in AECs undergo ER stress and develop exaggerated lung fibrosis after bleomycin without differences in lung inflammation (Lawson et al. 2011). CD151 is a tetraspanin expressed at the basolateral surface of AECs and is important to maintain epithelial integrity. A recent study showed downregulation of CD151 in AECs from IPF lungs (Tsujino et al. 2012). CD151-deficient mice spontaneously developed age-related lung fibrosis, while lung injury worsened AECs integrity and provoked severe fibrosis (Tsujino et al. 2012). Moreover, the lung epithelium in IPF shows decreased expression of phosphatase and tensin homolog (PTEN) (Miyoshi et al. 2013). The inactivation of PTEN causes disassembly of tight junctions with disruption of AECs integrity and destruction of the basement membrane, which results in exacerbated lung fibrosis. Interestingly, miR-21, a microRNA that is significantly increased in IPF, targets PTEN suggesting that epigenetic mechanisms may cause the loss of epithelial integrity (Zhou et al. 2013b; Liu et al. 2010c). Recent evidence demonstrated that the elongation of long-chain fatty acids family member 6 (Elovl6) is downregulated in IPF lungs and that lack of Elovl6 in mice is associated to spontaneous thickening of the alveolar walls and severe fibrosis after injury (Sunaga et al. 2013). Loss of epithelial integrity and lack of stable basement membrane scaffolding may change the spatial orientation required for AECs maintenance, spreading, and migration. Importantly, it may also influence the ability of the epithelium to contain the stress associated with mechanical stretch and may explain, at least partially, the characteristic basal and peripheral initiation of IPF (Selman and Pardo 2014).

3.3.2 Inflammatory Cells

Although the poor efficacy of immunosuppressive drugs in IPF has questioned the importance of inflammation in pulmonary fibrosis, it cannot be ignored that inflammation and immune activation are consistently found in IPF lungs (Todd et al. 2012).

3.3.2.1 Macrophages

Macrophages play an important role in the pathogenesis of pulmonary fibrosis (Boorsma et al. 2013). Elevated macrophage counts have been reported in IPF for many years (Murray and Wynn 2011). These cells are involved in ECM turnover by producing matrix metalloproteinases (MMPs) as well as tissue inhibitors of MMP (TIMPs). They regulate fibroblast proliferation, recruitment, and activation, the composition of ECM through a large variety of profibrotic cytokines and growth factors (Song et al. 2000; Atabai et al. 2009; Boorsma et al. 2013). It is critical to

consider that as macrophages are involved in many phases of tissue repair, they can adopt several phenotypes. M1 or "classically activated macrophages," develop after exposure to proinflammatory cytokines such as IFNγ, TNF-α, and LPS (Krausgruber et al. 2011). In fibrosis, as a result of epithelial cell damage, monocytes are recruited to the site of injury and differentiate into M1 macrophages. Once activated, M1 macrophages produce TNF-α, IL-1β, IL-12, and IL-23, which induce CD4+ T_h1 cell infiltration and activation (Herold et al. 2011). These CD4+ cells are responsible for the production of IFNγ, which may have antifibrotic effects (Herold et al. 2011). Several in vitro and animal studies suggest that M1 macrophages play a role in both the inflammatory as well as the resolution phase of pulmonary fibrosis (Herold et al. 2011; Boorsma et al. 2013).

The M2, or "alternatively activated macrophage" phenotype can be induced by IL-4, IL13, and granulocyte–macrophage colony-stimulating factor (GM-CSF) (Stein et al. 1992; Satoh et al. 2010). These cells are characterized by upregulated expression of mannose receptors and transglutaminase 2 (Stein et al. 1992; Martinez et al. 2009). M2 macrophages typically express molecules such as IL-10 and arginase I, which suppress the induction of CD4+ T_h1 cells and IFNγ. Depletion of M2 macrophages attenuated bleomycin or TGF-β1-induced pulmonary fibrosis in mice (Gibbons et al. 2011). Experimental studies show M2 macrophages are firmly associated with fibrosis development, but newer evidence suggests they may also contribute to resolution of fibrosis (Boorsma et al. 2013).

The categorization of macrophages as M1 or M2 probably explains the conflicting roles attributed to macrophages in fibrosis in the past literature. "M2-like macrophages" may be involved to a greater extent in the progression of fibrosis than "M2 macrophages." It remains to be established whether it is a particular macrophage subset with M2-type properties that preferentially infiltrates fibrotic tissue, or whether a profibrotic microenvironment drives macrophage polarization toward an M2-like phenotype. Targeting specific genes in macrophage subgroups may help to reveal the specific function and overall role of the macrophage in tissue fibrosis. It has become clear that macrophages are functionally important in the pathology of IPF, but many questions remain unanswered.

3.3.2.2 T Lymphocytes

Many studies have provided evidence for the presence of CD4+ and CD8+ T-cells in the lung tissue of IPF patients where CD4+ cells are typically found in higher proportions (Luzina et al. 2008). Increased CD8+ T-cell counts in the IPF lung tissue is associated with decreased lung function (Daniil et al. 2005). Also, in bronchoalveolar lavage fluid (BALF), higher CD4+/CD8+ ratios are correlated with an improved response to anti-inflammatory therapies (Fireman et al. 1998). The contribution of T lymphocytes to fibrotic disease seems to be context dependent. Athymic or T-cell depleted mice have lower degrees of bleomycin fibrosis (Schrier et al. 1983; Piguet et al. 1989; Sharma et al. 1996). In contrast, others reported that T-cell-deficient mice can develop substantial fibrosis (Luzina et al. 2008).

CD4+ regulatory T-Cells (FOXP3+) have the ability to regulate pulmonary fibrosis (Radstake et al. 2009), leading to the supposition that regulatory T-cells may in fact inhibit fibrogenesis (Trujillo et al. 2010). While some studies suggest that these cells can suppress pulmonary fibrosis in the bleomycin model (Trujillo et al. 2010; Kass et al. 2012; Boveda-Ruiz et al. 2013), others propose that they actually promote fibrogenesis in silica models (Liu et al. 2010a; Lo Re et al. 2011). Semaphorin $7a^{+}CD4+CD25^{+}FoxP3^{+}$ regulatory T-cells are associated with disease progression in IPF and induce fibrosis in the TGFβ1-exposed murine lung (Reilkoff et al. 2013). Regulatory T-cells may also promote M2 macrophage differentiation (Tiemessen et al. 2007). Taken together these results suggest a complex interplay between lymphocyte subsets, with regulatory T-cells themselves acting as profibrotic elements, whereas the role of CD4+ T-effector cells seems to depend on the presence or absence of the regulatory T-cells.

3.3.2.3 B Lymphocytes

IPF lungs express B-cell-related genes (Zuo et al. 2002) and have focal aggregates of activated T and B cells in proximity to fibroproliferative lesions (Marchal-Somme et al. 2006; Nuovo et al. 2012; Xue et al. 2013). Antibody production is the major function of B cells, and IPF patients sometimes display autoantibodies against alveolar epithelial cell antigens (Xue et al. 2013). Immune complexes can trigger cytotoxic and proinflammatory cascades (Mayadas et al. 2009) and have been described in the circulation of IPF patients (Dobashi et al. 2000), BAL (Dall'Aglio et al. 1988), and in IPF lung parenchyma (Kahloon et al. 2013; Xue et al. 2013). Circulating B-lymphocyte-stimulating factor (BLyS), a factor necessary for B-cell maturation and survival, is increased and correlates with acute exacerbations and survival in IPF (Xue et al. 2013). CD19 is a cell-surface molecule regulating B-cell function. Animal studies show that CD19 is necessary for B-cell aggregation and can promote bleomycin-induced pulmonary fibrosis (Komura et al. 2008). Another important molecule involved in B-cell regulation is the chemokine CXCL13, which is markedly expressed in both the plasma and lungs of IPF patients. Levels of CXCL13 have been suggested to be closely associated with the prognosis of IPF (Vuga et al. 2014).

3.3.2.4 Other Inflammatory Cells

Although it is widely recognized that monocytes, macrophages, and lymphocytes have important roles in the progression and resolution of fibrosis, other myeloid-lineage cells such as neutrophils, mast cells, eosinophils, and basophils have also been implicated and are potential therapeutic targets (Wynn and Ramalingam 2012). Mast cells are a group of less obvious immune cells involved in fibrosis, but they are increased in fibrotic lungs (Andersson et al. 2011). It is known that mast cells secrete a number of profibrotic mediators (Metcalfe et al. 1997); they directly

interact with fibroblasts and promote their activation (Rubinchik and Levi-Schaffer 1994; Levi-Schaffer and Rubinchik 1995; Wygrecka et al. 2013). However, studies in knockout mouse models are not consistent in elucidating a clear role for mast cells in pulmonary fibrosis (Veerappan et al. 2013; Reber et al. 2014).

Eosinophils are an important source of the profibrotic factors TGF-β1 and IL-13 (Minshall et al. 1997; Reiman et al. 2006). Eosinophils are associated with the development of pulmonary fibrosis (Humbles et al. 2004). Increased numbers of eosinophils have been linked with the activation of myofibroblasts in dermal fibrosis as well as retroperitoneal fibrosis (Levi-Schaffer et al. 1999; Reiman et al. 2006). In older studies, BALF eosinophilia has been identified as a predictive biomarker for progressive lung disease in IPF and pulmonary fibrosis associated with collagen vascular disorder (Peterson et al. 1987).

3.3.3 Fibroblasts and Myofibroblasts

Myofibroblasts, mobile cells with contractile ability and the main producers of collagen and other ECM proteins, are key effector cells in fibrotic disorders and as such a major target for antifibrotic therapies (Sivakumar et al. 2012; Ueha et al. 2012).

3.3.3.1 Characteristics of Myofibroblast

Fibroblastic foci, a hallmark of IPF, are regions of active fibrogenesis, and their presence in the IPF lung tissue is associated with poor prognosis (King et al. 2011; Raghu et al. 2011). Fibroblastic foci harbor alpha-smooth muscle actin (α-SMA)-positive myofibroblasts which can generate extracellular and intercellular mechanical forces via synthesizing ECM and contractile stress fibers composed of bundles of actin microfilaments that are regulated by myosin light-chain (MLC) phosphorylation (Tomasek et al. 2002). Although the healthy peripheral lung tissue does not contain myofibroblasts, they play critical roles in wound healing processes. In normal and controlled wound healing, once the active phase of repair is finished, myofibroblasts will progressively disappear via apoptosis. In contrast, in IPF these cells are relatively apoptosis resistant (Thannickal and Horowitz 2006). They persist and nourish the fibrotic process, contributing to the excessive deposition of ECM.

3.3.3.2 Source of Myofibroblasts

The exact source of myofibroblasts in IPF is unclear, and several cell types have been proposed as putative precursor, including tissue-resident fibroblasts, epithelial cells (via epithelial to mesenchymal transition; EMT), pleural mesothelial cells

(via mesothelial to mesenchymal transition; MMT), bone marrow-derived fibrocytes, and others (Shimbori et al. 2013). Resident fibroblast differentiation can be induced by fibrogenic cytokines (e.g., TGF-β1 and PDGF), mechanical stress, and different ECM proteins such as ED-A fibronectin (Hinz et al. 2007). Interestingly, "fibrotic fibroblasts" are described to have an invasive phenotype regulated by hyaluronan, a glycosaminoglycan component of ECM, and CD44+ (Westergren-Thorsson et al. 2004; Li et al. 2011). These cells can exhibit anchorage-independent growth characteristics similar to cancer cells (Torry et al. 1994). Indeed, progressive fibrosis has been compared to a benign tumor (Vancheri et al. 2010). This aggressive "tumorlike" behavior of fibroblasts is not observed when they are isolated and cultured on strata of different rigidity, suggesting that fibroblasts are influenced by the interaction with altered fibrotic ECM in the microenvironment (Noble et al. 2012).

During the process of EMT, epithelial cells lose their epithelial characteristics and acquire features distinctive for mesenchymal cells (Thiery and Sleeman 2006). EMT is a key process in embryogenesis, where it is a key mechanism in generating migratory mesenchymal cells that move along the primitive streak and populate new areas that develop into mesodermal and endodermal tissue (Moustakas and Heldin 2007). EMT is modulated by bone morphogenetic proteins (BMPs), which antagonize the effects of TGF-β and induce an inverse process called mesenchymal–epithelial transition (MET) (Thiery and Sleeman 2006). In lung fibrosis in mice, it has been estimated that EMT may account for one-third and bone marrow-derived progenitor cells for 20 % of myofibroblasts (Tanjore et al. 2009). However, these numbers widely depend on the experimental setting and methods used to induce fibrosis and quantify cells and should be interpreted with caution. Further, the concept of EMT has been challenged recently, and its exact role in fibrosis and its contribution to the myofibroblast pool remain unclear (Rock et al. 2011).

Fibrocytes are spindle-shaped mesenchymal progenitor cells (Bucala et al. 1994). Many disease models have shown that they respond to tissue-derived signals and migrate to sites of injury where they can differentiate into fibroblast-like cells (Phillips et al. 2004; Moore et al. 2006). Fibrocytes circulate in the peripheral blood and are capable of producing ECM, cross-linking enzymes, cytokines, and growth factors. In the tissue, they differentiate into fibroblasts and myofibroblasts, which contribute to the accumulation of ECM during the fibroproliferative process (Sivakumar et al. 2012; Maharaj et al. 2013). Fibrocytes express a variety of mesenchymal markers, e.g., collagen I, CD45+, and the hematopoietic stem cell marker CD34 (Quan et al. 2006). It is likely that both tissue-resident and bone marrow-derived progenitor cells contribute to injury repair and fibrosis in the lungs (Moore et al. 2006; Beers and Morrisey 2011; Mehrad and Strieter 2012). Small niches of progenitor cells resident to the lungs are involved in repair and reepithelialization after injury (Warburton et al. 2008). These mesenchymal progenitor cells demonstrate phenotypic plasticity which is affected by their niche environment (Maharaj et al. 2013; Shimbori et al. 2013). Recent studies suggest that other bone marrow-derived cells can produce collagen and contribute to

fibrosis (Lama and Phan 2006; Elkabets et al. 2011; Ding et al. 2013; Nakashima et al. 2013). Pericytes may also play a previously unrecognized role in fibrosis. Pericytes are specialized mesenchymal cells that share a common basement membrane with endothelial cells (Hung et al. 2013). Further, mesothelial cells have been suggested as a source of myofibroblasts in response to TGF-β (Karki et al. 2014). Pleural mesothelial cells are able to acquire myofibroblastic properties such as α-SMA expression, collagen production, and increased migration and thereby may contribute to fibrosis initiation and progression. Through a combination of all these mechanisms, the pool of myofibroblasts increases during fibrosis and promotes IPF.

3.3.4 Extracellular Matrix

The lung matrix is a complex and dynamic network composed of a large variety of collagens (mainly type I and type III), fibronectin, proteoglycans, glycoproteins, and others. It has long been regarded as a mere mechanical support for cells (Erler and Weaver 2009), but more recently, many studies have shown an important role of ECM in the transduction of biological signals critical for formation, development, and function of tissues and organs. From a broad perspective, the major components of the ECM are collagen and fibronectin, which are responsible for its structure. It has been suggested that the failure of the resolution of these provisional matrices during the wound healing process may underlie some of the pathological changes in the ECM in IPF. Collagen provides the tissue resistance to deformation, while elastin confers extensibility allowing the ECM to withstand repetitive mechanical strain. Fibronectin and laminin are components that are critical for cell adhesion. The links between ECM and cells are complex (Shimbori et al. 2013). Transduction of signals occurs via interactions with surface receptors and integrins, which are tethered to the intracellular actin cytoskeleton. Through these links, the ECM is a key player in the regulation of cellular mechanosensory pathways (Wipff et al. 2007; Hinz 2012; Huang et al. 2012, Brown et al. 2013, Byron et al. 2013). ECM also sequesters and acts as an endogenous reservoir for growth factors and cytokines (Bonniaud et al. 2004; Wipff et al. 2007; Wells and Discher 2008).

3.3.4.1 ECM Turnover

The most important enzymes in ECM turnover are metalloproteinases (MMP) (Cawston and Young 2010; Lu et al. 2011). Two main families of MMPs are the MMP and disintegrin and MMP with thrombospondin motifs (ADAMTS) families, which are specialized in degrading ECM. Serine proteases, e.g., plasmin and cathepsin G, also degrade ECM components (Cawston and Young 2010). Numerous cell types in the lung produce MMPs, including epithelial cells, fibroblasts, myofibroblasts, and macrophages (Page-McCaw et al. 2007). MMP3 and MMP10 target proteoglycans, fibronectin, and laminin. MMP1 targets collagen III, whereas

MMP8 and MMP13 selectively target collagen I and II. In addition, both MMP2 and MMP9 degrade denatured collagen (gelatin) (Cawston and Young 2010). TIMPs are potent MMP inhibitors (Lu et al. 2011). TIMPs can also inhibit the proteinases activity of the ADAM and ADAMTS families (Murphy and Nagase 2008).

It has long been considered that a decrease in MMP activity could explain a lot of the ECM accumulation in fibrosis. However, several studies have shown that this view is too simple. Indeed, the majority of MMP proteins are overexpressed in IPF. Although this finding seems counterintuitive, it can be explained by the diversity of substrates and other MMP functions. In IPF, MMP1, -2 and -9 co-localize to the epithelium surrounding fibrotic lesions, but TIMP2 levels were also increased in these areas, suggesting that the MMP activity may be inhibited and that the fibrotic tissue is not degraded (Fukuda et al. 1998). MMP3 is elevated in the IPF lung and overexpression of MMP3 leads to pulmonary fibrosis, while mice lacking MMP3 do not develop fibrosis (Yamashita et al. 2011). In vitro studies suggest a role for MMP3 in the activation of β-catenin signaling and the induction of EMT (Chen et al. 2013). MMP7 is overexpressed in IPF and is profibrotic by its action on cytokine release, including TGF-β1 (Pardo et al. 2008). IL-13 can directly induce a number of profibrotic MMPs, including MMP9, MMP12, and MMP7 (Lanone et al. 2002; Wadsworth et al. 2010). MMP3 and pro-MMP7 are elevated in the BALF of patients with IPF and are produced by hyperplastic alveolar and metaplastic bronchiolar epithelial cells (Fujishima et al. 2010). Membrane type-1 MMP (MT1-MMP or MMP14) has also been reported to serve as a key effector of type I collagenolytic activity in pulmonary fibroblasts (Rowe et al. 2011).

ADAMTS proteinases consist of 19 members of closely related MMPs. Being membrane proteins sitting at the cell surface, ADAMs are primarily involved with cytokine processing and growth factor receptor shedding (Murphy and Nagase 2008). ADAM-17, for example, can release TNF-α from the cell surface. ADAMTSs play a role in degradation of proteoglycans. Once activated, proteinases can be inactivated by permanent removal through degradation or temporarily by endogenous inhibitors (TIMPs, α2-macroglobulin, α1-proteinase inhibitor, and α1-chymotrypsin) (Nagase and Woessner 1999).

3.3.4.2 Biochemical Characteristics of ECM

Considering that scars are the main element in the IPF lungs, surprisingly little is known about the biochemical composition of the fibrotic tissue and the dynamics of its turnover. Early work showed that fibrotic ECM has a different amount of collagen subtypes, with collagen type I being present in mature and type III in newly formed scars (Laurent 1986; Laurent et al. 1988). Fibrotic lungs have substantial alterations in amount and distribution of glycoproteins and glycosaminoglycans compared to normal (Venkatesan et al. 2011; Booth et al. 2012). Increase in collagen I, periostin, fibrillins, hyaluronan, and LTBP (latency TGF-β binding protein)-1 are seen, all of which having a role in mesenchyme-ECM

interaction as discussed earlier (Lepparanta et al. 2012). Collagen has both inter- and intra-microfibrillar cross-links and binding sites for ECM molecules such as fibronectin (Nimni 1983; Dzamba et al. 1993) and proteoglycans (San Antonio et al. 1994). The accessibility of collagen degradation sites for proteolytic cleavage is important for the resolution of scar tissue, and in areas of fibrosis, collagen and other ECM components contain modified cross-links and thereby are resistant to degradation. In addition, there is an increased production of elastin and proteoglycans, intermingling with collagen in structurally unique patterns (Nimni 1983; Selman et al. 1986; Starcher et al. 1987; Bensadoun et al. 1996; Ebihara et al. 2000; Rozin et al. 2005; Li et al. 2011). The biomechanical property of scar tissue is influenced by a large number of cross-linking enzymes, including prolyl 4-hydroxylases (P4H), transglutaminases (TG), and lysyl oxidases (LOX/LOXL). P4H is a key enzyme in posttranslational collagen maturation, catalyzing hydroxylation of proline and thereby stabilizing the collagen triple helix. LOX was found to promote bleomycin-induced lung fibrosis through the modulation of inflammation (Cheng et al. 2014), and TG2 is increased in the bleomycin model (Olsen et al. 2011). Studies looking at serum LOXL2 levels and clinical parameters of IPF suggest that higher serum LOXL2 levels are associated with an increased risk of IPF progression (Chien et al. 2014). Previous studies have shown that P4H inhibitors can attenuate fibrosis in the liver (Sakaida et al. 1996, 1999), myocardium (Fielitz et al. 2007), and urinary bladder (Chung et al. 2012) and therefore should be an attractive treatment target for IPF. Mice deficient in TG2 or LOXL2 are resistant to bleomycin-induced lung fibrosis (Barry-Hamilton et al. 2010; Olsen et al. 2011). These studies highlight that the reduction of cross-linking enzymes could render collagen more accessible to normal homeostatic proteolytic degradation. Furthermore, neutralizing antibodies against LOXL2 showed promising preclinical therapeutic effects in the bleomycin model in mice (Barry-Hamilton et al. 2010). Targeting this mechanism of LOXL2 is explored in an ongoing phase II trial (NCT01769196). This evidence suggests that cross-linking enzymes are promising targets in current drug development for pulmonary fibrosis.

3.3.4.3 Mechanical Characteristics of ECM

Stiffening of lungs is one of the primary reasons for shortness of breath. In addition to their impact on respiratory physiology, the biomechanical characteristics of the lung matrix affect cell behavior in a major way. Myofibroblasts are the major producers of the fibrotic ECM which results in the characteristic stiffness of a fibrotic lung. In vitro studies showed that ECM stiffness can transform fibroblasts from a quiescent state to self-sustained activated state and thus become a major driver of fibrosis (Bellaye and Kolb 2015). The stiff fibrotic matrix promotes fibroblast proliferation and reduces fibroblast apoptosis with the suppressing of cyclooxygenase-2, and PGE2 (prostaglandin E2) seems to be a key link between matrix stiffness and fibroblast activation (Liu et al. 2010b). Matrix stiffness is also an important regulator of the proliferation of fibroblasts suggesting that

physiologically normal (soft) matrices attenuate differences in contractility and proliferation between normal and IPF fibroblasts (Marinkovic et al. 2012, 2013). Activation of rho kinase and focal adhesion kinase (FAK) by increased force tension appears to have a major role in this process, and the inhibition of these pathways prevents experimental fibrosis (Zhou et al. 2013a; Lagares et al. 2012). The activation of Rho/ROCK pathway in IPF fibroblasts showed that disruption of this mechanotransduction pathway with the ROCK inhibitor fasudil prevented myofibroblast differentiation and induced apoptosis (Bei et al. 2013; Zhou et al. 2013a). Booth et al. have developed an elegant model of a decellularized human lung, on which primary fibroblasts are seeded (Booth et al. 2012). Furthermore, our most recent study demonstrated that mechanical tissue stretch contributes to the development of pulmonary fibrosis via mechanotransduced activation of TGF-β1 in rodent and human pulmonary fibrosis (Froese et al. 2016). We also showed that this mechanically induced TGF-β1 activation in fibrotic lung tissue acts via rho kinase and αV integrin-mediated pathways (Froese et al. 2016). These studies indicate that lung stiffness and mechanotransduction might be a promising target for IPF therapy.

3.3.4.4 Growth Factor Storage in ECM

The ECM is a reservoir for growth factors, including connective tissue growth factor (CTGF), FGFs, platelet-derived growth factor (PDGF), IL-13, and TGF-β (Hynes 2009; Rozario and DeSimone 2010; Lu et al. 2011). As discussed, TGF-β is an important profibrotic growth factor (Tatler and Jenkins 2012) which is stored and bound to the matrix in its latent form (Wipff et al. 2007). The latency-associated peptide (LAP) is the crucial element coupling active TGF-β molecules within this large complex. LAP releases active TGF-β in response to a variety of stimuli, including integrin binding, proteases, pH changes, and oxidative stress (Biernacka et al. 2011). Even mechanical stress is able to release active TGF-β, particularly when the underlying matrix is stiff (Wipff et al. 2007; Klingberg et al. 2013, Froese et al. 2016). Integrins are important for stretch-induced TGF-β activation, especially αvβ8 (fibroblast specific) and αvβ6 (epithelial specific) (Munger and Sheppard 2011). In cells lacking LTBP-1, αvβ6 cannot activate TGF-β (Munger et al. 1999), showing that the activation of TGF-β by αvβ6 depends on the combination of latent TGF-β and LTBP with αvβ6 (Annes et al. 2004). The integrin αvβ8 can also bind and activate TGF-β, but the mechanism is different. The latent TGF-β is presented to MMP14 by the integrin αvβ8, which results in proteolytic cleavage of the LAP and the release of active TGF-β (Goodwin and Jenkins 2009; Mu et al. 2002). Integrin αv and β1 or 6 isoforms are directly involved in TGF-β1 activation and fibrosis in vivo (Henderson et al. 2013; Madala et al. 2014; Reed et al. 2015). Many other mediators are stored in fibrotic ECM. CTGF is a matricellular protein, which is expressed by activated fibroblasts (Leask and Abraham 2003) mediating the differentiation of mesenchymal stem cells to fibroblasts (Ponticos et al. 2009; Lee et al. 2010). BMPs belong to the TGF-β family and

regulate proliferation and differentiation of mesenchymal cells and epithelial cells (Rider and Mulloy 2010). Other growth factors such as FGF2 and PDGF can also induce the proliferation and activation of fibroblasts (Barrientos et al. 2008). Another important profibrotic mediator is IL-13, which can elicit a number of responses from fibroblasts in vitro, as well as promote fibrosis in vivo. IPF-derived fibroblasts are responsive to IL-13 stimulation, resulting in enhanced collagen production, differentiation, and TGF-β expression (Murray et al. 2008). It would be beyond the scope of this chapter to describe the biological functions of all these cytokines in detail, but many of them are current targets for antifibrotic therapies (Baroke et al. 2013).

3.3.4.5 Messenger Function of ECM

ECM also influences cell behavior as a biochemical mediator. Collagens have two types of receptors: integrins β1 (α1β1, α2β2, α10β1, and α11β1) and discoidin domain receptors (DDRs: DDR1 and DDR2). Integrins are non-tyrosine kinase collagen receptors, while DDRs are tyrosine kinase receptors. They are expressed in numerous cell types, including epithelial cells, fibroblasts, platelets, and smooth muscle cells and likely contribute directly to the development of fibrosis (Leitinger and Hohenester 2007; Yeh et al. 2012b). It has been demonstrated that reduced expression of integrin α3β1 ameliorates the progression of bleomycin-induced fibrosis (Kim et al. 2009). In addition, blocking integrin β1 inhibits TGF-β1-induced EMT and suppresses the progression of renal fibrosis (Yeh et al. 2010). Downstream signaling protein kinases of integrin β1, particularly ILK, mediate renal tubular EMT and interstitial fibrosis (Li et al. 2003, 2009). DDR2 has been identified as a novel receptor tyrosine kinase that utilizes type I collagen as major ligand. Activation of DDR2 by collagen I induces human lung fibroblast transmigration through collagen IV (Ruiz and Jarai 2012). Interestingly, integrin and DDR have opposing cell functions. Increase in integrin β1 expression disrupts E-cadherin-mediated cell–cell adhesion, whereas increase in DDR1 promotes junctional localization and membrane stability of E-cadherin. It has been suggested that these collagen receptors might regulate the fibrotic response (Yeh et al. 2012a).

3.3.5 Developmental Factors

It is well established that the recapitulation of developmental pathways is involved in fibrogenesis, such as BMPs, Wnt, Shh, and Notch pathways (Selman et al. 2008; Konigshoff et al. 2009; Aoyagi-Ikeda et al. 2011). BMPs antagonize the effects of TGF-β regarding EMT and induce the reverse process of MET (Thiery and Sleeman 2006). BMP-2 is decreased in IPF lungs (Selman et al. 2008). Gremlin, a BMP antagonist, is elevated in IPF and in experimental asbestosis (Koli et al. 2006; Myllarniemi et al. 2008b, Myllarniemi et al. 2008a). Transient overexpression of

Gremlin results in alveolar epithelial injury, followed by transient fibrosis with myofibroblast and collagen accumulation (Farkas et al. 2011). Wnt signaling regulates a wide range of developmental processes, and abnormal activation later in life can lead to disease (Veeman et al. 2003; Cadigan and Liu 2006). Several members of the Wnt signaling pathway are upregulated in IPF lungs (Zuo et al. 2002; Selman et al. 2006). Shh is expressed in the developing lung epithelium. In vitro and in vivo studies have shown that Shh increases the proliferation of lung mesenchymal cells and upregulates the expression of smooth muscle actin and myosin (Shannon and Hyatt 2004). In IPF lungs, the Shh pathway is activated with increased Shh expression in epithelial cells and upregulated receptors (Stewart et al. 2003; Coon et al. 2006). The exact role of these activated developmental pathways in fibrosis still needs to be explored, but they represent promising treatment targets.

3.3.6 microRNAs

Epigenetic mechanisms have a major influence on gene expression and activity. They cause reversible modifications in chromatin structure without changing the nucleotide sequence and include DNA methylation, histone modification, and regulation by noncoding microRNA (miRs). miRs are short RNA sequences of around 22 nucleotides which act as gene regulators by inhibition of posttranscriptional expression of target mRNAs. Several studies have revealed that a number of miRs are regulated differently in IPF lungs, effecting both fibroblasts and epithelial cells (Liu et al. 2010c; Pandit et al. 2011; Yang et al. 2012; Dakhlallah et al. 2013; Lino Cardenas et al. 2013; Yang et al. 2013). Let-7d, miR-200, and miR-326 are some examples, which target the TGF-β signaling and other fibrosis-related pathways and are decreased in IPF patients. In contrast, miR-21 promotes EMT that is increased in AECs from IPF patients (Pandit et al. 2011; Yang et al. 2012; Yamada et al. 2013). Increased expression of miR-21 in IPF fibroblasts enhances TGF-β1 activation and fibrosis by inhibiting the inhibitory Smad7 (Liu et al. 2010c). An interesting connection of miRs deregulation and the pathogenesis of IPF is related to the reactivation of developmental programs, such as Wnt/β-catenin and Shh signaling pathways which may contribute to an abnormal function of epithelial cells and fibroblasts (Selman et al. 2008). For example, upregulation of Wnt/β-catenin signaling may be associated with the decrease of miR-375 and miR-487b that target the Wnt receptor Frizzled and with the increase of miR-154 that targets Wnt inhibitors (Milosevic et al. 2012; Wang et al. 2013; Xi et al. 2013). While yet incompletely understood, differences in epigenetic modification play a major role in lung fibrosis and in many other chronic lung disorders and represent novel therapeutic targets.

3.4 Conclusion

The pathogenesis of IPF is very complex and despite considerable knowledge has been generated over the last decade; much more scientific work is required to better understand this disease. One of the major recent advances has been the revelation of some of the genetic predisposition for IPF, originating from GWAS data, which may become useful not only in exploring disease pathogenesis but also in helping early detection of IPF. It is crucial to understand the differences between initiation of disease in susceptible individuals and the phase of progressive fibrogenesis, which is the state in which most patients with IPF are seen when they first get diagnosed. The current paradigm is that not chronic inflammation, but repetitive alveolar microinjuries and loss of epithelial integrity play the most important role in fibrogenesis, together with uncontrolled myofibroblasts proliferation and accumulation of an abnormal fibrotic matrix. Once established, the fibrotic ECM could play a more active role in further driving the progression of fibrosis through complex interactions. Understanding how and when all these different elements contribute to fibrogenesis will eventually help to identify novel targets and offer new opportunities for therapies for IPF.

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Chapter 4
Pulmonary Alveolar Proteinosis: A Historic Perspective

Koh Nakata and Ryushi Tazawa

Abstract Since the first report, the pathogenesis of pulmonary alveolar proteinosis (PAP) had been mysterious. In 1999, we discovered granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibody in the blood and lung of idiopathic PAP, which consisted 90 % of acquired PAP and was later named as autoimmune PAP. Ten years later, Trapnell and his colleagues proved the hypothesis that the loss of GM-CSF bioactivity in the lung might lead PAP by developing a PAP model in nonhuman primates caused by injecting with a patient-derived GM-CSF autoantibody. The new technology for seroligical diagnosis revealed that most adult onset PAP is associated with GM-CSF autoantibody, and thus, the conventional name of "idiopathic PAP" was changed to "autoimmune PAP" of which a large cohort study was conducted in Japan by Inoue et al. reporting that PAP is distributed equally among subarctic to subtropical regions, with a 2:1 ratio of males to females and the mean age of 51 years, while efforts to develop novel treatment approaches for PAP have been continued at the same time based on the pathogenesis related to the deficiency of pulmonary GM-CSF bioactivity, aerosolized GM-CSF inhalation therapy to autoimmune PAP achieved a satisfactory success with an overall efficacy of more than 60 %. Rituximab therapy targeting reduction of GM-CSF autoantibody has been ongoing. Secondary PAP is a very rare lung disorder consisting approximately 8–9 % of acquired PAP. Hematological disorders are the most common underlying disease, of which 74 % cases demonstrated myelodysplastic syndrome in Japan and the prognosis was poor with a 2-year survival of <50 %. On the other hand, most pediatric cases of pathologically diagnosed PAP have been proven to have defects in a variety of genes involved in surfactant metabolism, such as surfactant protein B, surfactant protein C, ATP-binding cassette family of transporters, and thyroid transcription factor 1. Not only pediatric but also adult-onset PAP is caused by functional defects in the genes encoding the GM-CSF receptor (CSF2RA and CSF2RB). Thus, in the recent two-decade history of research on PAP, an outstanding progress has been achieved in a "bench-to-bedside" manner, which improved our understandings on

K. Nakata (✉) • R. Tazawa
Bioscience Medical Research Center, Niigata University Medical and Dental Hospital, 1-754 Asahimachi-dori, Niigata 951-8520, Japan
e-mail: radical@med.niigata-u.ac.jp

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the pathogenesis, enabled us to characterize clinical features, and increased the choice of the treatment, but efforts are still necessary to solve the mechanism for GM-CSF autoantibody production.

Keywords Pulmonary alveolar proteinosis • Granulocyte-macrophage colony-stimulating factor • Autoantibody

4.1 Introduction

Pulmonary alveolar proteinosis (PAP) is a rare disorder characterized by progressive accumulation of surfactant within the pulmonary alveoli resulting in respiratory insufficiency and, in severe cases, respiratory failure (Seymour and Presnell 2002; Trapnell et al. 2003). Since the first report of PAP, the pathogenesis of the disorder had remained a mystery for more than 35 years. However, light was cast on this mystery when Dranoff (Dranoff et al. 1994) and Stanley (Stanley et al. 1994) independently and serendipitously found pulmonary lesions similar to PAP in mice that they genetically engineered to encode and express granulocyte-macrophage colony-stimulating factor (GM-CSF). In a subsequent report by a research group in Cincinnati, it was revealed that defective surfactant catabolism in these mice is due to maturation arrest of alveolar macrophages (AMs) caused by lack of GM-CSF signaling (Ikegami et al. 1996). In 1999, Kitamura and Nakata discovered GM-CSF autoantibodies in the blood and lung of patients with idiopathic pulmonary alveolar proteinosis (Kitamura et al. 1999), which comprised 90 % of acquired PAP. This phenotypic observation was later termed autoimmune PAP. In a recent study, Trapnell and colleagues proved the hypothesis that loss of GM-CSF bioactivity in the lung might lead to PAP by developing a PAP model in nonhuman primates. They created the model by injecting nonhuman primates with patient-derived GM-CSF autoantibody (Sakagami et al. 2009, 2010) and subsequently developed a serological diagnosis method, which led to the conventional name of idiopathic PAP changing to autoimmune PAP. The autoimmune PAP serological diagnostic method was then used by Inoue et al. in a large cohort study conducted in Japan, which found that PAP is distributed equally across subarctic to subtropical regions, with a 2:1 ratio of males to females and with a mean age of 51 years (Inoue et al. 2008). Thus, over the last 15-years PAP research has made an outstanding progress in a “bench-to-bedside” manner, improved our understandings of PAP pathogenesis, enabled us to characterize PAP clinical features, and created a wealth of choices for PAP treatment. However, ongoing research efforts are still necessary to solve the mechanisms behind GM-CSF autoantibody production.

4.2 Lessons from PAP Mouse Models

The pathogenesis of PAP had been quite enigmatic until gene-targeted mice deficient in GM-CSF developed a phenotype of lung pathology similar to PAP in humans. Although the lungs of GM-CSF knockout (KO) mice at birth were indistinguishable from those of controls, differences appeared by 3 weeks: the lungs of GM-CSF KO mice filled with granular, eosinophilic, PAS-positive material and large, foamy macrophages in alveolar spaces. No differences were found in the levels of surfactant messenger RNA between GM-CSF KO mice and wild-type mice (Dranoff et al. 1994). Subsequent studies have revealed that neither production of surfactant by type II cells nor its uptake by alveolar macrophages was changed (Ikegami et al. 1996). Alveolar macrophages were described as abnormal in appearance, containing increased amounts of surfactant protein and lipids and showing impaired degradation of these elements (Ikegami et al. 1996; Yoshida et al. 2001). These abnormalities were attributed to disrupted catabolism of surfactant by alveolar macrophages (Yoshida et al. 2001).

In later reports, correction of GM-CSF expression was reported to reverse surfactant catabolism in alveolar macrophages either by chimeric gene expression in respiratory epithelial cells (Huffman et al. 1996), by local adenovirus-mediated gene expression (Zsengeller et al. 1998), or by GM-CSF aerosol inhalation (Reed et al. 1999).

In addition to surfactant catabolism, other miscellaneous alveolar macrophage functions found to be regulated by GM-CSF included cell adhesion, expression of Fc receptors and mannose receptors, phagocytosis, bacterial killing, and Toll-like receptor signaling (Shibata et al. 2001). GM-CSF elicits these multiple functions via activation of PU.1 in alveolar macrophages. In experiments where the PU.1 gene was transfected into cultured alveolar macrophages from GM-CSF KO mice, the mice recovered from macrophage abnormalities (Berclaz et al. 2002a, b; Shibata et al. 2001; Trapnell et al. 2003). In a parallel line of evidence, forced expression of PU.1 corrected the defective bactericidal activity of alveolar macrophages in vitro (Shibata et al. 2001).

Gene-targeted mice deficient in the GM-CSF receptor (GM-CSFR) common beta chain have also been reported to develop PAP (Robb et al. 1995) correctable by bone marrow transplantation (BMT) from wild-type mice, confirming that loss of GM-CSF signaling is the critical cause of PAP (Nishinakamura et al. 1996). BMT also restored bronchoalveolar lavage (BAL) fluid cellularity and the concentration of total protein, as well as improving the abnormal cellular morphology seen in PAP, including foamy macrophages. However, significant cellular infiltrates persisted in the lung tissue following BMT treatment, suggesting that BMT does not ameliorate the interstitial inflammatory component of PAP (Cooke et al. 1997).

4.3 History of Research on the Pathogenesis of Autoimmune PAP

Although the accumulated material in the alveolar spaces of PAP was quickly identified as pulmonary surfactant and a therapeutic trial targeting removal of the excess material by saline lung lavage was performed in the 1960s, it had remained an enigma as to whether the mechanism of surfactant accumulation in PAP involved an abnormality in the production or in the clearance of surfactant. Several reports showed that alveolar macrophages from PAP patients had, in addition to readily apparent short survival, excessive lipid accumulation, giant lysosomes, and impairment in adhesion, chemotaxis, and microbial killing. These abnormalities were reproduced in normal monocyte-derived macrophages cultured with proteinaceous material from a PAP patient, suggesting that the lung macrophages were defective as a consequence of an abnormal pulmonary environment (Golde et al. 1976). Some groups reported that a substance in the serum or BAL fluid (BALF) from a PAP patient inhibited the growth of mononuclear cells in response to mitogens, which was unlikely to be an immunoglobulin (Stratton et al. 1981). Later, a 40-kD protein was reported to be the macrophage-inhibiting factor previously identified in the BALF (Muller-Quernheim et al. 1987). Thus, several efforts were directed toward identifying various factors that inhibit alveolar macrophage function in the serum or BALF in PAP. However, the discovery of a GM-CSF murine PAP model and its receptor in mice redirected the researchers' attention from such soluble factors to defects of related genes in alveolar macrophages (Dirksen et al. 1997). However, tellingly, no patient has been identified to date as possessing any kind of GM-CSF deficiency (Bewig et al. 2000), and moreover, patients with idiopathic PAP have been found to express GM-CSF normally.

In 1998, Tchou-Wong et al. (1997) at New York University reported that human BAL cells from a patient with PAP expressed mRNA encoding GM-CSF following stimulation with lipopolysaccharides (LPS). However, GM-CSF protein release from BAL cells was undetectable, regardless of LPS treatment. This is in contrast to BAL cells from normal human controls, which release GM-CSF in abundance after LPS stimulation. Yet, in regard to the BAL cells from the patient with PAP, IL-10 production was upregulated, thus demonstrating that exaggerated IL-10 production in patients with PAP might suppress GM-CSF production from BAL cells.

We reproduced these experiments at the Tokyo University, Institute of Medical Science, but this time we analyzed the BAL fluid itself from patients with PAP instead of the BAL cells and found that some factor in the fluid suppressed GM-CSF-dependent growth of both monocytes and TF-1 cells, but not IL-3-dependent growth (Tanaka et al. 1999), indicating that the factor specifically blocked GM-CSF bioactivity in vitro (Fig. 4.1a, b). From this result, we supposed two possibilities: (1) the factor might interfere with GM-CSFR function or (2) it might neutralize GM-CSF per se. Fortunately, we could easily confirm that the factor directly bound GM-CSF (Fig. 4.2) but not the GM-CSFR. Therefore, we focused on the

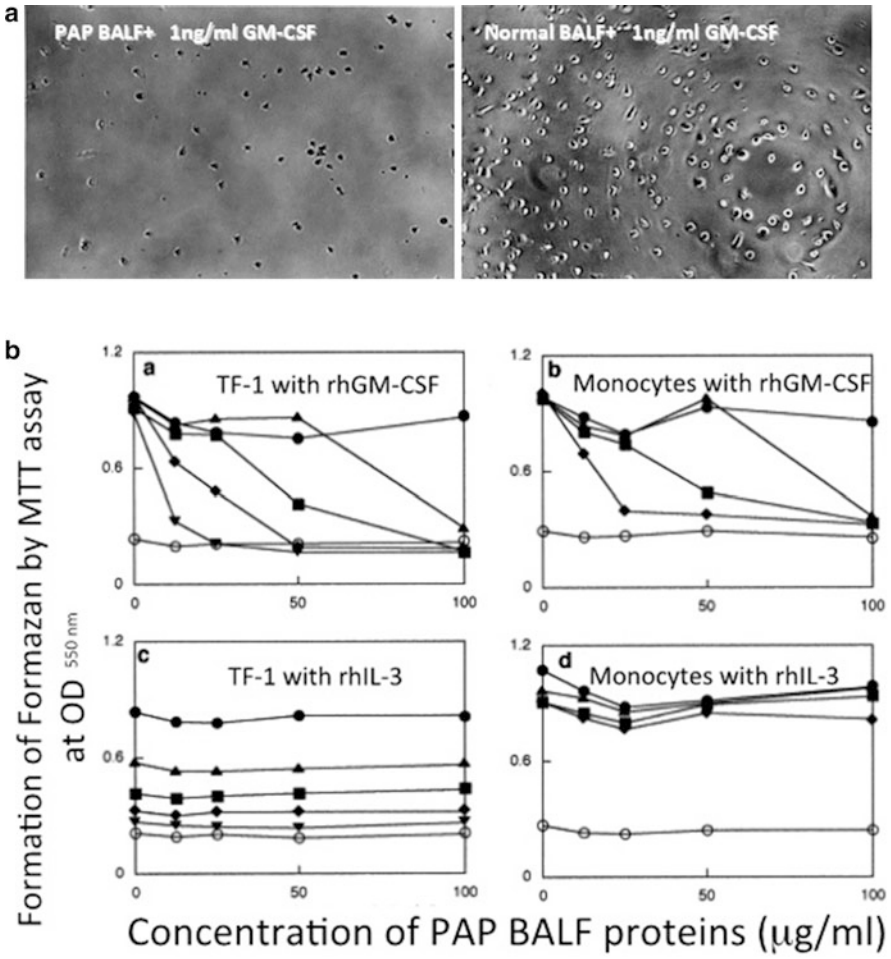

Fig. 4.1 (**a**) Freshly isolated monocytes were incubated with bronchoalveolar lavage fluid (BALF) protein extracts from patients with PAP (*left*) or normal subjects (*right*) and 1 ng/ml of GM-CSF. The cells died within 3 days in PAP-BALF, whereas the cells differentiated to macrophages within 3 days in normal BALF. (**b**) The growth inhibition of TF-1 cells or monocytes by the BALF from a PAP patient. The horizontal axis is the concentration of BALF proteins. (**a** TF-1 incubated with GM-CSF; **b** monocytes incubated with GM-CSF; **c** TF-1 incubated with IL-3; **d** monocytes incubated with IL-3). The *vertical axis* is the activity of viable cells examined by MTT assay. Cells were incubated with various concentrations of GM-CSF or IL-3 (10 ng/ml, **b**; 5, R; 2.5, F; 1.25, 8; 0.625, S; 0, **a**). In both TF-1 and monocytes, MTT uptake significantly decreased as the protein concentration of BALF increased (**a** and **b**). In contrast, BALF did not affect the bioactivity of IL-3 at all in either TF-1 cells or monocytes (**c** and **d**)

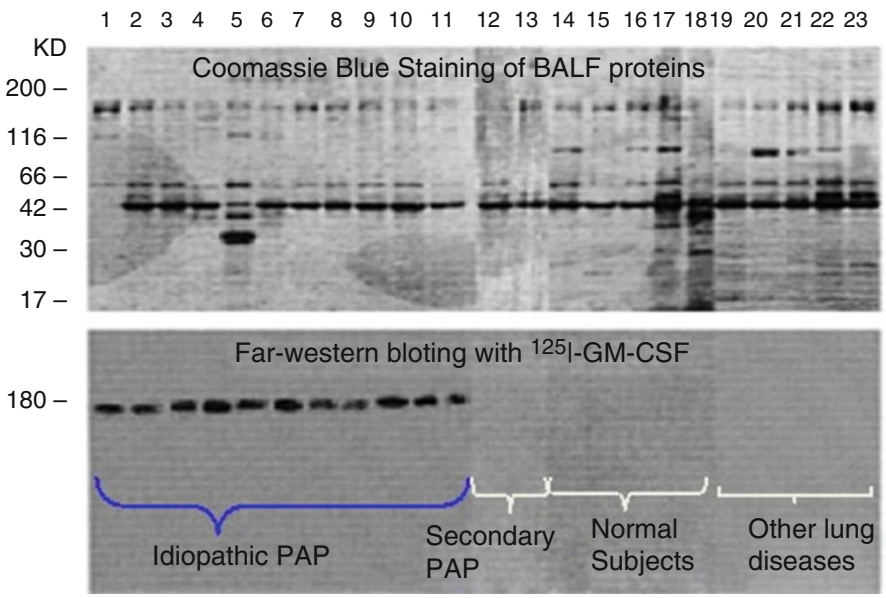

Fig. 4.2 Occurrence of GM-CSF binding factor in BALF from idiopathic PAP (iPAP) patients. Proteins in BALF from iPAP patients (lanes 1–11), secondary PAP (sPAP) patients (lanes 12–13), normal subjects (lanes 14–18), and patients with other lung diseases (namely: sarcoidosis, lane 19; collagen vascular lung disease, lane 20; interstitial pneumonitis, lane 21; hypersensitive pneumonitis, lane 22; and eosinophilic pneumonia, lane 23) were subjected to SDS-PAGE under nonreducing conditions, stained with Coomassie blue (*top panel*), and assayed with ^{125}I-GM-CSF (*bottom panel*). Molecular weight markers are shown on the *left* (kD). Radioactive 180-kD bands are seen in all iPAP samples but not in samples from sPAP patients, normal subjects, or patients with other lung diseases. No such band was detected in BALF from an additional 48 normal subjects or nine patients with other lung diseases

purification and identification of the GM-CSF binding substance in the BALF. However, this procedure ran into difficulties because of the abundance of insoluble phospholipids in the milky BALF solution obtained from patients with PAP. Following a 2-year struggle with the procedure, we discovered that the insoluble materials could be successfully removed from BALF by *n*-butanol extraction and the resulting aqueous layer preserved the GM-CSF binding activity. After concentrating the factor, five-step column chromatography was used to purify the GM-CSF binding factor, resolving it as a single band on SDS-PAGE (Fig. 4.3a, b). Based on molecular weight, N-terminal amino acid sequence, and Protein G binding activity, we identified the GM-CSF binding factor in the BAL fluid as an antibody to GM-CSF of an immunoglobulin G (IgG) isotype (Kitamura et al. 1999). Soon after, we found that the antibody abundantly occurred in the sera of patients with idiopathic PAP, but not with secondary PAP, other lung diseases, or healthy subjects (Kitamura et al. 1999, 2000). Although we did not realize it at that time, 1 year prior to this discovery, Svenson et al., a Danish group, serendipitously reported that GM-CSF autoantibodies are ubiquitously and consistently present in

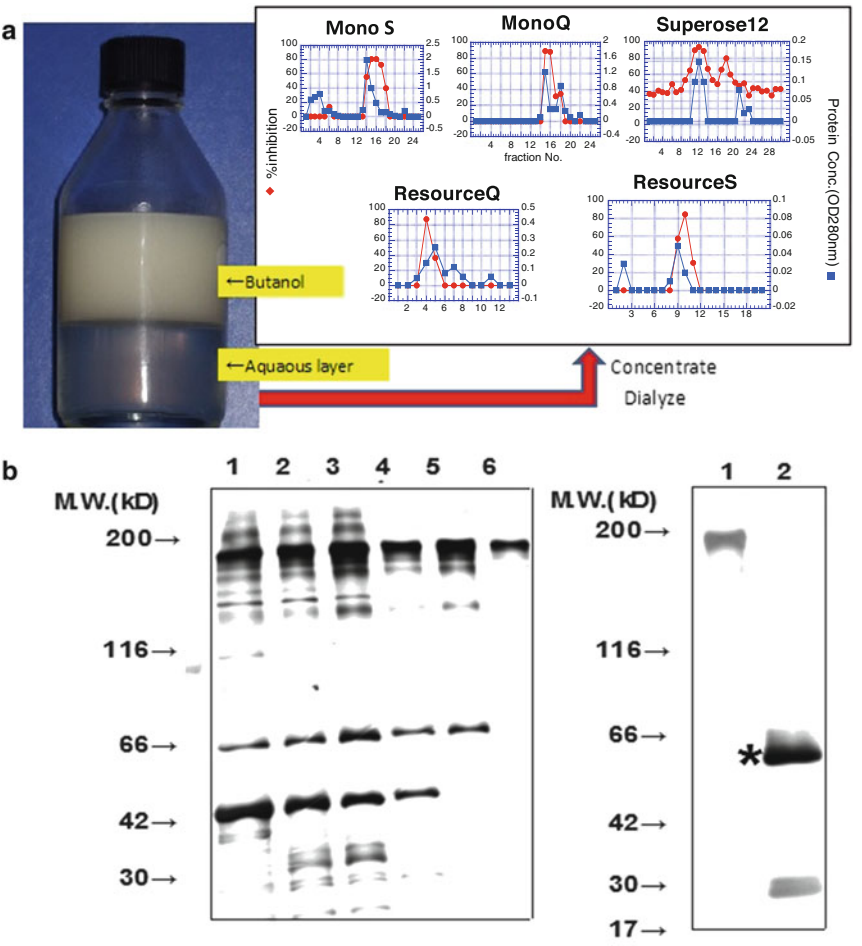

Fig. 4.3 (**a**) When BALF from a patient with idiopathic PAP was mixed with an equal volume of *n*-butanol and shaken in a bottle, phospholipids migrated to the butanol layer, while GM-CSF binding activity remained in the transparent aqueous layer. The delipidated solution was dialyzed against water, concentrated, and applied to Mono S, Mono Q, Superose 12, Resource Q, and Resource S. (**b**) SDS-PAGE protein profiles at each purification step (silver stain). *Left column*: Delipidated BALF (lane 1) was purified using HiTrap SP (lane 2), HiTrap Q (lane 3), Superose 12 (lane 4), Resource Q (lane 5), and Resource S (lane 6) columns. *Right column*: SDS-PAGE of the purified protein under nonreducing (lane 1) and reducing (lane 2) conditions was silver stained, resolving 57-kD and 28-kD proteins. The NH2 terminal sequence of the 57-kD protein coincided with that of human immunoglobulin heavy chain

pharmaceutical human immunoglobulin preparations (Svenson et al. 1998) at levels much lower than those in the sera of idiopathic PAP, suggesting that they may play a physiological role in healthy individuals (Uchida et al. 2009).

4.4 Characteristics and Role of the GM-CSF Autoantibody

We continued our research on the GM-CSF autoantibody at the International Medical Center in Tokyo, where we found that the autoantibody from PAP patients was polyclonal, bound to GM-CSF with high specificity and high avidity, and capable of neutralizing GM-CSF at levels up to many thousands of times higher than the normal physiological concentrations of GM-CSF in vivo (Uchida et al. 2004; Fig. 4.4). The GM-CSF autoantibody impairs not only alveolar

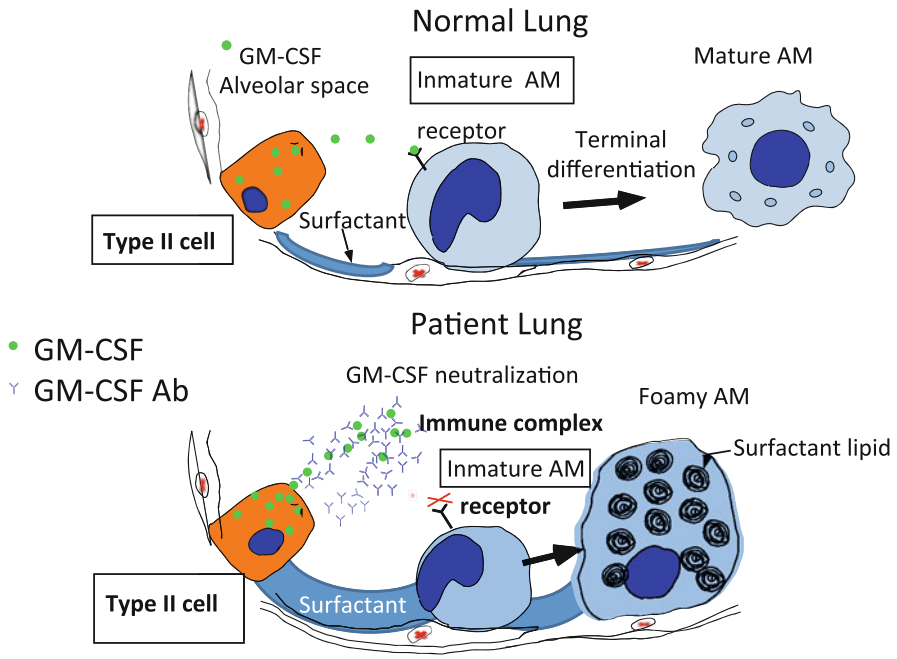

Fig. 4.4 The mechanism behind defects in terminal differentiation of alveolar macrophages in the lung of autoimmune PAP. In the normal lung, alveolar type II cells constitutively produce GM-CSF that binds to GM-CSFR on immature alveolar macrophages (AMs) to promote their terminal differentiation. Mature AMs catabolize surfactant to maintain their homeostasis (*upper panel*). On the other hand, autoimmune PAP is specifically associated with high levels of GM-CSF autoantibodies. GM-CSF autoantibodies neutralize the biological activity of GM-CSF and interfere with GM-CSFR binding, causing defects in the terminal differentiation of AM, and thus impair AM-mediated pulmonary surfactant clearance (*lower panel*)

macrophage function but also neutrophil functions in humans and mice (Uchida et al. 2007). Human monoclonal GM-CSF autoantibodies generated from PAP patients use multiple V genes and target several nonoverlapping epitopes on GM-CSF with a number of somatic mutations, suggesting that GM-CSF autoantibodies prevent signaling by blocking the interaction of GM-CSF with the GM-CSFR (Wang et al. 2013).

Direct evidence that GM-CSF autoantibodies cause PAP, however, was not obtained until highly purified anti-GM-CSF autoantibodies from patients with PAP were transferred to healthy primates, which then reproduced the PAP phenotype, milky BAL fluid containing increased surfactant lipids and proteins, and accumulation of foamy macrophages (Sakagami et al. 2009, 2010). High levels of GM-CSF autoantibodies are specifically associated with idiopathic PAP (Kitamura et al. 2000) and can be isolated in high purity from these patients (Kitamura et al. 1999). Importantly, GM-CSF autoantibodies cause pathological changes in the lung of patients with PAP, but not in other tissues. In this regard, our study demonstrated that this is due to the critical role that GM-CSF plays in the terminal differentiation of alveolar macrophages via PU.1 expression, but not in that of the macrophages of other tissues (Nakata et al. 2006; Fig. 4.5).

Serum GM-CSF autoantibodies are useful for the diagnosis of autoimmune PAP, but the concentrations do not correlate with disease severity (Inoue et al. 2008), which is inconsistent with the idea that there is a critical threshold for disease onset

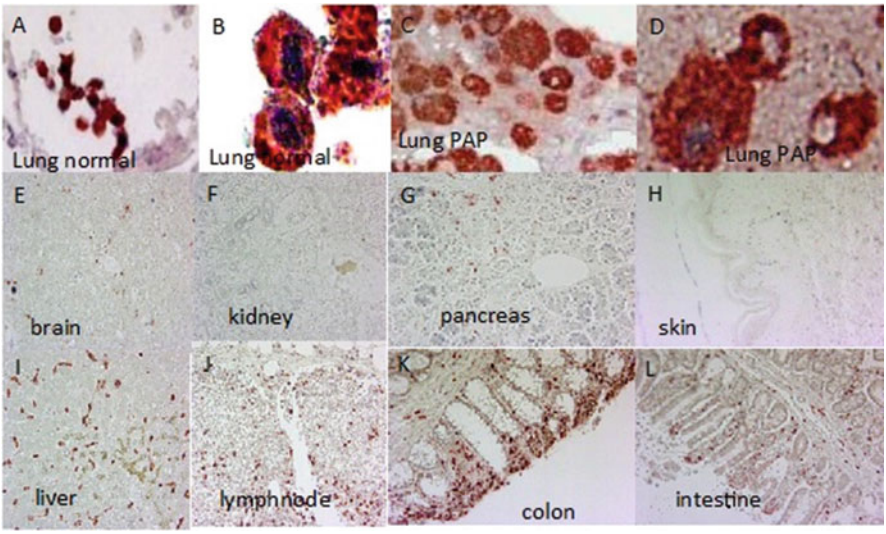

Fig. 4.5 PU.1 expression (*black or grey*) in various tissue macrophages (*red color*). Nuclei of AM were positive for PU.1 in the normal lung (40×, **a**; 400×, **b**), whereas staining was negative or weak in PAP lungs. PU.1 expression was also negative for macrophages in the brain (**e**), kidney (**f**), pancreas (**g**), skin (**h**), liver (**i**), lymph node (**j**), colon (**k**), and intestine (**l**) tissues

(Uchida et al. 2009). In this regard, Meager et al. reported that GM-CSF autoantibodies were detected in 4 out of 1258 healthy subjects (Meager et al. 1999). Bendtzen et al. further described that non-neutralizing autoantibodies and neutralizing autoantibodies against GM-CSF were detected in 38 out of 425 patients with autoimmune diseases, absent PAP, and that neutralizing antibodies were detected in 3 out of 425 patients (Bendtzen et al. 2007). Because the GM-CSF autoantibodies were polyclonal and thus capable of recognizing multiple target epitopes on GM-CSF molecules with variable binding avidity, the loss of GM-CSF bioactivity in the lungs of patients with autoimmune PAP was thought to be affected not only by the concentration but also by multiple properties of the antibody such as binding avidity, neutralizing capacity, and epitope targeting. Thus, little attention has been paid to the relationship between the antibody properties and the degree of hypoxemia. In this regard, we investigated the correlations between binding avidity, neutralizing activity, and light chain k/λ ratio with disease severity. We found that the k/λ ratio of the GM-CSF antibody was significantly correlated with the degree of hypoxemia. While the proportion of λ-type GM-CSF antibody per total λ-type IgG was significantly higher in severely affected patients than those in mildly affected patients, the proportion of k-type was unchanged (Nei et al. 2013).

4.5 Secondary PAP

Secondary pulmonary alveolar proteinosis (sPAP) is a very rare lung disorder comprising approximately 10 % of acquired PAP (Ishii et al. 2011, 2014). Secondary PAP has been reported in association with certain clinical disorders (hematological and immunological disorders, infections, lysinuric protein intolerance) and with several toxic inhalation syndromes. The pathogenesis of sPAP is not fully understood, and its disease association may be difficult to determine.

Hematological disorders are the most common underlying diseases of sPAP, of which 74 % of cases demonstrate a myelodysplastic syndrome (MDS) (Ishii et al. 2011). In contrast to previous reports on the prognosis of MDS, we found that the cumulative survival probability for mild MDS patients with sPAP was similar to that of severe MDS patients (Ishii et al. 2014). It has been speculated that alveolar macrophages derived from abnormal bone marrow precursor cells are defective in both surfactant homeostasis and host defense and thus causative of PAP progression and PAP-associated infections that may occur in cases with mild MDS. Previous studies have reported that the 5-year survival probability of patients with mild MDS is 74 % in the absence of complicating sPAP (Malcovati et al. 2005), whereas our sPAP cases with mild MDS had substantially poorer prognosis (46.2 %). Cordonnier et al. reported that PAP occurred in 5.3 % of patients with hematologic malignancies. Ten patients were neutropenic (Cordonnier et al. 1994), five had acute myeloid leukemia, four had chronic myelogenous leukemia, and one had acute lymphocytic leukemia. Three of these

patients had successfully undergone bone marrow transplantation, which led to PAP recovery.

Immunodeficiency associated with thymic alymphoplasia (Haworth et al. 1967), immunoglobulin A deficiency (Webster et al. 1980), heart-lung transplantation (Yousem et al. 1985), and acquired immunodeficiency syndrome (Israel and Magnussen 1989) have been reported as the underlying diseases of sPAP.

4.6 Hereditary PAP

In a recent report by Ishii et al. among 619 cases pathologically diagnosed as PAP, seven were unclassified cases in which neither GM-CSF autoantibodies nor the underlying diseases were confirmed (Ishii et al. 2014; Fig. 4.6). Of those, the structure of the GM-CSFR was defective in three cases. The GM-CSFR is composed of a binding α-chain and a common β-chain, which is also utilized by interleukin-3 (IL-3) and IL-5. Binding of GM-CSF initiates the Jak2, STAT5, and PI3K/Akt pathways.

Mutations of GM-CSFRα were initially described only in children. Patients with this disease presented with progressive dyspnea of insidious onset at 1.5–9 years of age. Interestingly, two patients who were sisters with identical impaired GM-CSFRα alleles demonstrated different clinical courses: one sister was symptomatic at 6 years of age and the other sister was completely asymptomatic until

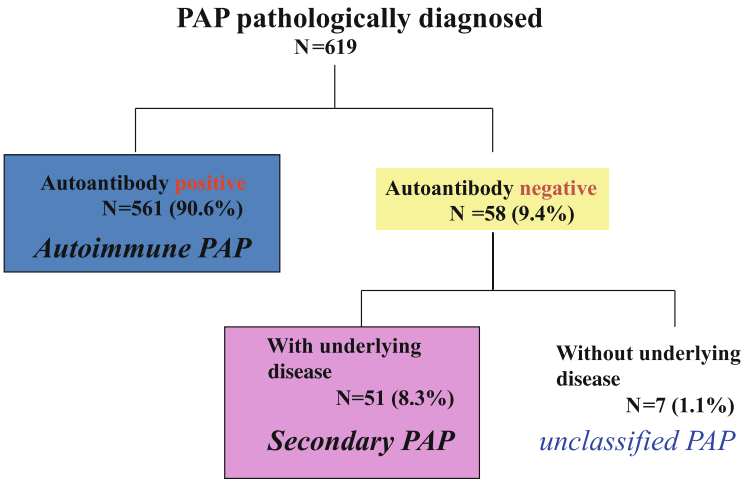

Fig. 4.6 From September 1999 to May 2013, we examined the sera of 619 Japanese cases that had been diagnosed with PAP for the presence of GM-CSF autoantibodies. Of those cases, 561 were positive for the antibody and 58 were negative. In the cases negative for GM-CSF antibodies, 51 demonstrated obvious underlying diseases such as hematological disorders, autoimmune diseases, and infectious diseases. Seven presented with neither GM-CSF autoantibodies nor underlying diseases and were thus termed unclassified PAP

8 years of age. The 6-year-old patient with defective GM-CSF signaling showed increased numbers of alveolar foamy macrophages in BALF and characteristic diffuse bilateral ground-glass opacifications on high-resolution computed tomography (HRCT) (Suzuki et al. 2008, 2010). In addition, this patient had a point mutation causing a single, nonconservative amino acid change (glycine to arginine) at position 174 of the mature protein in one allele and a 1.6-mb chromosomal deletion at Xp33.22 in the other allele, which resulted in reduced GM-CSF signaling in myeloid cells. Cloning and expression of the abnormal complementary DNA in cultured cells reproduced the defect. Another 4-year patient demonstrating one grossly intact X chromosome with a 0.41-mb chromosomal deletion at Xp33.22 and one short X chromosome truncated in the P arm, which is a characteristic of Turner syndrome, underwent bone marrow transplantation. However, this patient died of infection after transplantation (Martinez-Moczygemba et al. 2008). In contrast, we reported that one case of adult-onset genetic PAP was caused by defective GM-CSFRα expression, suggesting that impaired GM-CSF signaling via GM-CSFRα could be compensated by other signaling pathways and thus lead to adult-onset or asymptomatic PAP (Tazawa et al. 2014). Mutation of GM-CSFRβ was also suggested as the cause of neonatal PAP in three patients (Dirksen et al. 1997). More recently, two patients with PAP caused by partially functioning or complete absence of GM-CSFRβ presented with initial onset of symptoms at 9 (Suzuki et al. 2011) and 36 years of age (Tanaka et al. 2011), respectively.

Gene abnormalities in SFTPB, SFTPC, or ABCA3 are known to disrupt surfactant production and function, causing PAP (Garmany et al. 2008; Somaschini et al. 2007; Nogee 2004, 2006). SP-B and SP-C are surfactant apoproteins, which function to reduce surface tension of alveoli, whereas ABCA3 transports phospholipids, including phosphatidylcholine, cholesterol, sphingomyelin, and phosphatidylglycerol, into the lamellar bodies of alveolar type II cells where surfactant complexes are assembled, processed, and stored.

4.7 Molecular Pathogenesis of PAP

PAP is a rare lung disease that usually develops and progresses slowly. Cumulative studies have clarified that most PAP is caused by a series of events that arise from defects in GM-CSF signaling. First, GM-CSF signaling defects cause maturation arrest in alveolar macrophages. The macrophages become foamy within months and degrade to amorphous, granular materials in the alveoli in a matter of months to 1 year. Between 6 months and 1 year, the accumulated amorphous materials in the alveoli become recognizable in HRCT by their characteristic ground-glass opacity. They proceed to disrupt ventilation-perfusion valance by increasing physiological shunt causing reduced oxygen uptake (year to years).

Since macrophages internalize pulmonary surfactant by endocytosis and subsequently catabolize them in phagolysosomes, interruption of GM-CSF signaling likely blocks the translocation of endocytosed surfactant to lysosomes (Shibata

et al. 2001). Alternatively, maturation arrest of alveolar macrophages caused by lack of GM-CSF signaling may result in a key enzyme deficiency. This concept is supported by data regarding the peroxisome proliferator-activated receptor-γ (PPARγ), a ligand-activated transcription factor that regulates genes involved in lipid metabolism and other pathways (Ricote et al. 1999; Bonfield et al. 2003). PPARγ in alveolar macrophages is decreased in patients with PAP, along with a lipid scavenger receptor, CD36, that is regulated by PPARγ. Recently, Baker et al. showed that macrophage-specific PPARγ (MacPPARγ) knockout mice develop foamy alveolar macrophages with significant increases in cholesterol and phospholipid contents. MacPPARγ KO alveolar macrophages showed decreased expression of ABCG1 and a deficiency in ABCG1-mediated cholesterol efflux to HDL, suggesting that PPARγ mediates a critical role in surfactant homeostasis through the regulation of ABCG1 (Baker et al. 2010).

Alternatively, the pathogenesis of some PAP cases appears to involve a decrease in number or impairment of function of alveolar macrophages. Such abnormalities of the alveolar macrophage population may be associated with decreased clearance of surfactant in the lung, resulting in its accumulation. PAP secondary to various hematological disorders is likely to be involved in this mechanism. Inhalation of toxic fumes and dusts would reduce the ability of alveolar macrophages to clear surfactant. These disorders have a lung histopathology and clinical course that is markedly different from PAP caused by interruption of GM-CSF signaling. Further, the pathophysiology in these cases arises from surfactant deficiency rather than surfactant excess as in typical PAP.

4.8 Conclusion

PAP research has undergone a large, historical revolution during the last two decades. The first epoch was heralded by the discovery of murine PAP models. These models revealed that GM-CSF plays a critical role in surfactant homeostasis and that accumulation of PAP material is due to reduced surfactant catabolism in alveolar macrophages, not increased surfactant production. The second epoch was brought about by the identification of the neutralizing autoantibody against GM-CSF in idiopathic PAP. It established an autoimmune aspect to this disorder and provided us with novel insights and understandings of the mechanisms involved in maintaining lung homeostasis and differentiation of alveolar macrophages by GM-CSF.

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Chapter 5
Lymphangioleiomyomatosis

Kuniaki Seyama

Abstract Lymphangioleiomyomatosis (LAM) is a rare neoplastic disease, characterized by the proliferation of abnormal smooth muscle-like cells (LAM cells) in the lungs and along axial lymphatics, usually of females. Additionally, LAM-associated lymphangiogenesis is present in LAM lesions. LAM occurs as either a pulmonary manifestation of tuberous sclerosis complex (TSC-LAM) or a sporadic disease (sporadic LAM) often accompanying renal angiomyolipoma (AML). Previously, LAM was considered hamartomatous in nature but is now recognized as a neoplastic disease with either *TSC1* or *TSC2* mutations, both of which are tumor suppressor genes. TSC-LAM can occur due to a loss-of-function type mutation of either of these *TSC* genes, whereas sporadic LAM is solely a *TSC2* disease. A recent discovery was that not all LAM cells within LAM lesions harbor the *TSC* mutation; instead, only a small fraction of LAM cells bear the pathogenic mutation. Furthermore, the involvement of an alternative genetic basis other than that from the *TSC* genes has been suggested. Studies of surface markers on LAM cells in the blood and body fluids demonstrated that LAM cells are phenotypically heterogeneous dependent on where LAM cells exist. Loss-of-heterozygosity (LOH) analysis of LAM cells in the blood and body fluids determined that LAM cells are genetically heterogeneous; that is, about 20 % of LAM patients showed different extents of LOH regions in LAM cells isolated from multiple sites.

LAM-associated lymphangiogenesis is regulated by not only lymphatic endothelial cell growth factor, VEGF-D produced by LAM cells, but also the non-collagenous-1 domain (NC1 domain) of type IV collagen α5, named lamstatin. Lymphangiogenesis and estrogen seem to have cooperative roles in the progression of this disease. Estrogen operates in several intracellular signaling pathways that are relevant to the pathogenesis of LAM. In this milieu, estrogen enhances matrix metalloproteinase-2 production by LAM cells and promotes the survival of circulating LAM cells. LAM cells can enter the lymphatic stream either through activated mTORC1-mediated cellular and biochemical events or via lymphangiogenesis-mediated fragmentation of LAM lesions to shed LAM cell clusters.

K. Seyama, M.D., Ph.D. (✉)
Division of Respiratory Medicine, Faculty of Medicine and Graduate School of Medicine, Juntendo University, 2-1-1 Hong, Bunkyo-Ku, Tokyo 113-8421, Japan
e-mail: kseyama@juntendo.ac.jp

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Given that LAM cells are neoplastic, we must establish where such cells originate and identify their normal counterparts. The female genital tract including the uterus is an attractive candidate, since the majority of LAM patients examined had LAM lesions in the uterus and nearby lymph nodes. Another yet unsolved but important question is why LAM is limited to females; so far, we have no clues to the answer in terms of genetic background, biochemical composition, or cellular mechanisms.

Keywords Chyle leak • Loss-of-heterozyogosity • Lymphangiogenesis • TSC genes • Tumor suppressor gene

5.1 LAM Is a Neoplastic Disease with Dysregulated Intracellular Signaling of the Mammalian Target of Rapamycin Complex 1

5.1.1 LAM Cells Have Genetic Alterations in Either the TSC1 *or* TSC2 *Gene*

The evidence that LAM is a neoplastic disease caused by *TSC* mutations was first reported in 1998. Since LAM can occur in association with tuberous sclerosis complex (TSC), an autosomal dominant tumor suppressor gene syndrome, as well as without clinical manifestations of TSC (the former is TSC-LAM and the latter, sporadic LAM), a possible genetic cause was considered by questioning whether TSC and LAM have in common pathogenetic factors and mutations of either *TSC1* or *TSC2*. First, loss-of-heterozygosity (LOH) analysis in the regions of the *TSC1* (chromosome 9q34) and *TSC2* (chromosome 16p13.3) genes was performed by utilizing renal angiomyolipomas (AMLs) and retroperitoneal lymph nodes, both of which were obtained from patients with sporadic LAM (Smolarek et al. 1998). The results identified *TSC2* LOH in 54 % (7 of 13) of patients with renal AMLs and in all (4/4) of the retroperitoneal lymph nodes examined. Interestingly, no *TSC1* LOH was identified.

When the genetic study was further extended to focus on LAM cells themselves, somatic *TSC2* mutations were present in not only the LAM cells but also in AML cells (Carsillo et al. 2000). Renal AML tissues from seven patients were first examined for mutations of either the *TSC1* or *TSC2* gene, and somatic *TSC2* mutations were then identified in four of those with AML (57 %). Yet no such mutation existed in the corresponding normal renal tissue. Next, LAM cells in lung tissues were tested in four patients with sporadic LAM whose renal AML turned out to carry *TSC2* somatic mutations. These results indicated that AML and LAM cells had the same somatic *TSC2* mutation in each of four patients with sporadic LAM, suggesting that sporadic LAM is caused by a clonal expansion of neoplastic cells carrying a somatic *TSC2* mutation. Again, no mutation was apparent in the normal lung tissue or in lymphoblastoid cells from the four patients with sporadic LAM.

On the other hand, the other study revealed that no *TSC2* mutation was detected in the coding region of the *TSC2* gene when screening genomic DNA from 21 patients with sporadic LAM (12 of whom also had renal AML) (Astrinidis et al. 2000). Therefore, the likely cause of sporadic LAM is somatic *TSC2* mutations, whereas TSC-LAM occurs because of mutations in either the *TSC1* or *TSC2* gene. Similar findings for *TSC* genes were reported from studies of Japanese LAM patients (22 patients with sporadic LAM and six with TSC-LAM) (Sato et al. 2002). They demonstrated germline *TSC2* mutations in two (33.3 %) of six patients with TSC-LAM and a germline *TSC1* mutation in one (4.5 %) of 22 patients with sporadic LAM. Later, the condition of this TSC-LAM patient with a germline *TSC1* mutation was recognized as a forme fruste of TSC that mimics a sporadic LAM (Sato et al. 2004), thus supporting the opinion that sporadic LAM is a *TSC2* disease, whereas TSC-LAM can stem from either *TSC1* or *TSC2*.

A recent publication revealed that not all LAM cells, in fact only a small fraction of them, carry *TSC* mutations (Badri et al. 2013). In that study, LAM cells were microdissected from paraffin-embedded specimens of ten patients with sporadic LAM and then examined for exonic sequences of *TSC1* and *TSC2* genes by using a next-generation sequencer. Somatic mutations identified were nine different pathogenic *TSC2* mutations in eight of these patients. However, allelic frequencies of the mutations ranged from 4 to 60 % but, most often, less than a 20 % frequency. Four of these eight patients' samples showed *TSC2* two-hit inactivation. Interestingly, two patients with sporadic LAM showed no *TSC1*/*TSC2* mutation. In accordance with the results of genetic analysis, though, immunostaining of LAM lung tissues was positive for both hamartin and tuberin as well as negative for pS6K (phospho-S6 kinase), indicating no evidence of mammalian target of rapamycin complex 1 (mTORC1) activation. The important considerations as to the pathogenesis of LAM raised here are as follows. First, *TSC2* mutations may not be the primary driver of LAM development but occur in a subset of LAM cells after other unknown initiating events. Second, *TSC2*-mutant cells may recruit stromal cells to adopt a LAM cell phenotype. Third, the existence of the two individuals without *TSC1* or *TSC2* mutations suggests that alternative genetic mechanisms may be operative in some cases of LAM.

The results of detailed genetic analysis of renal AMLs were recently reported (Qin et al. 2011). cDNA sequencing was performed for the entire coding region of *TSC1*, *TSC2*, and *RHEB*. The *TSC2* mutation was then identified in seven of eight AMLs examined. In addition, 983 mutations in a set of 115 common cancer genes were assessed in five AMLs, but no mutations were found. Seven AMLs were further subjected to single nucleotide polymorphism (SNP) analysis to assess copy number variations of genomic fragments across the genome, but no abnormalities indicating somatic events were detected. Although the limited amount of samples precluded whole genome sequencing, the investigators concluded that *TSC2* mutations appear to be common but that no other genetic events are obvious in renal AML.

LAM and AML are categorized as a disease within the spectrum of perivascular epithelioid cell tumors (PEComas), defined as "mesenchymal tumors composed of

histologically and immunohistochemically distinctive perivascular epithelioid cells (PEC)" (Bonetti et al. 1994; Folpe and Kwiatkowski 2010; Zamboni et al. 1996). Since LAM and AML share a genetic basis when they coexist in a LAM patient (Crooks et al. 2004), the in-depth analysis of two diseases coexisting in a single patient by next-generation sequencing may reveal the precise genetic factor that contributes to these phenotypically different PEComas.

5.1.2 *LAM Cells Are Phenotypically and Genetically Heterogeneous*

One of the marked clinical features of LAM is its consistently progressive clinical course, although even the rate of progression varies considerably among individual patients. Since this disease course is likely to be the metastatic process, periodic CT scans surely find the number of pulmonary cysts to increase as time goes by. LAM cells carrying *TSC2* LOH have been identified in circulating blood and other body fluids including chylous pleural effusions, chylous ascites, bronchoalveolar lavage fluid (BALF), and urine (Crooks et al. 2004), but phenotypic surface markers of LAM cells appear to differ among each of these sources (Cai et al. 2010). In circulating blood, LAM cells with *TSC2* LOH were detected in more than 90 % of samples and were $CD90^+/CD45RA^-$ cells (with or without CD235a). In chyle, BALF, and urine, LAM cells with *TSC2* LOH occupied 50, 80, and 69 % of patient samples, respectively, and were $CD44v6^+/CD9^+$ cells. The patterns of *TSC2* LOH detected in LAM cells from most of these patients were identical despite the cells' locations at different sites, indicating a common genetic origin of LAM cells yet changes in their phenotypic expression according to the microenvironments. As recently demonstrated, the number of circulating LAM cells decreases when LAM patients are treated with sirolimus (Cai et al. 2014).

LOH analysis of LAM cells from various body fluids also revealed that LAM cells are genetically heterogeneous. In 8 of 29 (23 %) of patients with sporadic LAM and 2 of 8 (25 %) with TSC-LAM, LAM cells from different sites varied as to the extent of LOH regions in terms of informative microsatellite markers (Cai et al. 2010). The genetic heterogeneity of LAM cells could be explained by (1) a different second *TSC2* mutation in cells harboring the same first-hit mutation, thus enabling coexistence of two independent clones of LAM cells that originated from a single precursor cell with a first-hit mutation or (2) introduction of a new, independent genetic alteration in the existing LAM cells during the metastatic process, thus fostering sufficient chromosomal instability to allow the genetic divergence. The fact that each AML has different LOH patterns in TSC-LAM patients with multiple AMLs (Henske et al. 1995) could support the former hypothesis (#1), whereas the genetic divergence well documented among cancer cells appears to support the latter hypothesis (#2).

5.2 LAM Is a Disease of Lymphatics

5.2.1 LAM Manifests Lymphatic Involvements Likely Due to the Existence of Abundant Lymphatic Vessels in LAM Lesions

Peculiar clinical findings and manifestations noted in LAM are those related to abnormalities in lymphatic systems. Chylous pleural effusion and/or ascites occur in about 10–15 % of patients. Chylous sputum that frequently accompanies pulmonary lymphedema (lymphatic congestion) may be noted during the disease course or in advanced cases. Chyle can leak into urine, from the vagina, even from guts. Detailed pathologic analysis and the identification of lymphangiogenic growth factors, both of which are likely to be involved in LAM, clearly provide the basis for the findings and manifestations cited here. In this context, LAM can be labeled as a rare disease involving lymphatics.

As literally implied, the predominant histologic feature of LAM is the existence of abundant lymphatic vessels and the proliferation of LAM cells, which are morphologically benign-looking smooth muscle-like cells in the lungs and along axial lymphatics. The former used to be recognized as cleft- or slit-like spaces within LAM lesions. However, advances in identifying specific markers for lymphatic endothelial cells (LECs) brought realization that the abundance of lymphatic vessels, including not only cleft-like or slit-like spaces but also irregularly dilated lymphatic vessels, was associated with LAM lesions. In contrast, immunohistochemical study revealed that very few blood vessels were involved compared with the profusion of lymphatics (Kumasaka et al. 2005). Furthermore, round cell clusters consisting of α-smooth muscle actin (αSMA)- and HMB45-positive LAM cells in the core enveloped within a monolayer of LECs were often spotted within LAM-associated lymphatic vessels on histopathological specimen (Kumasaka et al. 2005). These clusters appeared in chylous pleural effusions, chylous ascites, and even chylous pericardial effusions as globular-shaped, voluminous aggregates (Mitani et al. 2009). Since these interior cells were LAM cells positive for α-SMA, HMB45, estrogen receptor, and progesterone receptor, they were termed LAM cell clusters (LCCs) (Fig. 5.1). LCCs are about 25–125 μm in size and have been present in all chylous samples tested. In all of 17 chylous fluid samples (eight from pleural effusions, eight from ascites, and one from pericardial effusion), the existence of LCC was confirmed, although the concentrations varied among LAM patients (Mitani et al. 2009).

This abundance of lymphatic vessels, i.e., lymphangiogenesis, within LAM lesions is a prominent pathologic feature of LAM. Generally, lymphangiogenesis is induced by various lymphangiogenic growth factors such as VEGF-C, VEGF-D, basic fibroblast growth factor, angiopoietin-1, etc. However, anti-lymphangiogenic factors are also likely to be involved. Thus, the balance between growth factors and anti-lymphangiogenic factors appears to be important for the regulation of

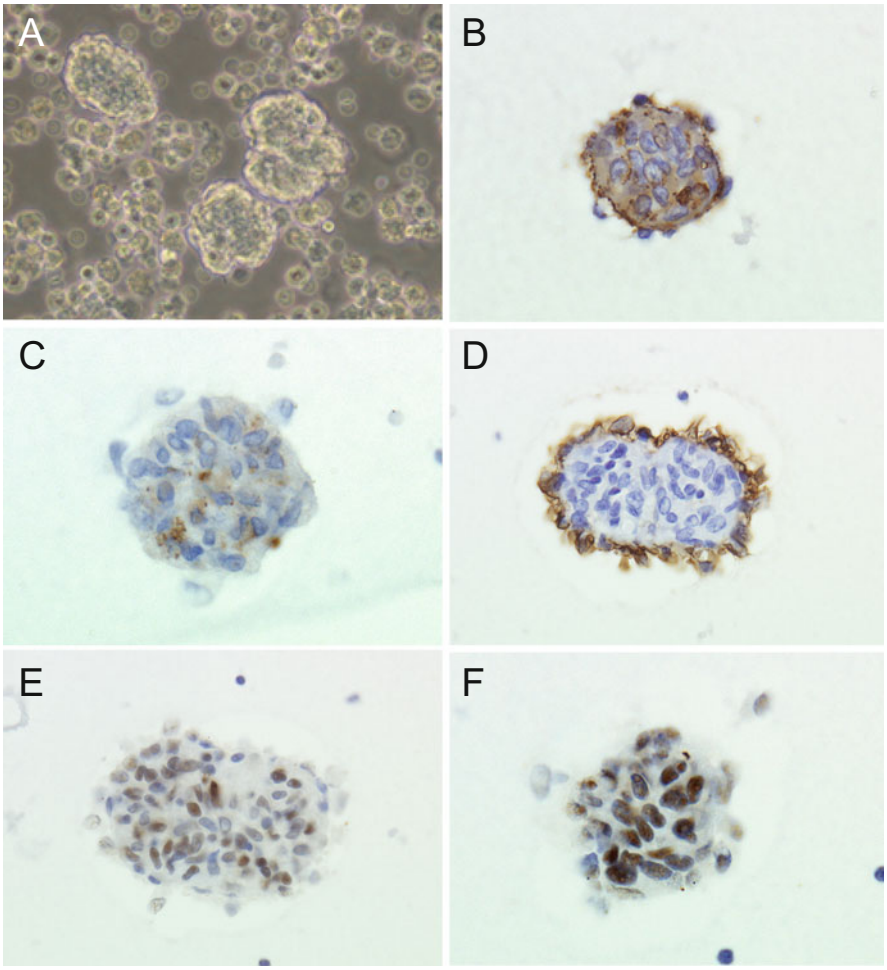

Fig. 5.1 LAM cell clusters found in chylous pleural effusions from LAM patients. Magnified view of LAM cell clusters (LCC) by inverted microscope (**a**). LCC in chylous ascites was collected in nylon mesh (40 μm pore size), suspended in phosphate-buffered saline and then observed by inverted microscope. LCC in the cell block preparation was subjected to immunohistochemical examination (**b**–**f**). LCC is composed of LAM cells immunopositive for α-smooth muscle actin (**b**) and for HMB45 (**c**). The surfaces of LCC are a monolayer of lymphatic endothelial cells that are immunopositive for podoplanin (**d**). Most cells within LCC are immunopositive for estrogen receptor (**e**) and progesterone receptor (**f**)

LAM-associated lymph vessel growth. A recent report stated that the expression of type IV collagen α3 and α5 non-collagenous-1 domain (NC1 domain) was specifically diminished in lung tissues of LAM patients (Weckmann et al. 2012). This NC1 domain of collagen type α5, named lamstatin, and its 17-amino-acid fragment identified as its theoretically active site and named CP17 were prepared and

examined for their ability to regulate lymphangiogenesis. In those assays, both lamstatin and CP17 clearly inhibited proliferation, migration, and cord formation of human microvascular lung LECs in vitro (Weckmann et al. 2012). Accordingly, LAM-associated lymphangiogenesis in the lung seems to be promoted by production of the lymphangiogenic growth factor, VEGF-D, by LAM cells, and also by reduction of the inhibitory extracellular matrix cleavage product of collagen IV α5, lamstatin. However, neither the regulatory mechanisms for VEGF-D production by LAM cells nor the means of reducing lamstatin content in LAM lungs has been determined.

5.2.2 How Does LAM Progress? Invasion-Dependent and Invasion-Independent Metastatic Pathways

The existence of circulating LAM cells clearly supports the conclusion that LAM is a neoplastic disorder, the progression of which can be mediated by a metastatic process. Several cellular and molecular mechanisms presumably drive this process. With respect to human cancers, two pathways appear to control disease progression: invasion-dependent or invasion-independent (Sugino et al. 2002; Sugino et al. 2004). Indeed, both also seem to govern the progression of LAM (Fig. 5.2). Like cancer cells, LAM cells' production of excess matrix metalloproteinases (MMPs) (Hayashi et al. 1997; Lee et al. 2010; Moses et al. 2006) disturbs the balance between proteases and protease inhibitors in the local milieu, leads to degradation of extracellular matrix proteins, and eventually facilitates invasion into lymphatics as well as blood vessels (invasion-dependent metastatic process). Considering the abundance of lymphatics within LAM lesions as well as the paucity of blood vessels (Kumasaka et al. 2005), lymphatic invasion by LAM cells is expected to be more frequent than blood vessel invasion. Alternatively, VEGF-D-driven LAM-associated lymphangiogenesis promotes the fragmentation of LAM lesions and their shedding of LCC into the lymphatic circulation (invasion-independent process). LCC is likely to be a main cellular source for the progression of lung involvement in LAM, since lymphatic circulation pours into pulmonary circulation via the left jugulosubclavian angle, where the thoracic duct drains directly into venous circulation. LCC can also generate a new LAM lesion along an axial lymphatic route (lymph nodes and thoracic duct). The metastatic process mediated in an invasion-independent manner is well documented for several human cancers including breast cancer, hepatocellular carcinoma, thyroid carcinoma, etc. (Sugino et al. 2004).

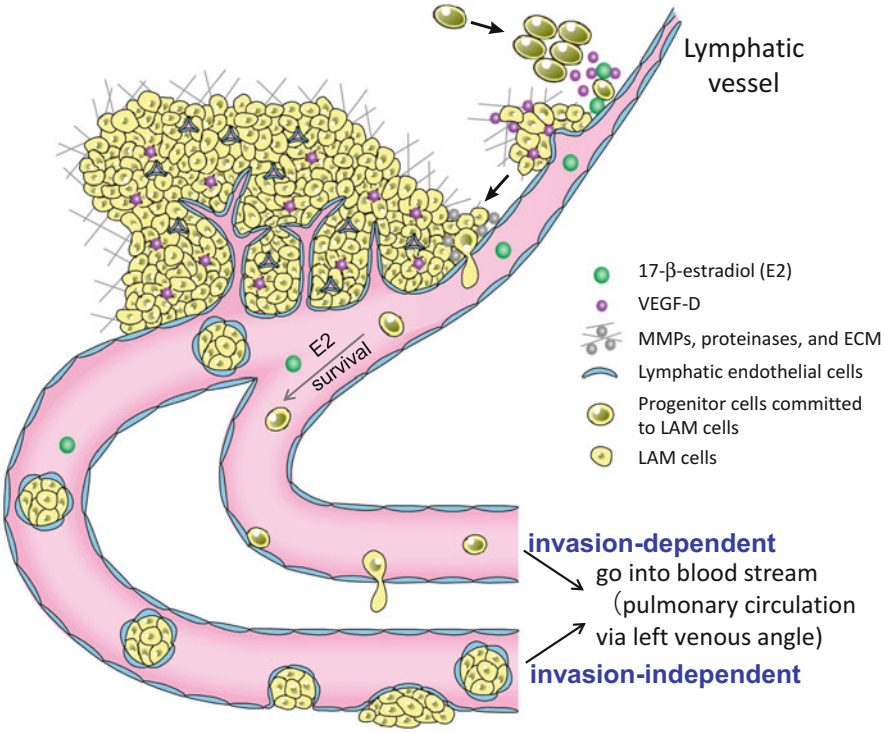

Fig. 5.2 A putative model of LAM progression through invasion-dependent and invasion-independent metastatic processes. Genetic analysis of LAM cells demonstrates that LAM cells harbor a loss-of-function type mutation on either the *TSC1* or *TSC2* gene leading to their proliferation through the dysregulated activation of mTORC1. The metastatic process is considered to be driven by several cellular and molecular mechanisms, i.e., matrix metalloproteinase (MMP) production by LAM cells, VEGF-D production and secretion that promote LAM-associated lymphangiogenesis and shedding of LCC, the enhancement of LAM cells' survival in circulation, etc.

5.3 Estrogen Is Surely Involved in the Pathobiology of LAM

LAM is a disease with an extreme gender predisposition and limited almost exclusively to females. Accordingly, the female sex hormone, estrogen, is considered an important player in the pathobiology of LAM. However, the many clinical studies that have evaluated various kinds of antihormonal therapy resulted in controversial results. On the other hand, experiments performed in vitro or with animal models utilizing *TSC*2-null cells from AML of LAM patients or *Tsc2*-null cells from knockout mice have consistently indicated that estrogen does have some role in the pathobiology of LAM.

When LAM-associated AML cells were utilized, estrogen and tamoxifen reportedly stimulated their growth in vitro through the activation of both genomic and nongenomic signaling pathways (Yu et al. 2004). ELT3 cells, *Tsc2*-null cells

derived from a uterine leiomyoma in the Eker rat, proliferated plentifully in the presence of estrogen. When ELT3 cells were injected into the flanks of male and ovariectomized female SCID mice (both implanted with estrogen), pulmonary metastases as well as the number of circulating ELT3 cells increased in mice of both genders (Yu et al. 2009). In addition, estrogen enhanced MMP-2 expression by ELT3 cells and augmented extracellular matrix breakdown in the tumor (Li et al. 2013). In this xenograft model, the activation of MEK-dependent pathways by estrogen turned out to be involved in promoting the survival of detached ELT3 cells in the circulation (Yu et al. 2009). The same group of investigators then identified other pathways in which estrogen was likely to be involved in the context of complete loss of *TSC2* function. The route recognized using a LAM-patient-derived cell line (*TSC2* null) was the ERK2 pathway, and its activation by estrogen increased transcription of the late-response gene Fra1, which is associated with epithelial-mesenchymal transition (Gu et al. 2013). This pathway is believed to collaborate with an activated mTORC1/S6K1 signaling pathway, leading to enhancement of translation efficiency for Fra1 mRNA transcribed by the estrogen-ERK2 pathway. An alternative route these investigators described was the pentose phosphate pathway to which estrogen promotes *TSC2*-null cells to become metabolically addicted to and the reactivation of Akt in the context of mTROC1 activation that prolongs survival of *TSC2*-null cells (Sun et al. 2014).

These accumulated findings clearly suggest that estrogen does have some role in the pathobiology of LAM but does not account for the remaining controversy in our real world of treating LAM in the clinical setting. A subanalysis of the MILES clinical trial described a monthly decline rate of forced expiratory volume in one second (FEV_1) that was significantly slower in postmenopausal LAM patients than in premenopausal LAM patients (-3 versus -17 ml/month, $P = 0.003$) (McCormack et al. 2012). Given that sirolimus stabilizes pulmonary function and keeps it around a baseline value rather than raising, estrogen-depletion therapy can be justified as an effective remedy to make a decline slower in this context. Merely, the magnitude of its effect on pulmonary function is not sufficient to sustain a baseline despite slowing its rate of decline. Still unclear is whether other antihormone therapies, such as the administration of progesterone or estrogen receptor blocker, provide a clinical effect as equivalent as estrogen depletion in patients afflicted with LAM.

5.4 What Is the Origin of LAM Cells?

If, as generally accepted, LAM cells are neoplastic, their normal counterparts must exist, but their identity and source of origin remain unknown. Since LAM cells express not only myogenic markers (SMA, desmin, etc.) but also melanocyte-related antigens (MiTF, MELAN-A, HMB45, etc.), some have speculated that LAM cells are derived from neural crest cells. On the other hand, LAM cells were postulated to originate from the myometrium of the uterus, since LAM occurs

exclusively in women of reproductive age, and LAM cells express estrogen and progesterone receptors. When reproductive organs obtained from women with LAM were subjected to pathological examination, the LAM lesions were highly prevalent throughout the genital tract, including the uterus, ovaries and adnexa, and in nearby lymph nodes (Hayashi et al. 2011). That analysis included uterine specimens from seven patients with sporadic LAM and three with TSC-LAM patients; of these ten LAM patients, nine had LAM lesions. Interestingly, the patients with TSC-LAM had more LAM lesions than those with the sporadic form of LAM. In support of those findings, others reported the incidental discovery of extrapulmonary LAM lesions in pelvic and para-aortic lymph nodes associated with uterine cancer (Iwasa et al. 2011). The three women described (ages 47, 59, and 71) underwent hysterectomy, bilateral salpingo-oophorectomy, and pelvic and para-aortic lymph node excision. In addition to their malignant lesions, all three patients presented with spindle cell proliferation that is best interpreted as LAM; 1–8 foci with 2–5 mm in size of LAM lesions were demonstrated in the pelvic and para-aortic lymph nodes.

Recently, experiments in a mouse model of uterine leiomyoma showed that the loss of *Tsc2* function in the uterine myometrium and stimulation by estrogen are crucial requirements for this tumor's development (Prizant et al. 2013). In these mice, *Tsc2* was designed to be knocked out in a uterine-specific manner when the animals reached puberty. Multiple uterine leiomyomas developed in 12–18 weeks during normal ovarian function; however, no leiomyomas developed when the mice were ovariectomized. Leiomyoma cells in the affected mice were immunopositive not only for estrogen and progesterone receptors but also for melanocyte-related antigens including HMB45. Interestingly, approximately half the mice were found to have pulmonary metastatic nodules of uterine leiomyoma. Conversely, in mice given rapamycin preceding puberty, the development of leiomyoma was prevented. Furthermore, the sizes of leiomyomas were markedly decreased if rapamycin was administered after the multiple leiomyomas were established. These conditions quite closely simulate those of clinical LAM, with the difference that murine model lacks cyst formation in pulmonary metastatic foci and tumor-associated lymphangiogenesis in leiomyomas. Nonetheless, this model clearly illustrates that an absence of *Tsc2* function in the myometrium and the presence of estrogen are significant factors for the genesis of neoplastic cells like LAM cells in humans.

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Part II
Treatment of Cystic Fibrosis

Chapter 6
CFTR Modulator Therapies in Cystic Fibrosis

David R. Spielberg, John P. Clancy, and Christopher Siracusa

Abstract Cystic fibrosis (CF), the most common life-shortening autosomal recessive disorder in Caucasians, results from mutations in the gene that encodes the cystic fibrosis transmembrane-conductance regulator (CFTR) protein. Nearly 2000 *CFTR* mutations have been identified as being associated with disease, with known effects of a given mutation on protein structure and/or function. Within the last 5 years, novel therapies called CFTR modulators have emerged that directly target the CFTR protein rather than treating only the clinical consequences of protein dysfunction. The categorization of mutations into classes has aided in the development of modulators that, in some instances, can restore CFTR function resulting from multiple different but related mutations. The development of CFTR modulators is rapidly expanding, with an increasing proportion of the CF population having genotype-specific therapies on the market or in clinical trials. As more patients become eligible for therapies and approvals are expanded to patients of younger ages, CFTR modulators have the potential to fundamentally change the way in which CF care is delivered.

Keywords Cystic fibrosis • Novel therapies • Modulators • CFTR • Pharmacology

6.1 Normal CFTR Structure, Function, and Relationship to CF Disease

Cystic fibrosis (CF) results from autosomal recessive mutations in the gene that encodes the cystic fibrosis transmembrane-conductance regulator (CFTR) protein, with nearly 2000 *CFTR* mutations identified that are associated with disease (http://www.genet.sickkids.on.ca/cftr/app). CFTR is a traffic ATPase, and members of this protein family are characterized by two transmembrane domains that span and secure the protein to the plasma membrane and two cytoplasmic nucleotide binding domains (NBD-1 and NBD-2). CFTR gating is accomplished by the two NBDs,

D.R. Spielberg • J.P. Clancy (✉) • C. Siracusa
Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA
e-mail: David.Spielberg@cchmc.org; John.Clancy@cchmc.org; Christopher.Siracusa@cchmc.org

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which form a heterodimer complex that binds and hydrolyzes ATP (Riordan 2008; Riordan et al. 1989; Rommens et al. 1989). CFTR is unique among traffic ATPases in that it possesses a regulatory (R) domain that imparts PKA-dependent regulation (Cheng et al. 1991). CFTR is a channel that conducts chloride and bicarbonate and regulates the epithelial sodium channel (ENaC), other chloride channels, and the transport of small molecules (e.g., glutathione and thiocyanate) (Anderson et al. 1991; Linsdell et al. 1997; Tang et al. 2009; Welsh and Smith 2001; Hudson 2001; Lorentzen et al. 2011; Moskwa et al. 2007; Rowe et al. 2005; Schwiebert et al. 1999; Stutts et al. 1995). CFTR expression in the lungs is highest in airway submucosal glands, but CFTR is also expressed in the pseudostratified epithelia of the medium and large airways and in cells lining the distal small airways (Engelhardt et al. 1992, 1994). Expression of CFTR in the airways, intestines, pancreatic ducts, bile ducts, vas deferens, and sweat glands relates directly to CF disease.

Studies from numerous laboratories support the hypothesis that CF lung disease results from defects in CFTR regulation of ion and fluid transport, including regulation of the airway surface liquid (ASL) volume and mucus hydration (Boucher 2007; Joo et al. 2002, 2006; Matsui et al. 1998a, b; Wine and Joo 2004). This work indicates that fluid and ion transport by the airway epithelia produces a carefully controlled fluid compartment (the periciliary liquid layer or PCL, a subcompartment of the ASL). Maintaining normal PCL volume supports full ciliary activity and mucociliary clearance (Boucher 2007; Matsui et al. 1998a, b). The gel layer of the ASL (comprised of hydrated MUC5AC mucins) traps particulates and serves as a fluid reservoir. Under normal conditions, the submucosal glands and surface epithelial cells together provide hydration to the PCL and ASL that promotes rapid mucus clearance without recruitment of secondary host defenses. In this model, ENaC activity promotes sodium followed by chloride absorption that maintains ASL homeostasis, including a PCL volume that approximates the height of extended cilia (7 μm). When volume expansion is needed, chloride secretion increases, which is primarily driven by CFTR and local regulatory molecules (Lazarowski et al. 2004; Tarran et al. 2006). Water follows passively in response to ion flux (Boucher 2007; Donaldson et al. 2006; Donaldson and Boucher 2007), thus providing locally integrated ion and fluid transport.

There continues to be debate regarding which CF ion-transport abnormalities are responsible for disease pathology. When CFTR is defective, ENaC activity and sodium absorption are increased, and fluid absorption dominates in primary human airway epithelial cell monolayers grown ex vivo (Boucher 2007). In this model, dehydration of the airway luminal surface reduces the PCL volume and increases the solid content of the ASL mucins. The resultant mucus stasis leads to the obstruction of small airways, creating an environment ripe for infection and inflammation. More-recent findings from the CF porcine model raise the question of whether these processes occur in vivo, as ENaC activity and ASL/PCL contraction are not observed in the airways of CFTR-knockout piglets compared with littermate controls (Chen et al. 2010). Increasing data support the hypothesis that bicarbonate transport via CFTR may be critical for normal unpackaging of mucin granules and

the formation of a normal mucin structure along the airway lumen (Ambort et al. 2012; Gustafsson et al. 2012). The pH of the ASL is reported to be reduced in CF, which has been attributed to the bicarbonate transport defect that results from absent CFTR activity and diminishes the bactericidal capacity of proteins that contribute to the innate immune system (Pezzulo et al. 2012). Studies in CF piglets have also provided evidence of defective mucus release from submucosal glands, potentially contributing to mucus stasis and infection (Welsh 2015). Finally, it long has been described that airway inflammation in CF is out of proportion to normal stimuli, producing high levels of cytokines that drive neutrophil influx into the airway (Banner et al. 2009; Konstan and Berger 1997; Sagel et al. 2007). Studies from the beta-ENaC-overexpressing mouse indicate that mucus plugging in the absence of infection is associated with airway neutrophilia, providing evidence that mucus plugging may be sufficient to initiate the airway disturbances that culminate in CF airway pathology (Zhou et al. 2011). In addition, transcriptional upregulation of mucus production is sufficient to produce an airway environment incapable of clearing viral pathogens following infectious challenge (Chen et al. 2009, 2014).

Therapeutic interventions aimed at restoring CFTR function offer the potential to address these early steps in the chain of CF lung disease, and the results from recent studies suggest that CFTR-targeted interventions may fundamentally change how we provide future care to our CF patients (Hoffman and Ramsey 2013).

6.2 Pathophysiology of CFTR Mutations

The 2000+ known CF disease-causing genetic mutations can be segregated into a number of mutation classes that share similar defects in CFTR function or localization (Rogan et al. 2011; Rowe et al. 2005; Welsh and Smith 1993). These classes are not mutually exclusive, but they do provide a useful framework to categorize CFTR-directed therapies (Table 6.1). Class I mutations impair the biosynthesis of CFTR at both the mRNA and protein level, often through a frame-shift or in-frame mutation leading to a premature termination codon (PTC), which results in the absence of CFTR at the plasma membrane. Class II mutations are characterized by defects in protein folding and maturation that are accompanied by accelerated degradation, which also result in minimal CFTR levels at the plasma membrane. Class III and class IV mutations are capable of localizing normally to the plasma membrane, but are characterized by defective gating (class III) or defective chloride conductance by CFTR (class IV). Class V mutations are typically splicing defects that reduce the efficiency of *CFTR* transcription, leading to reduced but measurable CFTR levels at the plasma membrane. Finally, class VI mutations accelerate the turnover of CFTR protein at the cell surface, leading to lower levels of CFTR at the plasma membrane. Class I–III mutations are typically considered nonfunctional, while class IV–VI mutations retain some CFTR function. Class I and II mutations elicit little if any CFTR measurable at the plasma membrane, while class III–VI

Table 6.1 Examples of genetic mutations causing cystic fibrosis and treatment strategies

Class	I	II	III	IV	V	VI
Defect	Impaired biosynthesis	Abnormal folding and trafficking	Gating defect	Reduced conductance	Reduced synthesis	Shortened time at plasma membrane
Examples of mutations	G542X R553X W1282X R1162X	F508del N1303K	G551D[a] G551S[a] Others[a,b]	R117H R334W	2789+5G>A	Corrected F508del
Modulator strategy	Suppressor[c]	Corrector/ potentiator[c]	Potentiator[c]	Potentiator[c]	Potentiator[c]	Unknown
Goal of treatment	Suppress premature termination codon	Restore folding and increase gating	Increase gating	Increase gating; increase channel pore size[d]	Increase gating; increase splicing efficiency[d]	Stabilize protein at plasma membrane

Adapted from van Barneveld et al. (2008), Jurkuvenaite et al. (2010), Clancy and Jain (2012)

[a]FDA approved for potentiator therapy

[b]Includes G1244E, G1349D, G178R, S1251N, S1255P, S549N, S549R

[c]Studies currently in progress

[d]Theoretical modulator strategy based on underlying mutation class defect

mutations produce some CFTR (of variable function) that is available at the cell surface.

6.3 Targeting CFTR Mutations

Since the number of mutations that cause CF is quite large, it can be difficult to envision the development of clinically meaningful mutation-specific CFTR drugs. However, despite the high number of known *CFTR* mutations, only a handful of them are responsible for the vast majority of CF disease. The most prevalent mutation, *F508del-CFTR*, is a class II mutation, and over 80 % of CF patients have at least one copy of *F508del*. The deletion of phenylalanine at position 508 interrupts normal CFTR protein folding and processing, with accelerated degradation that results in little or no detectable F508del-CFTR at the plasma membrane (Riordan 2008).

The class I mutation *G542X-CFTR*, which is the second most common CF-causing mutation, is found in approximately 4–5 % of CF patients. It is caused by a single base-pair mutation that creates a PTC, resulting in a biosynthetic defect. PTCs are responsible for the highest number of *CFTR* mutations, and in principle, members of this mutation class may be amenable to common restorative strategies. Another example is *G551D-CFTR*, which is the third most common CFTR mutation and is found in approximately 4 % of CF patients. It is a class III mutation with normal plasma membrane levels, but defective gating. A number of *CFTR* mutations share similar gating defects and thus may be responsive to common approaches to restore function (Yu et al. 2012; Rogan et al. 2011; Rowe et al. 2005). Therefore, strategies that target common mutations or can be extended to mutations with common fundamental defects may be sufficient to reach the vast majority of CF patients, including patients with very rare *CFTR* mutations.

6.3.1 Activating CFTR at the Plasma Membrane: CFTR Potentiators

Programs undertaken by academic laboratories and pharmaceutical companies (often in collaboration with the Cystic Fibrosis Foundation) were initiated 10–15 years ago to identify small molecules that restore function to different *CFTR* mutations (Caci et al. 2003; Galietta et al. 2001; Pedemonte et al. 2005; Van Goor et al. 2009, 2011; Welch et al. 2007). As one example, high-throughput screening efforts by Vertex Pharmaceuticals examined thousands of compounds from molecular libraries to activate G551D-CFTR in a heterologous expression system (Van Goor et al. 2009). Ivacaftor (VX-770, Kalydeco) is one result from these efforts and is a “potentiator” of CFTR (use of potentiators aims to improve the gating of CFTR).

Recent in vitro studies have demonstrated that PKA-phosphorylated CFTR is the target of ivacaftor, which opens the channel independent of ATP binding and hydrolysis (Eckford et al. 2012). Ivacaftor is capable of increasing chloride secretion tenfold in cultured human-CF bronchial epithelia cells expressing the *G551D-CFTR* mutation, and this activation approximates 50 % of that seen in non-CF human bronchial epithelia cells. In addition, ivacaftor can reduce the amiloride-sensitive current (via ENaC) to attenuate fluid absorption in G551D CF airway epithelia, increasing the ASL volume and ciliary activity (Van Goor et al. 2009). Together, these results supported the rationale for clinical trials of ivacaftor in CF patients possessing the *G551D-CFTR* mutation. Several recent publications summarize the safety and efficacy of ivacaftor (Accurso et al. 2010; Davies et al. 2013; Flume et al. 2012; Ramsey et al. 2011). The two pivotal, 48-week phase III trials of ivacaftor in CF patients with the *G551D-CFTR* mutation included patients over the age of 12 years (Ramsey et al. 2011) and patients 6–11 years of age (Davies et al. 2013). Both studies were randomized, double-blinded, placebo-controlled trials with a parallel design, and both demonstrated sustained improvement in a variety of key clinical outcome measures and CFTR biomarkers accompanied by an acceptable safety profile. The first study (that enrolled CF patients over the age of 12 years with the *G551D-CFTR* mutation) included patients with lung function measured as an FEV_1 of 40–90 % predicted, with 84 patients randomized to ivacaftor and 83 randomized to placebo. Adherence was >90 %, and the primary endpoint was the change in absolute FEV_1 % predicted from baseline through 24 weeks. Additional secondary endpoints included the change in FEV_1 through 48 weeks, time to first pulmonary exacerbations, and changes in the respiratory domain of the CFQ-R, weight, and sweat chloride concentration. Patients randomized to ivacaftor demonstrated significant improvements in all key primary and secondary endpoints at weeks 24 and 48 versus placebo. The change in mean FEV_1 was +10.5 % predicted at week 48 for the ivacaftor subjects versus placebo ($P < 0.001$), with a >50 % reduction in the risk of pulmonary exacerbation ($P < 0.001$), an increase in mean weight of +2.7 kg ($P < 0.001$), and a reduction of the mean sweat chloride concentration of 48.1 mMol ($P < 0.001$). Patient-reported outcomes (CFQ-R respiratory domain) improved in the ivacaftor group above the minimal clinically important difference (MCID) ($P < 0.001$), and these patients demonstrated a similar adverse events (AE) profile relative to the placebo group (Ramsey et al. 2011).

More recently, the results of the second study (that enrolled CF patients aged 6–11 years with the *G551D-CFTR* mutation) were reported with generally similar findings observed (Davies et al. 2013). Fifty-two patients were randomized 1:1 to either ivacaftor treatment (150 mg every 12 h) or placebo. Despite high baseline FEV_1 % predicted values of 84.7 and 83.7 % in the ivacaftor and placebo groups, respectively, the ivacaftor-treated patients demonstrated an increase in FEV_1 % predicted of +12.5 % ($P < 0.001$) through 24 weeks compared with the placebo group. This effect occurred by week 2 and continued through 48 weeks. The ivacaftor group gained an average of 2.8 kg of weight more than the placebo group at week 48 ($P < 0.001$), and their relative reduction in sweat chloride concentrations through week 48 was −53.5 mMol ($P < 0.001$). Again, ivacaftor

was well tolerated with an AE profile that was similar between the two groups. Based on these results, the FDA approved the use of ivacaftor monotherapy (150 mg every 12 h) for the treatment of CF patients >6 years of age with the *G551D-CFTR* mutation. This was followed by similar regulatory approvals in other countries.

During this same time period, in vitro studies demonstrated that ivacaftor also potentiates CFTR with additional gating mutations, including *G178R*, *S549N*, *S549R*, *G551S*, *G1244E*, *S1251N*, *S1255P*, *G1349D*, and *G970R* (Yu et al. 2012). Ivacaftor can increase the activity of these mutations to 30–118 % of normal CFTR, which supported additional clinical trials in patients with these rare, non-*G551D-CFTR* gating mutations. The KONNECTION trial was a phase III, randomized, double-blinded, placebo-controlled crossover study that investigated the use of ivacaftor for patients with these gating mutations and demonstrated efficacy comparable to that seen in patients with G551D-CFTR (De Boeck et al. 2014). This led to FDA approval of ivacaftor for use in patients with these mutations. Subsequently, ivacaftor safety and efficacy has been studied for use in multiple gating mutations for patients aged 2–5 years with FDA approval for this younger population (Davies et al. 2016).

Ivacaftor has also demonstrated clinical benefit in CF patients with a conductance mutation, specifically the class IV *R117H-CFTR* mutation. This particular mutation is notable in that the specific number of thymidine repeats within an intron affects splicing efficiency and phenotypic expression. If five thymidine repeats are present (5T genotype), aberrant gene splicing leads to the absence of exon 9 of CFTR, tending towards a disease phenotype. Conversely, the 7T and 9T genotypes are associated with normal gene splicing a milder phenotype in association with R117H-CFTR (Chu et al. 1991, 1993).

A case report demonstrated improvement in sweat chloride levels in a CF individual possessing the 5T *R117H-CFTR* allele. This was followed by a randomized, double-blinded, placebo-controlled clinical trial of ivacaftor in CF patients with R117H-CFTR. The change in mean FEV_1 % predicted at 24 weeks for all subjects was +2.1 % compared to placebo ($P = 0.20$), but notably adults ($n = 50$) demonstrated a +5.0 % treatment benefit compared to placebo ($P = 0.01$). Ivacaftor treatment produced an improvement in both the CFQ-R respiratory domain score (8.4 points favoring ivacaftor compared to placebo, $P = 0.009$) and a significant reduction in sweat chloride levels in all subgroups. Notably, sweat chloride levels improved compared to placebo for those with both the 5T genotype (treatment difference −25.1 mmol/L, $P < 0.0001$) and 7T genotype (treatment difference −20.6 mmol/L, $P = 0.0003$). These results led to FDA approval of ivacaftor therapy for use in CF patients >6 years of age with an *R117H-CFTR* allele irrespective of poly-T genotype.

An important phase II trial examined ivacaftor (150 mg every 12 h) in CF patients possessing two copies of the *F508del-CFTR* mutation (Flume et al. 2012). This mutation produces defects in trafficking of CFTR to the plasma membrane, but some studies suggest that small amounts of F508del-CFTR may reach the plasma membrane in some patients, thus raising the question of whether

ivacaftor monotherapy may be appropriate for a subgroup of *F508del* homozygotes (van Barneveld et al. 2010). CF patients >12 years of age were enrolled in a 16-week double-blinded trial with 4:1 randomization to ivacaftor versus placebo (Part A). 140 patients were enrolled; 104 completed ivacaftor treatment and were compared to 26 in the placebo arm. Safety and adverse event (AE) frequency were comparable between the two treatment groups, and the difference in the change of FEV_1 % predicted through week 16 between the ivacaftor and placebo groups was +1.7 % ($P = 0.15$). Sweat chloride levels in the ivacaftor group declined by −2.9 mMol ($P = 0.04$) compared to the placebo group (baseline through week 16). The results indicated that ivacaftor monotherapy was insufficient to produce clinically meaningful benefits to CF patients who were homozygous for the *F508del-CFTR* mutation. Subjects in Part A of the trial who met predefined increases in FEV_1 % predicted or reductions in sweat chloride were enrolled in Part B of the study, which was an open-label 96-week extension assessing the sustainability of potential drug effects. No meaningful improvements were observed in either outcome measure during the extension. The results support the conclusion that ivacaftor monotherapy is not indicated for *F508del*-homozygous CF patients and laid the foundation for investigation of ivacaftor as a co-therapy with F508del-CFTR correctors in CF patients with the *F508del-CFTR* mutation.

6.3.2 *Modulating CFTR That Fails to Localize to the Plasma Membrane*

6.3.2.1 Restoring Biosynthetic Defects by Suppressing Premature Termination Codons

PTCs are caused by a single base-pair substitution that creates an in-frame stop or nonsense codon (Rowe and Clancy 2009; Bedwell et al. 1997; Peltz et al. 2009). When the ribosome and associated translational proteins reach the PTC, the shortened protein is released, resulting in little or no protein function. Agents that suppress PTCs bind the eukaryotic ribosome and can allow the insertion of a near cognate amino-acyl tRNA into the ribosomal A site (Rowe and Clancy 2009). This process of PTC suppression allows the ribosome to continue translation to the authentic termination codon, producing some full-length protein. Many aminoglycoside antibiotics have been shown to exhibit this property, and PTC suppression by aminoglycosides has been demonstrated both in vitro and in vivo, including in CF patients (Clancy et al. 2001; Wilschanski et al. 2000; Wilschanski et al. 2003; Sermet-Gaudelus et al. 2007; Dranchak et al. 2011; Malik et al. 2010a, b; Mendell et al. 2010). One oral compound (PTC124 or ataluren, a non-aminoglycoside developed by PTC Therapeutics) has entered clinical trials to suppress PTCs and treat nonsense-mediated CF. Three phase II, randomized, dose-ascending clinical trials of ataluren have been completed in CF, and all have been open-label trials. In the first study, 23 subjects with CF due to nonsense-

mediated mutations were treated with two doses of oral ataluren for two 2-week treatment periods (Kerem et al. 2008). The safety profile of ataluren was tolerable and no subjects discontinued treatment. One CFTR biomarker [CFTR-dependent chloride transport as measured by the nasal potential difference (NPD)] showed improvement during ataluren treatment compared with baseline and washout values that were statistically significant. There was a mild increase in mean lung function (FEV_1 % predicted) during the low-dose treatment, but the relevance of this finding is uncertain in the absence of a control group. In a related study, 19 subjects were enrolled in a 3-month open-label study of ataluren that assessed longer-term safety, tolerability, and NPD responses (Wilschanski et al. 2011). CFTR activity, as monitored by the NPD, improved over the course of the study by a mean of −5.4 mV ($P < 0.001$), and many "nonresponding" subjects from the shorter trial demonstrated NPD improvements after longer ataluren treatment. In addition, the study reported trends of increased lung function and decreased coughing over 3 months. A subsequent phase II trial of ataluren in pediatric patients with nonsense-mediated CF was completed in France and Belgium (Sermet-Gaudelus et al. 2010). Thirty CF children (ages 6–18 years) were randomly assigned to treatment with low-dose or high-dose ataluren [using a similar dosing scheme as (Kerem et al. 2008)] for two 2-week treatment periods. Improvements in CFTR function by NPD while on ataluren were reported compared with baseline and washout values, with nearly 50 % of subjects crossing threshold responder criteria for several CF-causing PTCs. Brushed nasal cells demonstrated detectable CFTR at the plasma membrane of several patients during ataluren treatment. The results from these two studies differ from those of a third phase II study performed in parallel to the Kerem and colleagues' (2008) study, but in the USA [$n = 6$ centers, (Clancy et al. 2006)]. Although ataluren again demonstrated an acceptable safety profile, the US study did not demonstrate improvements in CFTR function (by NPD) or improvements in FEV_1 % predicted compared with baseline or washout values (Clancy et al. 2006). A phase III double-blinded, placebo-controlled trial evaluating the efficacy, safety, and tolerability of ataluren compared with placebo for 48 weeks was recently completed with similar findings to the 2006 phase II study (Kerem et al. 2014). NPD and sweat chloride did not improve on therapy, nor was there improvement in FEV_1 % predicted. A subgroup analysis did note improvement in FEV_1 % predicted compared to placebo in those subjects who did not concomitantly use inhaled aminoglycoside antibiotics. NPD and sweat chloride data were not reported for this subgroup. At the time of this publication, there is an ongoing phase III trial of ataluren in CF patients who are not using inhaled aminoglycosides (NCT02107859).

Separate from ataluren, there is interest in exploiting the known PTC-suppressing effects of aminoglycosides through development of other novel compounds. Preclinical studies of synthetic aminoglycoside derivatives (lacking antimicrobial activity) have provided evidence for PTC suppression in heterologous expression systems, primary human airway epithelial cells (AECs), and CF mice expressing a human *CFTR-G542X* transgene (Xue et al. 2013). These data suggest that the synthetic derivatives produce greater PTC suppression compared with

authentic aminoglycosides (such as gentamicin) and restored CFTR function can be augmented with ivacaftor in vitro.

6.3.2.2 Addressing Folding Defects in F508del-CFTR: CFTR Correctors, Alone and in Combination with Ivacaftor

Parallel high-throughput screening efforts by Vertex Pharmaceuticals sought to identify molecules capable of increasing F508del-CFTR levels at the plasma membrane, and one identified lead compound was subsequently developed through iterative medicinal chemistry into VX-809 (Van Goor et al. 2011). The screening was completed in Fischer rat thyroid (FRT) cells stably transduced with human *F508del-CFTR* cDNA, and promising molecules were subsequently confirmed in human AECs derived from *F508del*-homozygous CF patients.

VX-809 studied in *F508del*/*F508del* human AECs demonstrates reasonable potency ($EC_{50} = 81 \pm 19$ nM), a two-log dose window of correction, specificity for F508del-CFTR (without correction of other protein-folding defects in P glycoprotein), and enhancement of F508del-CFTR activity at the plasma membrane (including improved membrane half-life and open-channel probability) (Van Goor et al. 2011). VX-809 monotherapy achieves F508del-CFTR activity that approximates 15 % of wild-type CFTR in AECs. When VX-809 is combined with ivacaftor ex vivo, F508del-CFTR function is doubled, achieving approximately 30 % of wild-type CFTR activity. This co-treatment effect is the foundation for the co-development of VX-809 (lumacaftor) with ivacaftor therapy in CF patients possessing *the F508del-CFTR* mutation.

The first phase II clinical trial of lumacaftor assessed monotherapy in CF patients possessing two copies of the *F508del-CFTR* mutation (Clancy et al. 2011). In this randomized, double-blinded, placebo-controlled trial, 89 CF patients (>18 years of age) were randomized to one of four doses of lumacaftor (25, 50, 100, 200 mg once daily) compared with placebo for 28 days. Patients treated with lumacaftor demonstrated an acceptable safety profile, with a similar AE and serious AE incidence compared with the placebo group. Pharmacokinetic analysis supported once-daily dosing, and pharmacodynamics showed that lumacaftor treatment produced a rapid, dose-dependent drop in sweat chloride values compared with both baseline values and the placebo group. Sweat chloride reductions were modest (peak change of −8.12 mMol versus placebos following 28 days of treatment, $P = 0.009$). The reductions were dose dependent, observed within 7 days on lumacaftor, sustained through 28 days, and returned to baseline during drug washout. Clinical efficacy measures (FEV_1 % predicted, pulmonary exacerbations, CFQ-R) and other CFTR biomarkers (NPD measures of chloride transport, mature CFTR protein in rectal biopsies) were not different compared with the placebo controls. The power of the study was insufficient to detect NPD improvements, and a minority (one third) of participants chose to undergo the optional rectal biopsies (including only one in the highest-dose group). However, the results supported a follow-up phase II trial that examined increasing doses of

lumacaftor (200, 400, and 600 mg) combined with ivacaftor (250 mg) in adult CF patients with either one or two copies of the *F508del-CFTR* mutation (Boyle et al. 2014). Adult subjects ($n = 109$) were enrolled with approximately 20 subjects assigned to each treatment group. Three cohorts of *F508del*-homozygous patients were randomized to treatment with 28 days of once-daily lumacaftor (200, 400, or 600 mg) followed by 28 days of lumacaftor + ivacaftor (250 mg every 12 h). In addition, a cohort of *F508del-CFTR*-heterozygous subjects received 600 mg lumacaftor daily for 28 days followed by lumacaftor + ivacaftor (250 mg every 12 h) for 28 days. A placebo group (56 days) was included for comparison. Patients in the 600 mg lumacaftor group showed small improvements in FEV_1 % predicted as well as in sweat chloride levels. Importantly, the lower lumacaftor dose groups demonstrated smaller treatment effects, as did the *F508del*-heterozygous patients treated with 600 mg lumacaftor combined with ivacaftor. These findings led to the phase III TRAFFIC and TRANSPORT trials of lumacaftor combined with ivacaftor in *F508del-CFTR*-homozygous CF patients >12 years of age, which ultimately led to the approval of the two-drug combination as Orkambi (Wainwright et al. 2015). These parallel studies together enrolled 1122 subjects, making this the largest phase III program to date evaluating a new therapy for CF; the designs were identical for the two studies except for assessing ambulatory electrocardiography data only in the TRAFFIC study and adolescent pharmacokinetic data only in the TRANSPORT study. Two dosing regimens were assessed, with lumacaftor either 600 mg daily or 400 mg twice daily and the latter dosing regimen representing the current dosing of Orkambi. In this dosage group, subjects had statistically significant improvement in mean FEV_1 % predicted compared to placebo (absolute change from baseline +2.8 %, $P < 0.001$), as well as a reduced hazard ratio for pulmonary exacerbations compared to placebo (HR = 0.61, $P < 0.001$). In addition, compared to placebo, patients receiving lumacaftor-ivacaftor demonstrated CFQ-R respiratory domain scores with small but statistically significant improvements (+2.2, $P = 0.05$), and there was a small increase in BMI relative to baseline (+0.24 kg/m^2, $P < 0.001$). Orkambi was approved by the FDA in 2015 for patients 12 years of age and older who are homozygous for the *F508del-CFTR* mutation.

A second F508del-CFTR corrector developed by Vertex (VX-661) is currently being evaluated in combination with ivacaftor. Initial results from phase II studies have been reported in abstract form and press releases (Donaldson et al. 2013). A phase III program is now underway (NCT02347657, NCT02392234, NCT02412111, and NCT02516410), studying VX-661 in combination with ivacaftor in patients with a variety of different CFTR genotypes.

Within the past 2 years, a number of new compounds have been brought into clinical trials for CF, representing various molecular approaches to addressing CFTR dysfunction. Riociguat (Adempas®, developed by Bayer Healthcare Pharmaceuticals) is a soluble guanylate cyclase agonist that increases the synthesis of cyclic GMP, a pathway potentially linked to CFTR regulation. Riociguat is currently approved for use in adults with pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension and is being investigated in a phase II study of CF patients homozygous for *F508del-CFTR* (NCT02170025). Several

trials are also underway with a drug called N9115 (from Nivalis Pharmaceuticals, Inc.), an *S*-nitrosoglutathione (GSNO) reductase inhibitor; this follows a previous trial of an earlier compound of the same class, N6022 (NCT01746784). In vitro, GSNO reductase inhibition reverses the GSNO activation seen in CF and is proposed to have additive effects in combination with CFTR potentiators and correctors (Peter et al. 2015; Tarran et al. 2006). The current clinical trials span phase Ib to phase II designs in combination with Orkambi (NCT 02227888, NCT02275936, and NCT 02589236). Other active early studies include two studies of QR-010 (ProQR Therapeutics—NCT02532764 and NCT02564354), a single-stranded RNA editing strategy. Studies in animal models have reported systemic distribution following airway administration and improved CFTR function in F508del-CFTR mice (Noreen et al. 2015). QBW251 (Novartis Pharmaceuticals, NCT02190604) is a proposed CFTR potentiator that is currently being studied in patients with at least one class III–VI CFTR mutation.

6.4 Summary and Conclusions

The development of CFTR modulators that target the underlying cause of CF is rapidly expanding, with an increasing proportion of the CF population having genotype-specific therapies on the market. The dramatic improvements observed in a variety of clinical efficacy measures following ivacaftor treatment in patients with gating mutations are unprecedented and suggest that small molecules targeting CFTR mutations are a viable strategy to treat CF. With the approval of Orkambi bringing genotype-directed treatment to a much larger proportion of the population, future research is striving to improve the clinical benefits achieved with new medications as well as develop additional novel treatments that can be used for other classes of CFTR mutations. As more patients become eligible for therapies and approvals are expanded to patients of younger ages, CFTR modulators have the potential to fundamentally change the way in which CF care is delivered.

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Chapter 7
Drug Therapies that Augment Airway Surface Liquid

Evangelia Daviskas, Sheila Sivam, Mark R. Elkins, Tiffany J. Dwyer, Ruth Dentice, and Peter T. Bye

Abstract This chapter briefly describes the airway surface liquid (ASL) and the critical role of its hydration status and reviews the pathophysiology initiated by insufficient hydration of ASL. It then presents detailed evidence about medications intended to restore ASL and their effectiveness in people with CF. Inhaled osmotic agents are one class of ASL-augmenting medications. Nebulized hypertonic saline significantly improves lung function, reduces exacerbations, and improves the quality of life of people with CF. Dry powder mannitol also significantly improves lung function and reduces the incidence of exacerbations in this population, but it does not significantly improve quality of life. Ion transport modifiers are another class of inhaled medications that augment the ASL. These medications address the underlying ion transport defects by either inhibiting sodium absorption (e.g., amiloride) or by stimulating chloride secretion (e.g., denufosol). Despite promising preliminary research, ion transport modifiers have not demonstrated clinical benefits to date. In summary, osmotic agents, such as hypertonic saline and dry powder mannitol, have clinical worthwhile benefits in people with CF and are currently used in clinical practice.

Keywords Airway surface liquid • Cystic fibrosis • Hypertonic saline • Dry powder mannitol • Osmotic agents

7.1 Introduction

Airway diseases such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) have common features including abnormal clearance of mucus, persistent inflammation, and recurrent infections. People with these diseases may benefit from therapies that augment the volume of airway surface liquid (ASL). An

E. Daviskas, M.Biomed.E., Ph.D. (✉) • S. Sivam • M.R. Elkins • T.J. Dwyer • R. Dentice • P.T. Bye
Department of Respiratory and Sleep Medicine, Royal Prince Alfred Hospital, Sydney, NSW 2050, Australia
e-mail: Evangelia.daviskas@sydney.edu.au

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understanding of the ASL is essential when applying target-specific therapies that aim to augment ASL in diseases with abnormal clearance of mucus.

In the normal lung, the ASL is a thin layer that lines the luminal surface of the airway epithelia. It consists of two distinct layers, the periciliary layer (PCL) and the mucus layer. It is part of the innate defense system of the respiratory tract because it maintains airway hygiene by clearing mucus that has trapped inhaled particles, inflammatory products, and pathogens. Mucus clearance is achieved by the cilia, which are cellular projections of the epithelial cells, beating in the PCL in order to propel the mucus toward the mouth. The PCL was originally thought to be a watery fluid that provides a favorable environment for the beating of the cilia and separates the mucus from the epithelial cells. Recent work proposed a gel-on-brush model according to which the PCL has membrane-spanning mucins and mucopolysaccharides tethered to the cilia, microvilli, and epithelial surface (Fig. 7.1a) (Button et al. 2012).

The tethered macromolecules in the PCL were shown to have several functions. First they form an extracellular brush that prevents the mucins of the mucus and the inhaled deposited particles from penetrating into the PCL. Second they stabilize the PCL by intermolecular repulsion against compression by the osmotically active mucus layer. Finally they generate an osmotic pressure, through the intermolecular repulsion of the tethered macromolecules, that regulates the hydration of the PCL (Button et al. 2012). The stabilization of the PCL is required for the formation of the distinct mucus layer and for effective mucus clearance. The gel-on-brush model describes the forces that govern the distribution of water between the PCL and mucus layer in health and disease (Button et al. 2012). It predicts that water distributes between the two layers according to the osmotic moduli (Fig. 7.1b).

The large macromolecules of the mucus layer (secreted mucins that do not penetrate the PCL) are the osmotically active molecules. In health, the osmotic modulus of the mucus is lower than that of the PCL and mucus acts as a reservoir for water (Fig. 7.1b). When there is decreased water at the airway surface, such as in CF (Boucher 2007), or excessive mucins, such as in COPD (Hogg et al. 2004), the osmotic modulus of the mucus increases, the PCL collapses, and the cilia stop beating (Button et al. 2012). Basically, insufficient hydration of the airway surface fluid induces destabilization of the two-layer system, which results in failure of the mucus to clear. The gel-on-brush model can explain the pathogenesis of the airway diseases that have in common mucus stasis, inflammation, and infection.

The depth of the PCL is 5–7 μm, just under the length of the cilia, and it is critical for the normal function of the mucociliary clearance (MCC). The depth of the PCL is normally regulated by the active transport of Na^+ and Cl^- ions. The movement of Cl^-, Na^+, and water is controlled directly and indirectly by the cystic fibrosis transmembrane regulator (CFTR) protein, located in the apical membrane of the epithelia. Sodium absorption occurs through the epithelial sodium channel (ENaC). Abnormalities of the CFTR and ENaC channels lead to abnormal regulation of the volume of the ASL.

Airway mucus is a complex biopolymer gel. It consists mainly of glycoproteins (called mucins), globular proteins, lipids, and water. In health, water constitutes

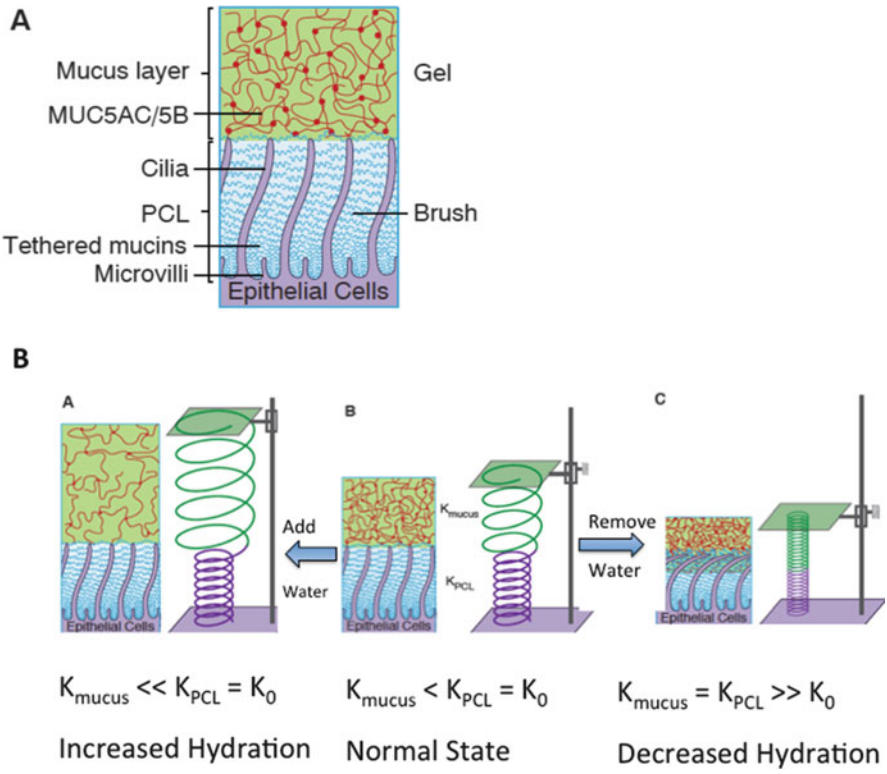

Fig. 7.1 (**a**) Schematic representation of the brush-on-gel model. (**b**) Schematic illustration showing the effects of the relative water-drawing powers of the mucus gel and the PCL quantified by the osmotic modulus *K* and represented by a spring in three states: increased water (**a**), normal (**b**), and dehydrated (**c**). The osmotic modulus is defined by the rate of osmotic pressure variation with polymer concentration. K_0 is the osmotic modulus of fully hydrated PCL as in normal state (Button et al. 2012)

95–97 % of the mucus. The glycoproteins are joined linearly by S:S bonds to form long chains (mucins) that are entangled to form a three-dimensional matrix through many weak electrostatic and hydrogen bonds. MUC5AC and MUC5B are the main primary mucins in the airway mucus. The mucins are secreted from the mucous cells of the submucosal glands and the goblet cells where they are stored in granules. The granules provide the necessary environment for the mucins to remain in a condensed state, i.e., low Na^+, high Ca^{2+}, and low pH. Upon exocytosis, mucus is rapidly hydrated and swells to form an entangled matrix gel, a process governed by the Donnan equilibrium. In health, the airway fluid provides the necessary environment for the swelling of the mucus to occur, i.e., low Ca^{2+} and high Na^+. An exchange of Na^+ for Ca^{2+} occurs within the mucus and this Na^+/Ca^{2+} exchange is necessary for the mucus to swell. In addition, the released Ca^{2+} is chelated by HCO_3^-. The swelling of the mucus is necessary for it to reach the required properties to flow. The degree of swelling depends on the water available and the

composition of the fluid at the luminal surface of the airway. Regardless of the water availability, however, for the mucus to swell, Ca^{2+} needs to be removed from the mucus matrix and chelated by HCO_3^- (Verdugo et al. 1987). In summary, control of Na^+, pH, Ca^{2+}, HCO_3^-, and water is necessary for mucus to swell, to achieve the necessary flow properties (such as viscoelasticity), and to achieve normal MCC.

7.2 Pathophysiology of Airway Surface Liquid in Cystic Fibrosis

In people with CF, the volume of the ASL is greatly reduced as a consequence of CFTR mutations. This can be a mutation in a single gene that encodes a protein that controls Cl^- transport. The CFTR defect makes the apical membrane impermeable to Cl^- and highly permeable to Na^+. The consequences of these ion transport abnormalities are reduced hydration, greatly reduced PCL, dehydrated mucus, collapse of the cilia (which allows the mucus layer to adhere to the airway surface), and ciliostasis. The clearance of mucus becomes abnormally low as a result. Inhaled pathogens continue to be trapped by the exposed surface of the mucus layer. Bacterial colonies within the adhered dehydrated mucus are stimulated to form biofilms. These biofilms protect the colonies against endogenous and inhaled antimicrobial agents (Matsui et al. 2006). This enhances the progression of the disease by increasing inflammation and bacterial growth. The concept that airway dehydration is the initiating event in CF airway disease has been tested in a mouse model (Mall et al. 2004). Studies in mice have shown that overexpression of the beta subunit of the sodium channel leads to increased Na^+ absorption (3 times) without disturbing the Cl^- secretion. This in turn causes depletion of the ASL leading to reduced MCC, neutrophilic inflammation in the absence of infection, and goblet cell hyperplasia, which are characteristics of CF airway disease (Mall et al. 2004). Recent imaging studies, in patients with CF, have made quantitative measurements confirming that airway liquid absorption is increased thus contributing to the reduction of the ASL (Locke et al. 2014).

In CF, the mucus itself is abnormal by failing to swell upon exocytosis. Defective swelling of the mucus upon exocytosis results not only from the insufficient water at the airway lumen but also from the high Ca^{2+} and reduced HCO_3^-. The CFTR-dependent HCO_3^- secretion is defective and defective HCO_3^- transport causes mucoviscidosis (Quinton 2010).

Improving the hydration of the ASL is therefore a clear treatment target to improve mucociliary and cough clearance and thus intervene in the vicious cycle of the disease. The obvious therapy in CF would be to target the CFTR, indirectly improving the airway surface liquid and subsequently the clearance of mucus. Studies with ivacaftor, which targets a single-gene mutation, have been recently completed in adults and children >6 years of age with a G551D-CFTR mutation (Ramsey et al. 2011; Davies et al. 2013). Regulatory approval has been obtained for

its use. However, as there are many CF mutations with diverse effects on the CFTR, ivacaftor will only benefit the 4 % of patients with CF that have the G551D-CFTR mutation. Other treatments that directly increase the ASL have also proven beneficial to people with CF.

7.3 Inhaled Therapies that Increase Airway Surface Liquid

Defects in electrolyte transport and consequently water transport lead to abnormal ASL and the clearance of mucus. Therapies that increase the hydration of the ASL either directly or indirectly can benefit diseases such as CF. These therapies include the osmotic agents such as hypertonic saline and dry power mannitol, sodium channel blockers, Ca^{2+}-activating chloride channel agents, and the CFTR correctors or potentiators. The latter are addressed separately.

7.3.1 Osmotic Agents

The airway surface fluid is normally isotonic and the epithelia are highly permeable to water. Therefore an acute increase in the osmolarity of the PCL can create an osmotic drive for water efflux into the airway lumen. Water moves transcellularly through water channels called aquaporins. Consequently inhalation of osmotic agents such as hypertonic saline and dry powder mannitol has the capacity to rapidly increase water movement to the airway surface, increasing hydration of the PCL and mucus with subsequent improvement in the clearance of mucus. Hyperosmolarity of the airway fluid in response to the osmotic stimulus is transient, as equilibrium is established quickly. However, the magnitude and duration of the increase in ASL volume would depend on the strength of the osmotic stimulus and how quickly it clears from the airways. Osmotic agents increase the hydration of mucus and improve its physical properties without denaturing its structure.

7.3.1.1 Hypertonic Saline

The ASL in patients with CF is isotonic, as it is in healthy individuals (Knowles et al. 1997; Matsui et al. 1998). Saline concentrations of 3 % or higher are generally referred to as hypertonic saline (HS) solutions. HS is delivered as an aerosol using a nebulizer and it is inhaled tidally. When HS deposits on the airway surface, it increases the osmotic pressure of the ASL above isotonicity. Therefore, it creates an osmotic gradient that causes the drawing in of water from airway epithelial cells, thus increasing the hydration of the ASL, restoring the PCL height, and promoting recoupling of the mucociliary clearance mechanism (Tarran et al. 2001; Boucher 2004; Donaldson et al. 2006). Hypertonic saline was first shown to increase MCC

by Pavia et al. (1978) in chronic bronchitis. In the 1980s ultrasonically nebulized HS was developed for use as a bronchial provocation test to identify bronchial hyperresponsiveness as an objective measure of the severity of asthma (Schoeffel et al. 1981; Smith and Anderson 1989). It was not until the early 1990s when HS was used again for MCC, and it was shown to stimulate MCC above normal in healthy and mild asthmatics and to improve MCC in CF subjects (Daviskas et al. 1996; Robinson et al. 1996).

In vitro studies of CF airway epithelia indicate that the equalization of the osmotic stimulus of HS by a rapid influx of water is very rapid (i.e., seconds to minutes) and that the restored PCL height is likely to be sustained for longer than normal (i.e., minutes to hours) (Tarran et al. 2007). In addition to increasing ASL osmotically (Tarran et al. 2001), other postulated mechanisms by which HS may increase mucociliary and cough clearance include inhibition of ENaC (Hebestreit et al. 2007) and a decrease in mucus viscoelasticity (King et al. 1997; Wills et al. 1997). A decrease in viscoelasticity in response to HS may be partly due to a change in the ionic bonding between the macromolecules of the mucus thereby reducing the entangled network (Dasgupta et al. 1995; King et al. 1997). In addition, an increase in mucus viscosity in CF could be due to an increase in the concentration of anionic glycosaminoglycans (GAGs) that interact with inflammatory mediators and antimicrobial peptides (Hilliard et al. 2007; Reeves et al. 2011). HS may disrupt the electrostatic interactions between GAGs and inflammatory mediators such as IL-8 (Reeves et al. 2012). Mucus viscosity in CF is also increased by DNA and actin copolymers released by necrotic neutrophils; however, this can be treated with dornase alfa (Zahm et al. 1995), although an increase in MCC with dornase alfa has not been demonstrated (Robinson et al. 2000). Also, cough is acutely increased after each dose of HS (Robinson et al. 1996, 1997), which further aids the mechanisms described above.

Restoration of the mucociliary mechanism is not, however, the only mechanism by which HS may induce clinical improvement in people with CF. Recent work in vitro has shown that HS may reduce *Pseudomonas aeruginosa* motility (Havasi et al. 2008) and viability (Behrends et al. 2010) and may improve bactericidal efficiency of some peptides (Bergsson et al. 2009). HS appears to disrupt established bacterial biofilms and inhibit formation of new ones (Anderson and O'Toole 2008). HS was also shown in vitro to increase two CFTR-dependent thiols that are protective against oxidative lung injury (Gould et al. 2010).

The optimal dose of HS is yet to be determined and studied doses vary from twice-daily use of nebulized 7 % HS (4 mL) to 4 times a day administration of nebulized 7 % HS (5 mL) (Elkins et al. 2006; Donaldson et al. 2006). Concentrations as high as 12 % have been evaluated but were found to be at the upper limit of tolerability, with oropharyngeal irritation frequently reported (Robinson et al. 1997). Twice-daily dosing is more practical. In the Robinson study (1997), improved clearance with HS appeared to last throughout the study period of 90 min, and with regular use of 7 % HS, 4 times per day, the effect on clearance was still evident 8 h after the last dose (Donaldson et al. 2006). It is expected that the deposited NaCl will be retained for longer in the CF airway lumen compared to

normal, as cellular absorption of Cl^- via the CFTR channel is absent and absorption of Cl^- would be via the paracellular route which is slower (Tarran et al. 2007). In addition, HS may prolong the decrease in ENaC activity that persists after the hyperosmolarity is resolved (O'Riordan 2016). No significant improvement in MCC has been demonstrated in children (median age 10.5 years) with CF (Laube et al. 2011). This is postulated to relate to the reduced degree of airway disease in children. There was also no benefit afforded by HS for most outcomes in infants and children younger than 6 years with CF (ISIS trial) (Rosenfeld et al. 2012). However, in a subgroup analysis on infant lung function testing, the forced expiratory volume in 0.5 s was improved significantly by 38 mL with HS (ISIS trial) (Rosenfeld et al. 2012).

Premedication with bronchodilators is recommended, as HS can induce transient airway narrowing in some patients. Data from a series of 175 adults and children with CF shows that when bronchodilator is used as premedication, marked airway narrowing occurs in ~3 % of cases and this narrowing is sustained for more than 15 min in fewer than 1 % of cases (Elkins et al. 2006, plus unpublished data from our center). Repeating the test dose after a few days usually elicits a much milder response, allowing these patients to commence therapy with HS.

Evidence of Clinical Benefits with Hypertonic Saline

In a randomized controlled trial by Eng et al. (1996), 6 % HS (10 mL) ultrasonically nebulized twice daily led to a 12.2 % improvement in FEV_1 within 2 weeks compared to control in 52 CF subjects. The effect in FEV_1 was lost within 2 weeks of ceasing the inhalations, suggesting that ongoing therapy is required for sustained benefit.

Later, a 1-year multicenter double-blind placebo-controlled randomized trial in Australia demonstrated further clinical benefits with 7 % HS (4 mL) nebulized with a jet nebulizer. A significant change in absolute difference in lung function [68 mL (3.2 %) in FEV_1 and 82 ml (2.8 %) in FVC] between the HS ($n = 82$) and control group (0.9 % saline; $n = 80$) was noted (Elkins et al. 2006). Although the increase in lung function was sustained throughout the trial, the rate of change in lung function did not significantly differ between the groups. Importantly there was a marked decline in pulmonary exacerbations requiring intravenous antibiotics (relative reduction, 56 %; $p = 0.02$; Fig. 7.2a; Elkins et al. 2006). HS also caused a similar significant reduction in the number of pulmonary exacerbations defined by symptom severity alone, with a reduction of 1.42 exacerbations per person per year ($p = 0.02$; Fig. 7.2b, Elkins et al. 2006). HS was not associated with worsening bacterial infection or inflammation.

With regular use, HS significantly reduces CF-related symptoms and significantly improves quality of life. In the 2-week study by Eng and colleagues (1996), HS significantly improved symptom scores for the perception of a clear chest after treatment, exercise tolerance, and sleep quality. In the long-term trial by Elkins et al. (2006), in which quinine was used to mask saltiness, HS significantly

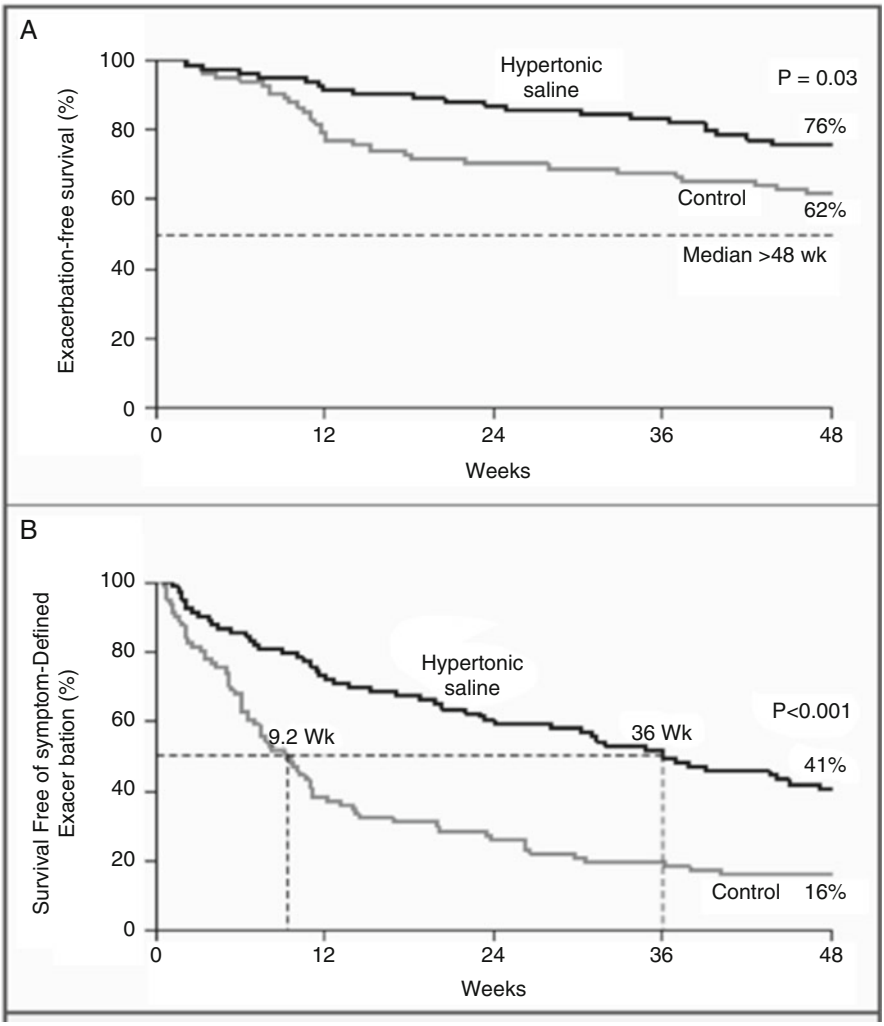

Fig. 7.2 Percentage of participants in each group remaining free of exacerbations during the HS trial (Elkins et al. 2006)

improved five of the eight domains of quality of life as assessed by the Cystic Fibrosis Questionnaire (SF-36). The mental health domain of the SF-36 was a mean of 5 points higher in the HS group than the control group ($p = 0.02$). In subjects $\geq$14 years, HS improved the role domain by 7 points, the emotional domain by 5 points, and the health domain by 5 points (all $p < 0.05$).

With regard to tolerability of HS, a recent multicenter, randomized controlled trial comparing 7 % HS alone versus HS with hyaluronic acid (HA) in 40 patients, as young as 8 years of age, demonstrated a reduced prevalence of cough, throat irritation, and saltiness with HS plus HA (Ros et al. 2014). The addition of HA has

the potential to improve tolerability and thus compliance in patients who were previously intolerant to HS (Ros et al. 2014). However, it is not yet known whether the reduction in coughing due to HA reduces the clinical efficacy of HS. In a meta-analysis of five published studies reporting tolerance to regular use of 6 or 7 % HS by people with CF, fewer than 8 % were intolerant (Elkins and Bye 2006). Therefore the addition of other compounds to improve tolerability does not need to be considered in the majority of people with CF.

A recent study (Dentice et al. 2016) was designed to address the equipoise regarding whether hypertonic saline is harmful or beneficial during standard in-patient management of a pulmonary exacerbation in adults with CF, with respect to tolerability, length of hospital stay (primary outcome), rate of resolution of clinical signs and symptoms of the exacerbation, and time to next exacerbation. One hundred thirty-two adults with an exacerbation of CF were randomized to inhale three nebulized doses per day of either 4 mL 7 % saline or a taste-masked control, throughout the hospital admission. All participants tolerated their allocated saline solution. Length of stay was 12 days in the hypertonic saline group and 13 days in control, with a mean between-group difference (MD) of 1 day (95 % CI 0–2). The likelihood of regaining pre-exacerbation FEV_1 by discharge was significantly higher in the hypertonic saline group (75 versus 57 %), number needed to treat 6 (95 % CI 3–65). On a 0–100 scale, the hypertonic saline group had significantly greater reduction in symptom severity than the control group at discharge in sleep (MD 13, 95 % CI 4–23), congestion (MD 10, 95 % CI 3–18), and dyspnea (MD 8, 95 % CI 1–16). No significant difference in time to next hospitalization for a pulmonary exacerbation was detected between groups ($p = 0.13$). Hypertonic saline speeds the resolution of exacerbation symptoms and allows patients to leave hospital with greater symptom resolution.

In summary, HS is a low-cost effective treatment modality that improves mucociliary clearance and lung function, reduces exacerbations, and improves the quality of life of people with CF.

7.3.1.2 Dry Powder Mannitol

Mannitol is a naturally occurring nonionic sugar alcohol ($C_6H_{14}O_6$, MW 182.2) that was originally developed to identify the bronchial hyperresponsiveness associated with asthma (Anderson et al. 1997) (Aridol™, Pharmaxis Ltd., Frenchs Forest, Sydney, Australia). Mannitol is not readily metabolized, is poorly absorbed, and is not transported across the epithelium. Mannitol, in its application to respiratory disease, is prepared for inhalation as a dry powder and delivered from capsules using a dry powder inhaler. Mannitol dry powder particles resist absorption of water even at a high relative humidity and therefore are stable and suitable for encapsulation. Mannitol is inhaled with a close to full inspiration, with an inspiratory flow above normal (>45–60 l/min or more), followed by a breath-hold of 5 s. Each capsule can contain up to 40 mg of mannitol. For enhancing the clearance of mucus, a standard dose of 400 mg is taken as ten capsules inhaled sequentially.

Mannitol will clear from the airways either via the mucus or the paracellular route. Its clearance via the paracellular route is very slow due to its high molecular weight. Therefore the osmotic effect of mannitol in the ASL may be sustained for a longer time compared to HS. The formulation of dry powder mannitol, its physicochemical characteristics, and its relatively fast delivery from an inhaler make mannitol an attractive option for augmenting ASL and improving airway clearance.

An increase in the hydration of ASL implies an increase in the hydration of both the mucus layer and the PCL. The first evidence suggesting that mannitol increases ASL came from studies demonstrating an increase in mucociliary clearance (MCC) in patients with asthma, bronchiectasis, and CF (Daviskas et al. 1997, 1999, 2001, 2008; Robinson et al. 1999). Inhaled mannitol (160–480 mg) has been shown to significantly improve mucociliary and cough clearance in a dose response manner both acutely (Daviskas et al. 1999, 2001, 2008; Robinson et al. 1999) and over 24 h in patients with bronchiectasis (Daviskas et al. 2001). Mucus clearance in bronchiectasis and asthma increased from a mean of 12 % per hour at baseline to 34 % per hour with mannitol, a value similar to that measured in healthy subjects. After mannitol, subjects cleared in 2 h the equivalent amount of mucus cleared over 24 h without mannitol (Daviskas et al. 2001).

Changes in the mucus layer in vivo, in response to an osmotic agent, are studied through the changes in the physical properties of sputum. Consistent with an increase in hydration, sputum studies have shown that the percentage of solids in sputum from patients with CF and non-CF bronchiectasis, as well as asthma, decreased after mannitol inhalation (Daviskas et al. 2007, 2010a, b). As a result of the increase in hydration, the interfacial tension (surface tension) of the sputum primarily responsible for the cough clearance was also reduced in CF and non-CF bronchiectasis in several studies (Daviskas et al. 2007, 2010a, b). The effect of mannitol on sputum properties has been measured at various time points ranging between 15 and 60 min to 24 h and has been shown to be dose independent over the range of 160–635 mg (Daviskas et al. 2001, 2007, 2010a, b; Daviskas and Rubin 2013).

Studies in patients with CF have demonstrated a sustained reduction in the solid content for at least 12 h after the last dose of regular mannitol treatment (420 mg, twice daily), administered over a 2-week period (Daviskas et al. 2010a). As with sputum solids in CF, the decrease in surface tension was also sustained 12 h after the last dose of mannitol and correlated significantly with improvement in FEV_1 over 2 weeks (Daviskas et al. 2010a).

While significant changes in viscoelasticity were observed in bronchiectasis when inhaled mannitol was followed 30 min later by repetitive voluntary coughing, no change in viscoelasticity after 2 weeks of treatment with mannitol was demonstrated in CF (Daviskas et al. 2010a). As CF sputum may have more pus than mucus (Shah et al. 2005; Rubin 2007) and viscoelasticity can be variable, perhaps the reduction in surface tension, following an increase in hydration, is the most important change for improving cough clearance in CF.

Changes in CF sputum properties in response to mannitol were similar regardless of treatment or not with dornase alfa, suggesting that the effect of mannitol is

independent of the effect of dornase alfa (Daviskas et al. 2010a). Dornase alfa reduces the viscosity of sputum by depolymerizing DNA filaments and reduces surface tension by increasing the surface-active phospholipids (Griese et al. 1997; Zahm et al. 1995, 1998). In contrast, mannitol decreases the surface tension by increasing the hydration at the airway surface. The additive effect of mannitol to dornase alfa in CF is evident in phase III clinical studies showing an increase in FEV_1 in those treated with both mannitol and dornase alfa (Aitken et al. 2012; Bilton et al. 2011).

The presence of mannitol at the airway surface not only creates a drive for water thus increasing the ASL but sustains this increase in water. This is supported by the sustained reduction in the percentage of solids in the CF sputum (Daviskas et al. 2010a) and the ease in expectorating sputum in CF and non-CF bronchiectasis (Bilton et al. 2011, 2013b). In addition, recent data in vitro demonstrated that the hyperabsorption of liquid, characteristic in CF, is reduced in the presence of mannitol suggesting that it inhibits the ENaC activity (Corcoran et al. 2013).

A clinical trial measuring the effect of mannitol and hypertonic saline on the liquid absorption in the airways of patients with cystic fibrosis is in progress (http://clinicaltrials.gov/ct2/show/NCT01887197).

Evidence of Clinical Benefits with Dry Powder Mannitol

Although low doses of mannitol were effective in improving mucus clearance in asthmatic and bronchiectasis subjects, a dose-ranging study of 40–400 mg of mannitol twice daily for 2 weeks in CF subjects showed 400 mg produced the largest improvement in lung function (Teper et al. 2011). Subsequently, the dose of 400 mg mannitol twice daily was used in phase III clinical trials. Unlike HS, mannitol has been through the regulatory process and the dose of 400 mg has been approved in Australia and Europe for treatment of CF (Bronchitol™ Pharmaxis Ltd. Frenchs Forest, Sydney, Australia).

An improvement in FEV_1 with mannitol treatment has been demonstrated in subjects with CF. This improvement has been a consistent and persistent finding after short-term (2 weeks) or long-term (26–52 weeks) treatment and irrespective of concomitant treatment with dornase alfa (Aitken et al. 2012; Bilton et al. 2011; Jaques et al. 2008). An improvement in FEV_1 of 92.9 mL ($p < 0.001$) and 3.60 % predicted ($p = 0.008$) was reported in subjects receiving mannitol compared to control that was obvious in the first 6 weeks and was sustained over 26 weeks in the first phase III clinical trial ($n = 295$, in Australasia, Ireland, and the UK) (Bilton et al. 2011). In the second phase III clinical trial ($n = 305$, in North and South America and Europe) (Aitken et al. 2012) while the absolute improvement from baseline (54.1 mL) in the mannitol subjects compared to control did not reach statistical significance ($p = 0.059$), the relative change in % predicted FEV_1 was 3.59 % ($p = 0.029$). Both studies reported results in subjects 6 years and over; however, in the Bilton et al. study, approximately two thirds were $\geq$18 years, while in the Aitken study, only half were $\geq$18 years. When the results of the two

randomized placebo-controlled double-blind phase III studies were pooled and analyzed together, a significant improvement in the % predicted FEV_1, both in absolute (73 mL) and relative (3.6 %) change, was reported (Fig. 7.3a; Bilton et al. 2013a). However, when the effects were analyzed by age group, improvements in FEV_1 % predicted compared to control were evident in all groups but statistically significant only in the adult CF population (>17 years) (Fig. 7.3b; Bilton et al. 2013a). However, the significant absolute change in FEV1 % predicted from baseline favors mannitol over control in adults (2.55 %, $p = 0.002$) and children (3.14 %, $p = 0.03$), but not in adolescents (0.60 %, $p = 0.615$). Significant improvements in FVC with mannitol compared to control were also reported in both clinical trials (Aitken et al. 2012; Bilton et al. 2011). Pooled analysis by age group showed that the largest improvement in FVC (6 %) was in children (Aitken et al. 2012). Improvement in lung function in the control group (mannitol 50 mg twice daily versus 400 mg twice daily in the mannitol group) was also observed, which may have affected the difference in the primary end point between groups. More positive results in children are in the process of being published following completion of a clinical trial to determine the efficacy of mannitol to improve lung function in subjects aged 6–17 years (http://clinicaltrials.gov/ct2/show/NCT1883531) (Pharmaxis Ltd.).

Despite the substantial improvement in FEV_1, mannitol did not improve the quality of life of people with CF (Bilton et al. 2011; Aitken et al. 2012). Mannitol compared to control did not show significant improvement in quality of life in any of the eight domains of the Cystic Fibrosis Questionnaire-R in either of the long-term trials in people with CF.

Mannitol treatment over 26 weeks compared to control significantly reduced the incidence of exacerbations by 35 % and increased the time to the first exacerbation in the CF subjects in the Bilton et al. (2011) study. These results did not achieve statistical significance in the Aitken et al. study (2012). Further, there was no significant reduction in the rate of exacerbations with mannitol treatment in either of these clinical trials (Bilton et al. 2011; Aitken et al. 2012). As the studies were not adequately powered to show an effect on the exacerbation rate, more data would be required for firm conclusions.

Osmotic agents could potentially provoke airway narrowing in persons with hyperresponsive airways (Brannan et al. 2005). Prior to initiation of therapy, all patients have an assessment with one dose to identify and exclude subjects with airway hyperresponsiveness. Bronchospasm following inhalation of mannitol or HS however is usually mild and short lived and responds well to treatment with a bronchodilator (Briffa et al. 2011; Rodwell and Anderson 1996). The percentage of CF subjects who had significant airway narrowing in response to the mannitol dose [mannitol tolerance test (MTT)] and were excluded in participating in the trials was 7 and 4 % in the Bilton et al. (2011) and Aitken et al. (2012) trial, respectively. In addition, very few patients reported bronchospasm during the mannitol treatment period (Aitken et al. 2012; Bilton et al. 2011; Jaques et al. 2008).

Further, osmotic stimuli usually provoke cough after inhalation as hyperosmolarity stimulates the sensory nerves (Jia and Lee 2007). However, coughing

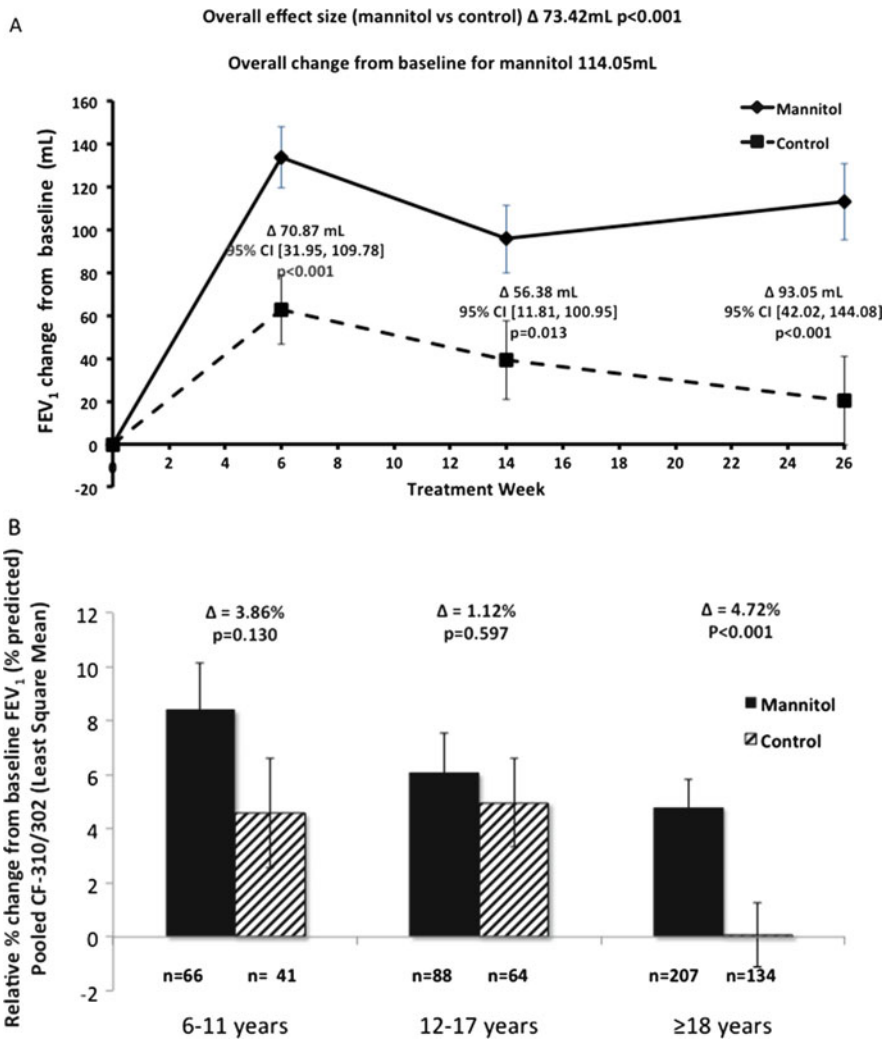

Fig. 7.3 Pooled data from CF301 and CF302 trials. (**a**) Showing mean absolute change from baseline in FEV_1 in the mannitol (400 mg) (*solid line*) and control (50 mg) (*broken line*) treatment group. (**b**) Showing the percentage change in % predicted FEV_1 by age in the mannitol (*black bar*) and control (*striped bar*) group (Bilton et al. 2013a)

has been tolerated well by most patients in all studies. Patients with excessive secretions rely on cough to clear secretions but cough is not effective when mucus is dehydrated and adheres to the airway surface. Therefore, effective coughing, following inhalation of an osmotic stimulus, should be regarded as beneficial to patients as it promotes the clearance of mucus.

Inhaled mannitol has an acceptable safety profile, and it is well tolerated by most subjects as reported in the phase III clinical trials (Aitken et al. 2012; Bilton

et al. 2011). In the Bilton et al. study (2011), a total of 15.8 % (mannitol group) versus 8.5 % (control group) withdrew from the study due to adverse events (AEs); of these 13.6 and 5.1 % were AEs related to the treatment, respectively. In the Aitken et al. study (2012), the proportion of subjects withdrawn from the study due to treatment-related AEs was 6.7 and 1.7 % in the mannitol and control group, respectively. The most common AEs leading to withdrawal were cough, hemoptysis, condition aggravated, and pharyngolaryngeal pain (Aitken et al. 2012; Bilton et al. 2011). Withdrawals from the trials due to subject decision were similar in the mannitol and control groups. An 80 % adherence was observed, based on returned medication and empty blister packages, suggesting that the use of ten capsules per dose was acceptable.

Notably, there was no quantitative or qualitative change from baseline in the sputum pathogens, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, in both groups (Aitken et al. 2012; Bilton et al. 2011). More importantly, fewer patients receiving mannitol treatment had infective exacerbations requiring antibiotics compared to control. Mannitol could potentially be a substrate to bacteria; however, it is evident from the clinical trials that mannitol does not promote the growth of bacteria in vivo (Aitken et al. 2012; Bilton et al. 2011). In addition, recent data suggested that it could improve the effectiveness of antibiotics in the eradication of gram-positive bacterial persisters and biofilms (Allison et al. 2011; Barraud et al. 2013).

As mannitol is inhaled from a disposable, capsule-based, dry powder inhaler, it has the potential to be more convenient than HS, which requires nebulization and cleaning of the nebulizer. The clinical data to date supports chronic dosing of osmotic agents to get the best effect and a dose response curve; higher doses of both mannitol and HS have been proven to be more effective than lower doses. It therefore follows that careful consultation with the patient to select a form of osmotic therapy that they can tolerate at optimum doses and frequencies is of utmost importance. There has been no large randomized control study comparing mannitol with HS, and all studies have excluded enrollment if participants were currently taking the other osmotic agent. The only comparison between mannitol and HS was an acute study showing that both osmotic agents improved MCC to a similar extent (Robinson et al. 1999). One has to keep in mind that improved clearance of mucus in response to the osmotic stimuli contributes significantly toward breaking the vicious cycle of the disease thus leading to clinical benefits.

7.3.2 Ion Transport Modifiers

7.3.2.1 Sodium Channel Blockers

In CF, the epithelial sodium channel (ENaC) is dysregulated due to the mutations of the CFTR gene causing hyperabsorption of sodium that leads to hyperabsorption of water from the airway surface. The mechanism of interaction between CFTR and

ENaC is still uncertain. However, as dehydration of the airway surface has been realized to be the initiating event in CF airway disease, treatments that increase the airway surface liquid have been developed, including the ENaC blockers. ENaC is sensitive to the sodium channel blocker and K^+-sparing agent, amiloride, which is now often used as a diuretic particularly in refractory hypertension, because of its effect on sodium transport in the kidney.

Amiloride has been investigated as an inhaled sodium channel blocker; however, there is no convincing evidence to support its use in CF. In a recent meta-analysis of four randomized controlled trials comparing amiloride to placebo, no significant improvement in lung function was demonstrated. Instead, the results were in favor of the placebo (Burrows et al. 2012). These negative results were thought to be related to rapid clearance and low potency of the study drug. A 2-week clinical trial applying amiloride pretreatment to HS aiming to prolong the retention of HS and increase its effect also showed no benefit to lung function or MCC (Donaldson et al. 2006). Amiloride blocked the increase in lung function and MCC in response to HS (Donaldson et al. 2006). It has been suggested that amiloride not only blocks the ENaC, but it blocks the water transport by blocking the aquaporin 5, a water channel in the apical membrane (Tarran et al. 2007). Several amiloride derivatives, more potent than amiloride that do not block aquaporins, e.g., P-552-02 (Parion Sciences) and GS-9411 (Parion Sciences and Gilead Sciences), were investigated. Preliminary data showed promising results in decreasing liquid absorption, an effect that potentiated when combined with HS (Hirsh et al. 2008, 2009). However, further studies showed they were not clinically suitable (Clunes and Boucher 2008; O'Riordan et al. 2014). The ENaC inhibitor P-1037/VX-371 (Parion Sciences/ Vertex Pharmaceuticals) is currently under development. A phase II clinical trial (CLEAN-CF) is currently investigating the effect of P-1037 in combination with HS in patients with cystic fibrosis (http://clinicaltrials.gov/ct2/show/NCT2343445).

7.3.2.2 P2Y2 Receptor Agonists

Correctors of underlying ion transport defects given by nebulization not only have the capacity to inhibit sodium absorption but can also stimulate chloride secretion, increase ciliary beat frequency, stimulate surfactant production, and enhance mucin secretion, independent of CFTR (Morse et al. 2001; Kim et al. 1996; Devor and Pilewski 1999; Knowles et al. 1991; Gobran et al. 1994). Purinergic receptors are plasma membrane molecules expressed on the surface of airway epithelium. Denufosol is a P2Y2 receptor agonist. P2Y2 receptors are a subtype of the purinoceptors. Denufosol stimulates chloride secretion via calcium activated chloride channels, inhibits sodium reabsorption via epithelial Na^+ channels (ENaC), and stimulates ciliary beat frequency (Kellerman et al. 2008). While phase II trials appeared promising (Deterding et al. 2005, 2007), 24- to 48-week phase III studies did not demonstrate either a significant improvement in lung function or a reduction in pulmonary exacerbations (Ratjen et al. 2012; Accurso et al. 2011). This failure was postulated to be related to the short half-life of the drug in vivo, even though it

has greater metabolic stability than other P2Y2 receptor agonists. Chest tightness was also a common side effect among adults and children in these trials.

7.4 Conclusion

Reduced hydration of the ASL in the CF airway initiates the events that lead to chronic infection and inflammation, with progressive lung damage and impairment of respiratory function. Recent work by Button et al. (2012) proposed a gel-on-brush model which explains the two distinct layers of the ASL (PCL and mucus) and how water is distributed between them so that PCL is restored first to its functional height and any excess water is then taken up into the mucus layer. This indicates that agents that augment the hydration of the ASL have the potential to restore the mucociliary mechanism in the CF airway. Osmotic agents have been shown to work in this way, although they may have other modes of action such as changing the physical properties of mucus and disrupting bacterial biofilms. Hypertonic saline significantly improves lung function and substantially reduces respiratory exacerbations, with consequent sustained improvements in several domains of quality of life. Dry powder mannitol is another inhaled osmotic agent with clinical benefits that has the advantage of being conveniently delivered. Mannitol also significantly improves lung function and reduces the incidence of exacerbations, although it does not improve quality of life. ASL can also be augmented by ion channel modifiers in benchtop models. Although older compounds in this class of medications have not shown any benefit in clinical trials, newer compounds are under development and further clinical trials are in progress.

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Chapter 8
Anti-Inflammatory Therapies for Cystic Fibrosis

Elliott C. Dasenbrook and James F. Chmiel

Abstract Cystic fibrosis (CF) lung disease is characterized by airway obstruction, chronic bacterial infection, and a self-propagating inflammatory response. These three factors are interrelated and feed into each other resulting in structural damage to the airway wall architecture, loss of lung function, and ultimately the death of the patient. Current maintenance regimens targeted toward treating the lung disease typically include airway clearance measures to relieve the airway obstruction and inhaled antibiotics to treat the chronic bacterial infection. Few patients receive chronic anti-inflammatory therapy. The first studies of anti-inflammatory drugs for CF began approximately 30 years ago with the first study of oral corticosteroids. Unfortunately, chronic systemic corticosteroid use was associated with significant side effects and therefore cannot be recommended for long-term use in treating CF airway inflammation. High-dose ibuprofen is the only anti-inflammatory drug currently recommended for the treatment of CF airway inflammation. Unfortunately, it has never been widely adopted primarily due to the requirement to obtain pharmacokinetic studies prior to initiating therapy and concerns over potential side effects. Clearly, new anti-inflammatory drugs are desperately needed in CF. Because massive neutrophil influx is the hallmark of airway inflammation in CF, any new anti-inflammatory drug must either directly or indirectly address the neutrophil and its damaging inflammatory products. In this chapter, the history of anti-inflammatory drug development in CF will be reviewed first, and then an update on current studies of anti-inflammatory drugs in the CF pipeline will be provided.

Keywords Cystic Fibrosis • Anti-inflammatory • Ibuprofen • Corticosteroids • Inflammation

Anti-inflammatory therapeutics are key to maintaining lung health in patients with cystic fibrosis (CF). Systemic inflammation is a consequence of the dysfunctional

E.C. Dasenbrook, M.D., M.H.S. (✉) • J.F. Chmiel, M.D., M.P.H.
Department of Pediatrics, Case Western Reserve University School of Medicine, Rainbow Babies and Children's Hospital, Cleveland, OH 44106, USA
e-mail: elliott.dasenbrook@case.edu; james.chmiel@uhhospitals.org

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cystic fibrosis transmembrane conductance regulator (CFTR) protein (Davis et al. 1996). The primary source of morbidity and mortality in CF is progressive loss of lung function and destruction of the airways (O'Sullivan and Freedman 2009). Clinically, this is manifest as increased pulmonary exacerbations, decreased lung function, and reduced quality of life. Current treatment regimens address multiple aspects of the pathophysiologic cascade of CF lung disease with a multitude of medications, but this has saddled patients with a tremendous treatment burden. As drugs become available that target the basic defect in CF, there is hope that this will eliminate the downstream consequences of abnormal CFTR function and reduce treatment burden. However given the unrelenting inflammatory response in CF, it is possible that some amount of inflammation may persist which will continue to damage airway wall architecture. Therefore the development of new anti-inflammatory drugs is desperately needed. While many medications, such as antibiotics or those that treat the basic defect, may modulate inflammation, this review will focus on currently available anti-inflammatory CF therapies and the recommendations of the CF Foundation Pulmonary Clinical Practice Guidelines Committee as well as discuss anti-inflammatory therapeutics under development.

8.1 High-Dose Ibuprofen

The CF Pulmonary Guidelines Committee recommends the chronic use of oral ibuprofen, at a peak plasma concentration of 50–100 mg/ml, to slow the loss of lung function for CF patients between 6 and 17 years of age, with a forced expiratory volume in one second (FEV_1) >60 % predicted (Mogayzel et al. 2013). For patients 18 years of age and older, the evidence is not sufficient to recommend for or against the use of chronic ibuprofen to slow loss of lung function or prevent exacerbations. In addition, a Cochrane review also concluded that high-dose ibuprofen can slow the progression of lung disease in people with CF, especially in children, thus suggesting that strategies to modulate lung inflammation can be beneficial for people with CF (Lands and Stanojevic 2007).

Nonsteroidal anti-inflammatory drugs (NSAIDs) in CF are thought to be associated with a reduction in neutrophil migration into the lung when a dose is given that results in a peak plasma concentration >50 μg/ml (Konstan et al. 2003). A landmark study published in 1995 found that high-dose ibuprofen was associated with a slowing in the progression of lung disease (Konstan et al. 1995). The study design was a single-center, double-blind, randomized, placebo-controlled trial that enrolled 85 subjects with CF who were followed for 4 years. Patients aged 5–39 years at enrollment had a FEV_1 of 60 % predicted or better. Importantly, the dose of ibuprofen was titrated to result in peak plasma concentrations of 50–100 μg/ml. Patients in the treatment arm had an annual rate of change in FEV_1 % predicted of approximately 2 % per year, while those in the placebo arm had an annual rate of change in FEV_1% predicted of approximately 3.5 % per year. In addition, weight was superior in the ibuprofen group compared to the placebo group. High-dose

ibuprofen was not associated with a significant increase in adverse events. A 2-year Canadian safety and effectiveness trial of high-dose ibuprofen was undertaken in young CF patients with mild lung disease and found high-dose ibuprofen to be well tolerated and associated with slowing of lung function decline (Lands et al. 2007).

To evaluate the potential benefit of high-dose ibuprofen in a real-world setting, an analysis of the US Cystic Fibrosis Foundation Patient Registry (CFFPR) was undertaken (Konstan et al. 2007). The authors studied the subgroup of patients aged 6–17 years with an FEV_1 % predicted >60 % to evaluate differences in lung function decline and adverse events compared to patients of similar age and disease severity who did not receive high-dose ibuprofen. The authors found that patients receiving ibuprofen had an annual rate of change in FEV_1 % predicted of approximately 1.4 % per year, while those in the placebo arm had an annual rate of change in FEV_1% predicted of approximately 2 % per year. The overall risk of GI bleeding was double in the ibuprofen group, but the overall risk in patients not treated with ibuprofen was approximately 1 %. The study evaluated the years 1996–2002. During this period there was most likely more aggressive care of CF patients given newer therapeutics than what was available during the original randomized controlled trial, yet the benefits of ibuprofen were still seen. These results suggest treatment with ibuprofen should be considered for CF patients.

Despite multiple studies suggesting efficacy and safety of ibuprofen as an anti-inflammatory therapeutic in CF, the overall use of ibuprofen in the USA is low (Cystic Fibrosis Foundation, Patient Registry 2014 Annual Data Report 2015). Administering subtherapeutic doses not only may expose patients to side effects but may also worsen pulmonary inflammation by increasing neutrophil influx to the site of infection (Konstan et al. 2003). Therefore, one reason for the low acceptance of ibuprofen as a maintenance therapy in CF is the requirement for frequent pharmacokinetic monitoring to establish the correct dose. In addition, caregivers are concerned about the gastrointestinal and renal side effects of high-dose ibuprofen (Aitken et al. 2012; Bell et al. 1999; Kovesi et al. 1998). The clinical trials and observational studies do not suggest side effects that would change the risk/benefit ratio away from high-dose ibuprofen (Konstan et al. 1995, 2007; Lands et al. 2007). To minimize renal toxicity, consideration should be given to holding high-dose ibuprofen when patients are receiving systemic aminoglycoside therapy or are having symptoms related to dehydration. To minimize gastrointestinal bleeding complications, strong consideration for all patients receiving high-dose ibuprofen therapy should be given to concomitantly prescribing proton pump inhibitors, misoprostol, or histamine antagonists. Finally, many practitioners stop or will not initiate ibuprofen therapy in patients 18 years of age and older with CF. There is no biologic reason why a patient would suddenly stop responding to therapy after their 18th birthday. It may be that some patients would not benefit from ibuprofen therapy, but there is no way currently to make this determination. The lack of a demonstrated statistically significant response in the adult patients in the original trial may be due to study design. The original ibuprofen study was conducted approximately 25 years ago at a time when there were few drugs available for maintaining lung health. Inflammation was likely not a major contributor to the

minimal lung disease in the CF adults who were enrolled in this study. Consideration should be given to continuing high-dose ibuprofen therapy in patients after the age of 18 years as long as they are not experiencing side effects. Furthermore, initiation of ibuprofen in adult CF patients, especially those with significant arthritis or nasal polyps, can be considered if the patient has early-stage CF lung disease.

8.2 Corticosteroids

The CF Foundation recommends against the chronic use of oral corticosteroids to improve lung function and quality of life or reduce exacerbations for individuals with CF who do not have an asthma phenotype or allergic bronchopulmonary aspergillosis (ABPA) (Mogayzel et al. 2013). Similar to ibuprofen, oral corticosteroids have been shown to improve important outcomes; however, in contrast to ibuprofen, the side effect associated with oral corticosteroids outweighs the benefits.

Corticosteroids were the first anti-inflammatory drugs studied in CF. Oral corticosteroids have many beneficial anti-inflammatory properties including reducing the formation of mucus and edema and inhibiting chemotaxis, adhesion, and activation of leukocytes. Multiple randomized controlled trials have evaluated oral corticosteroids in CF. The first was a single-center, double-blind, randomized, placebo-controlled trial that enrolled 45 patients that were followed for 4 years (Auerbach et al. 1985). Patients aged 1–12 years had mild-to-moderate lung disease. Patients received 2 mg/kg of prednisone every other day. At the conclusion of the study, patients receiving prednisone had significantly less hospitalizations and higher FEV_1 % predicted compared to patients who received placebo. In this study the authors tracked glucose abnormalities, cataracts, and growth abnormalities and found no significant side effects. In a subsequent study, Eigen and colleagues conducted a multicenter (15 sites in North America), randomized, placebo-controlled, double-blind trial comparing every other day the administration of 1 mg/kg prednisone, 2 mg/kg prednisone, or placebo (Eigen et al. 1995). The authors reported that patients in the 1 mg/kg group had improvements in lung function over the 4 years of study, but this occurred primarily in patients infected with *Pseudomonas aeruginosa*. Furthermore, in contrast to the Auerbach study, significant complications from oral corticosteroids were noted. Significantly lower heights and excess glucose metabolism abnormalities were noted. A follow-up study demonstrated that these side effects persisted 7 years later (Lai et al. 2000). These results in combination with concerns about the long-term impact of corticosteroids on bone health significantly dampened the enthusiasm for prolonged courses of oral corticosteroids outside of asthma or ABPA.

Due to their familiarity with them, many CF care providers have prescribed inhaled corticosteroids (ICS) for the chronic treatment of CF airway inflammation. ICS have the advantage of being delivered directly to the lung while minimizing systemic exposure, thereby minimizing many of the side effects of oral

corticosteroids. Given the high rates of use of ICS in the UK and the USA and previous observational studies suggesting potential benefit (De Boeck et al. 2011; Ren et al. 2008), Balfour-Lynn and colleagues performed a multicenter randomized controlled trial of withdrawal of ICS in CF (Balfour-Lynn et al. 2006). The study showed that after stopping ICS therapy, there was no change in pulmonary exacerbations or lung function. Of note, patients with an asthmatic component to their CF were not enrolled in the study. A Cochrane review identified 13 trials that used ICS in 506 CF patients between the ages of 6 and 55 years (Balfour-Lynn and Welch 2012). The review concluded that it was not possible to establish benefit and ICS may impair growth. Therefore, just as with oral corticosteroids, the use of chronic ICS as an anti-inflammatory therapy in CF to improve lung function or reduce exacerbations for individuals with CF, who do not have an asthma phenotype or ABPA, is not recommended by the CF Pulmonary Guidelines Committee (Mogayzel et al. 2013).

8.3 Azithromycin

The CF Foundation recommends the chronic use of azithromycin to improve lung function and reduce exacerbations in CF patients aged 6 years or older with persistent detection of *P. aeruginosa* in the airways. The guidelines state that in similar patients without persistent *P. aeruginosa*, chronic use of azithromycin should be considered (Mogayzel et al. 2013).

Azithromycin is a macrolide antibiotic that is thought to have pleomorphic properties suggesting that it may also act as an anti-inflammatory therapeutic. Ratjen and colleagues examined systemic inflammatory markers in a double-blind, randomized controlled trial of oral azithromycin in patients aged 6–18 years who did not have *P. aeruginosa* detected in their sputum (Ratjen et al. 2012). The group treated with azithromycin had significant reductions in neutrophil counts and serum inflammatory markers within 28 days of treatment. In addition, two major clinical studies have been performed looking at important clinical outcomes in patients chronically infected with *P. aeruginosa* (Saiman et al. 2003) and those without *P. aeruginosa* (Saiman et al. 2010). The strongest evidence for efficacy of azithromycin is in patients chronically infected with *P. aeruginosa*. In a multicenter, double-blind, randomized controlled trial, subjects treated with chronic azithromycin had significant decreases in pulmonary exacerbations and improvements in lung function. A follow-up in patients uninfected with *P. aeruginosa* was performed in 40 CF centers in the USA and Canada (Saiman et al. 2010). There was no change in lung function in this study population with mild lung disease; however there was a clinically and statistically significant 50 % reduction in pulmonary exacerbations. Compared to other anti-inflammatory therapies, azithromycin has an excellent side effect profile and is generally well tolerated.

8.4 Other Anti-Inflammatory Drugs: Leukotriene Modifiers, Oral *N*-Acetylcysteine, and Inhaled Glutathione

The CF Foundation has determined that there is not enough evidence to recommend for or against leukotriene modifiers, oral *N*-acetylcysteine, or inhaled glutathione in patients with CF (Mogayzel et al. 2013).

Leukotriene modifiers exhibit anti-inflammatory effects through modification of cysteinyl leukotrienes. Two small studies have suggested that leukotriene modifiers are associated with decreased sputum and serum inflammatory markers and a trend toward improvement in respiratory outcomes (Conway et al. 2003; Stelmach et al. 2005). However, larger studies powered to examine important clinical outcomes are still needed before they could be recommended for routine clinical use. Chronic glutathione (GSH) deficiency in the CF lung may contribute to excess inflammation. Therefore, repleting GSH in CF patients may be a therapeutic option. *N*-acetylcysteine (NAC) has generated interest as an anti-inflammatory medication since it increases glutathione levels. In a phase I study of 18 CF patients, high doses of oral NAC were safe and significantly decreased sputum elastase activity (Tirouvanziam et al. 2006). These investigators published a follow-up phase II multicenter, double-blind, randomized, placebo-controlled study of oral *N*-acetylcysteine and reported that NAC recipients maintained their lung function, while the placebo group had a decrease in their lung function (Conrad et al. 2014). Dauletbaev and colleagues performed a single-center, double-blind, randomized, placebo-controlled phase II study of low-dose compared to high-dose NAC and reported that NAC was well tolerated and increased extracellular GSH in induced sputum (Dauletbaev et al. 2009). In addition to oral preparations to replete GSH, it is possible to inhale glutathione. Based on success in case reports and a phase I study of inhaled glutathione (Bishop et al. 2005; Visca et al. 2008), an investigator-initiated study of inhaled glutathione was undertaken. The investigators found that 6 months of inhaled glutathione resulted in increased GSH levels in the sputum, but was not associated with any improvements in clinical outcomes or surrogate markers of inflammation. It was noted that the study population was already receiving intense treatment regimens, and clinical improvements may have been difficult to detect when inhaled GSH is given as an "add-on" therapy. However due to the lack of sufficient evidence, the chronic use of inhaled GSH as an anti-inflammatory drug cannot be recommended at this time.

8.5 Anti-Inflammatory Drugs Under Development

In addition to the above therapies, there are many anti-inflammatory medications in the early stages of development for CF. KB001 is a PEGylated, recombinant, anti-*Pseudomonas*-PcrV antibody FAB fragment that inhibits the function of the

P. aeruginosa type III secretion system (TTSS) (Milla et al. 2014). The TTSS results in increased inflammation through modulation of NFkB, and its inhibition has been shown to be protective in animal models of acute *P. aeruginosa* infection and to reduce inflammation (Epelman et al. 2000; Frank et al. 2002; Imamura et al. 2007; Sawa et al. 1999). Based on the promising preclinical data, a double-blind, randomized, placebo-controlled, single-dose, dose-escalation study of KB001 in CF patients chronically infected with *P. aeruginosa* was undertaken at ten US sites (Milla et al. 2014). Following a one-hour intravenous infusion, KB001 was found to be safe and resulted in a 0.5 log reduction of free neutrophil elastase, which is a similar reduction seen with the administration of intravenous antipseudomonal antibiotics. The next step is to evaluate if repeated doses of KB001-A can maintain an anti-inflammatory impact as well as improve patient reported outcomes in patients chronically infected with *P. aeruginosa*. The results from this clinical trial were presented in 2015. KB001-A was safe and well tolerated, but had no timely effect as treatment of pulmonary exacerbations. KB001-A was associated with a trend toward improved FEV_1 and a decrease in pro-inflammatory markers measured in sputum. IL-8 had a statistically significant decrease after a 16-week treatment period ($p < 0.0484$) (Chmiel et al. 2015). This phase II study suggests that reducing the stimulus for the inflammatory response may have positive downstream effects in CF.

Alpha-1-antitrypsin (AAT) for inhalation is another anti-inflammatory medication in the CF pipeline. In the airways of CF patients, AAT defenses are overwhelmed by the massive quantities of free elastase present in the airway (Birrer et al. 1994). Thus, augmenting AAT via inhalation is a potential therapeutic strategy in CF. Administration of AAT for treatment of CF lung disease has been under consideration for over 20 years. However, difficulties in producing the drug in large enough quantities to completely bind all of the free elastase present in the CF airway have limited its usefulness in CF. Fortunately, many of these production problems have been overcome, and there is renewed interest in AAT therapy in CF. Recently, investigators performed a multicenter, open-label randomized trial at eight sites in Germany (Griese et al. 2007). After 4 weeks of AAT inhalation, there was a significant reduction in neutrophils, pro-inflammatory cytokines, and levels of elastase activity. This preliminary study did not include a placebo group, and future studies with a placebo group to evaluate the impact on clinical outcomes will be critical. A recently completed phase IIa clinical trial of purified A1AT delivered by inhalation to subjects with CF found no significant concerns with safety or tolerability. This study also demonstrated delivery of high concentrations of the drug to the CF airway using a modern, ultrahigh efficiency nebulizer (Gaggar et al. 2015). Based upon the results of these studies, A1AT is ready to be advanced to larger safety and efficacy clinical trials.

In addition to novel therapeutics, there is also an interest in developing CF medications already being used in other diseases. Sildenafil is a phosphodiesterase-5 inhibitor that possesses anti-inflammatory functions. In addition, sildenafil has been shown to potentiate CFTR activity and facilitate CFTR placement into the apical membrane of epithelial cells (Leier et al. 2012). Thus, sildenafil has both

potentiator and corrector properties, which would be a desirable combination in CF patients with two copies of F508del. There is concern that the doses of sildenafil required to achieve clinical improvements would result in significant side effects. A phase II, single-center study is currently underway to test the safety and efficacy of sildenafil in CF (Taylor-Cousar 2008).

Tumor necrosis factor-alpha (TNF-α) is present in high concentrations in the CF lung and plays a role in propagating the inflammatory response by increasing neutrophilic inflammation as well as facilitating chemotaxis of additional neutrophils (Gibson et al. 2003). Etanercept is a biologic agent that binds TNF-α and blocks its interaction with cell surface receptors, thus subsequently decreasing inflammation. Etanercept is already being used to treat autoimmune diseases like rheumatoid arthritis and Crohn's disease, and there is interest in using it to treat CF lung disease. However, there are concerns that etanercept may be too potent in CF. Given the key role TNF-α plays in controlling the host's response to infection, inhibiting TNF-α may result in infectious complications including increased pulmonary exacerbations as was seen in a study of an inhibitor of LTB_4 (Konstan et al. 2014). However, case reports demonstrating improvements in lung function without infectious complications in CF patients treated with etanercept for rheumatoid arthritis or Crohn's disease suggest that etanercept may have some utility in CF (Visser et al. 2012). Obviously, controlled trials with close monitoring of potential infectious complications must be performed to further delineate the risks and benefits of etanercept.

Omega-3 fatty acids may be an effective treatment in CF. Fatty acids are located on cellular membrane phospholipids and help curtail inflammation (Oliver and Watson 2013). CF patients have abnormalities in the concentrations of fatty acids which may contribute to the excess inflammation in CF. Since absorption of fatty acid may not be ideal in CF, treatment with supplemental omega-3 fatty acids, which can only be found in the diet, may be beneficial. A recent update to a Cochrane review includes four small studies that suggested that there may be some benefit from omega-3 supplementation (Oliver and Watson 2013). However, the authors concluded that there is limited evidence to support changes to clinical practice and that larger, long-term, randomized controlled trials are needed to evaluate the optimal dose and potential efficacy of omega-3 fatty acids. In addition, there is concern whether omega-3 fatty acids can be given in high enough doses in patients with pancreatic insufficiency to maintain a therapeutic level sufficient enough to control lung inflammation.

Thiazolidinediones activate the peroxisome proliferator-activated receptors (PPARs) and are used as a treatment for type II diabetes because they increase insulin sensitivity. Studies suggest that CF tissues may be deficient in PPAR which leads to exuberant inflammation (Ollero et al. 2004; Perez et al. 2008). A reversible defect in PPAR signaling in CFTR-deficient cells has been corrected with rosiglitazone with improvements in the severity of CF disease in mice (Harmon et al. 2010). A 28-day clinical trial of pioglitazone did not demonstrate a beneficial effect on sputum inflammatory mediators but the dose of pioglitazone may not have been adequate or the study was too short in duration to detect an improvement

(Konstan et al. 2009). Further evaluation of this class of medications in CF is merited.

Many other anti-inflammatory drugs have been studied in CF. Interferon-γ, another cytokine with anti-inflammatory effects used in other non-CF inflammatory diseases, did not improve pulmonary function or change inflammatory mediators in a multicenter clinical trial (Moss et al. 2005). Hydroxychloroquine, a dihydrofolate reductase inhibitor that increases intracellular pH, was evaluated in a small 28-day study in CF and, while well tolerated, did not change inflammatory markers (Williams et al. 2008). Methotrexate increased FEV_1 in five CF patients after 1 year of treatment (Ballmann et al. 2003). However follow-up studies suggest that methotrexate was not well tolerated by CF patients and was associated with an increased need for IV antibiotics (Oermann et al. 2007). Although none of these drugs were approved to treat CF lung disease, valuable lessons were learned from these studies that will be applied to future trials as the search for new anti-inflammatory drugs continues.

To date, most anti-inflammatory drugs that have been considered in CF have targeted the activation arm of the inflammatory response. These drugs aim to downregulate the pro-inflammatory mechanisms of CF airway inflammation. However, a newer approach is to upregulate counter-regulatory pathways and allow the body's own anti-inflammatory pathways to move the inflammatory response back toward equilibrium. Several of these new drugs are being considered for CF. Resunab (JBT-101), an oral drug that triggers the resolution of inflammation by binding to and activating the cannabinoid receptor type 2 on immune cells, is set to begin safety and tolerability studies in CF in 2015. Also, analogs to the anti-inflammatory molecule lipoxin A_4 (LXA_4) are undergoing preclinical evaluation and could be ready to enter the clinic within the next 1–2 years. A clinical trial in CF of CTX-4430, a drug that blocks the conversion of LTA_4 to the neutrophil chemoattractant LTB_4 by inhibiting LTA_4 hydrolase, is currently underway. By blocking LTA_4 hydrolase, this drug would also upregulate counter-regulatory pathways by shunting substrate to LXA_4. It is possible in CF that drugs which augment the termination arm of the inflammatory response may be more effective and result in fewer side effects than drugs that seek to downregulate the activation arm of the inflammatory response.

8.6 Conclusion

CF lung disease begins at birth and continues throughout life. While the pathogenesis of CF lung disease is complex, excessive inflammation is a key contributor to the vicious cycle of airway infection and obstruction that eventually leads to irreversible lung damage. Treatment with anti-inflammatory medications is an important component of a CF patient's regimen for maintaining lung health. Unfortunately, many anti-inflammatory therapeutics are associated with untenable side effects (e.g., oral corticosteroids) despite their benefit or have been shown to be

efficacious, but dosing and monitoring are challenging (e.g., high-dose ibuprofen). Therefore the search for safe, efficacious anti-inflammatory drugs continues. Future anti-inflammatory agents must provide improvement in important patient outcomes while minimizing side effects. It is possible that gene-modifying agents will eliminate the downstream pathophysiologic consequences of defective CFTR. However, it is more likely that gene-modifying agents will reduce the pathophysiologic consequences in the airway, particularly the exaggerated inflammatory response, but not completely eliminate it. Therefore, future treatment regimens for CF lung disease will likely employ the administration of one or more gene-modifying drugs in combination with an anti-inflammatory drug. This will reduce lung damage, preserve pulmonary function, maintain quality of life, and prolong survival until that time when a cure is found.

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Chapter 9
Anti-Infective Therapies in Cystic Fibrosis

Patrick A. Flume and Donald R. VanDevanter

Abstract A major feature of cystic fibrosis lung disease is chronic infection. This is thought to contribute to an exaggerated inflammatory response resulting in chronic cough productive of purulent sputum and progressive loss of lung function. There are also episodic times of worsening of symptoms and acute loss of lung function identified as CF pulmonary exacerbations. A major contributor to improved survival over the last few decades is believed to be the increased use of antibiotics targeting pathogens found in sputum cultures. This includes the use of systemic and inhaled antibiotics against these pathogens to treat pulmonary exacerbations and suppression of chronic infection. Despite recent exciting advances in the treatment of CF lung disease, the treatment of infection with anti-infectives remains an essential part of the standard treatment regimen, and there are additional questions yet to be answered.

Keywords Antibiotics • Bacteria • Cystic fibrosis • Exacerbations • Inhaled • Pseudomonas

9.1 Introduction

As described in an earlier chapter, the lung disease of cystic fibrosis (CF) is manifested as chronic infection, an exaggerated inflammatory response, and obstruction of the airways with retention of inflammatory and infected phlegm. The result is chronic airway symptoms, such as cough productive of purulent sputum, and progressive loss of lung function with early demise as a consequence of respiratory failure. There are also episodic times of worsening of symptoms and acute loss of lung function identified as CF pulmonary exacerbations, typically

P.A. Flume, M.D. (✉)
Departments of Medicine and Pediatrics, Medical University of South Carolina, Charleston, SC, USA
e-mail: flumepa@musc.edu

D.R. VanDevanter, Ph.D.
Department of Pediatrics, Case Western Reserve University School of Medicine, Cleveland, OH, USA

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treated with systemic antibiotics. A major contributor to improved survival over the last few decades is believed to be the increased use of antibiotics targeting pathogens found in sputum cultures. This includes the development and use of better intravenous (IV) and oral antibiotics against these pathogens to treat the pulmonary exacerbations, innovative use of IV antibiotic formulations by aerosol route, and eventual development of antibiotics specifically for inhalation by CF patients. Despite recent exciting advances in the treatment of CF lung disease, the treatment of infection with anti-infectives remains an essential part of the standard treatment regimen, and there are additional questions yet to be answered.

9.2 Airway Infection in Cystic Fibrosis

It has long been recognized that chronic bacterial infection develops in the airways of patients with CF. As can be seen in Fig. 9.1, *Pseudomonas aeruginosa* is the most common bacterial opportunist isolated by selective culture from airway secretions from adult patients, but is also found in a large percentage of patients early in life. We have considerable data that suggest that *P. aeruginosa* is particularly pathogenic, associated with worse symptoms, more pulmonary exacerbations, and earlier death (Amin et al. 2011; Ballmann et al. 1998; Demko et al. 1995; Emerson et al. 2002; Henry et al. 1992; Hoiby et al. 1977; Kosorok et al. 2001; Kozlowska et al. 2008; Mayer-Hamblett et al. 2012; Pamukcu et al. 1995; Parad et al. 1999; Taccetti et al. 2005). It is because of this knowledge that we have taken a greater interest in treating *P. aeruginosa* as the primary pathogen in the last few decades; more recently there is growing interest in the other pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA). Importantly, it has become increasingly clear from culture-independent molecular analyses of microbiota from CF airway secretions (Rogers et al. 2004) that our emphasis on selective culture to identify bacterial pathogens in the lung has been just that: selective. Further, comparisons of culture-dependent and culture-independent surveillance of respiratory secretions suggest that detection of "traditional" CF microbial pathogens by selective culture is highly influenced by relative microbial density (Mahboubi et al. 2015). Our cumulative knowledge of the importance of *P. aeruginosa* in disease progression only highlights how little we know about other bacterial opportunists found in these complex infections (Zhao et al. 2012).

Our current understanding of the development of lung infection by *P. aeruginosa* in the CF airways can be seen in Fig. 9.2. We believe that the CF airways are sterile at birth, or at least the pathogens typical of CF disease are not present, particularly *P. aeruginosa*. If we were to implement the strategy of periodically obtaining a culture of the airways, whether by oropharyngeal swab, sputum, or lower airway samples obtained by bronchoscopy, we would likely find the early cultures repeatedly to be negative. There may come a time, however, when a culture will be positive for *P. aeruginosa*, and we define this observation as first infection, although it is more accurately named first positive culture. In the absence of treatment, repeated subsequent cultures may be intermittently positive for

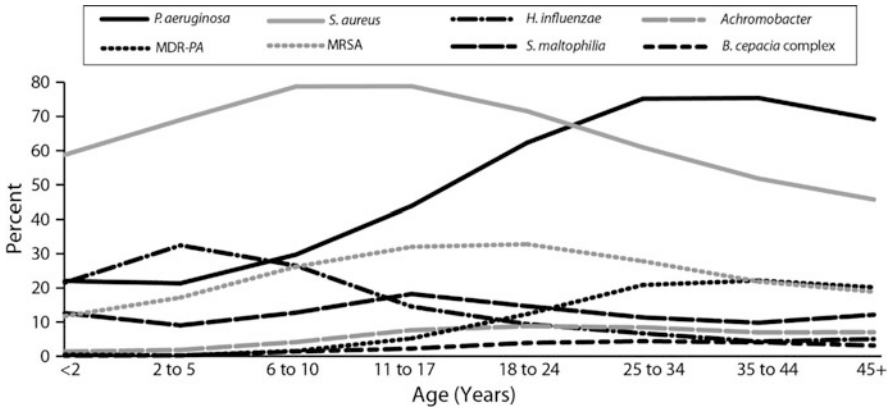

Fig. 9.1 Pathogens identified in a CF population prevalence of bacterial species cultured from respiratory specimens in CF patients (Cystic Fibrosis Foundation Patient Registry 2013)

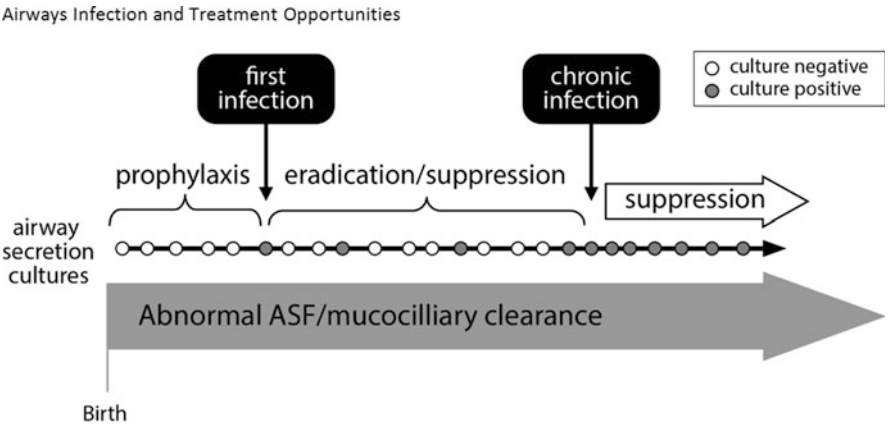

Fig. 9.2 Airway infection and treatment opportunities. This represents the strategy of periodic culture of the airways, monitoring for the presence of bacterial pathogens. The first positive culture is defined as first infection, which is followed by a period when subsequent cultures are intermittently positive. Chronic infection is defined as when >50 % of cultures are positive or when the *P. aeruginosa* is described as mucoid producing (Lee et al. 2003). Antibiotic strategies are demonstrated relative to the definition of infection. Prophylaxis is intended to prevent the acquisition of infection. Eradication is an attempt to clear acquired infection with the intent of delaying the time to chronic infection. Once chronic infection is established, antibiotics are used to suppress the infection

P. aeruginosa (Burns et al. 2001). This could be because some patients are able to clear the infection spontaneously or the amount of infection present is intermittently below the level of detection using standard culture techniques, a possibility supported by a recent comparison of culture-dependent and culture-independent *P. aeruginosa* detection in CF sputum samples (Mahboubi et al. 2015). However, at

some point repeated airway cultures will become mostly, or always, positive for *P. aeruginosa*, and when more than 50 % of cultures are positive, then we have reached the definition of chronic infection (Lee et al. 2003). Classically, we have thought of chronic infection as impossible to eradicate; this led to the hypothesis that earlier infection may be more vulnerable to antibiotic therapy and perhaps could be eradicated before a chronic infection is established. Recent treatment strategies have tested this hypothesis, finding that patients can be converted back to "culture negativity," meaning either eradication of the pathogen or at least reduced in numbers below the level of detection.

9.3 Anti-Infective Treatment Options for *Pseudomonas aeruginosa* in Cystic Fibrosis

The description of *P. aeruginosa* infection over time in patients with CF (Fig. 9.2) is a useful one when considering anti-infective treatment options and expected outcomes. For example, antibiotic use intended to prevent the acquisition of bacterial infection is considered prophylactic; treatment after identification of first infection is performed with the intent of eradication of the infection, while treatment of chronic infection is better described as suppressive therapy, with no, or little, expectation of eradication of the pathogen.

9.3.1 Suppression

Persistent *P. aeruginosa* infection promotes inflammation and progression of injury to the airways and is associated with considerable morbidity and early mortality (Ballmann et al. 1998; Demko et al. 1995; Emerson et al. 2002; Henry et al. 1992; Hoiby et al. 1977; Kosorok et al. 2001; Pamukcu et al. 1995; Parad et al. 1999). The notion of aerosolizing antibiotics as a means of suppressing respiratory infection actually dates back to the 1940s, when there was ad hoc use of penicillins and polymyxins (Geddes 1997). Delivery of antibiotics directly to the airway by inhalation has been rationalized as a method to bypass associated systemic toxicities and also to allow accumulation of extremely high quantities of antibiotic at the site of infection: the lumen of the lung (Di and Andersen 1946). The first randomized trial of inhaled antibiotics in CF patients occurred in the 1980s, when IV preparations of carbenicillin and gentamicin were administered by air-jet nebulizer and compared to nebulized placebo in a crossover study design (Hodson et al. 1981). Patients experienced improved lung function and fewer IV antibiotic courses when on inhaled antibiotics. These observations eventually led to the use of chronic inhaled antibiotics at CF care centers, primarily as extemporaneous IV formulations with empiric dosing regimens (Touw et al. 1995). Further

Table 9.1 Inhaled antipseudomonal antibiotics

Antibiotic class	FDA approved	In development	Used off-label
Aminoglycosides	Tobramycin inhalation solution Tobramycin inhalation powder	Liposomal amikacin	Gentamicin
Beta-lactams	Aztreonam inhalation solution		Ceftazidime
Fluoroquinolones		Levofloxacin aerosol Liposomal ciprofloxacin Ciprofloxacin powder	
Phosphonic acids		Fosfomycin/tobramycin aerosol	
Polymyxins			Colistimethate

investigation of tobramycin pharmacokinetics led to the eventual development of tobramycin inhalation solution (TIS), the first aerosolized antibiotic approved for use in CF patients (http://www.pharma.us.novartis.com/product/pi/pdf/tobi.pdf). The regimen of TIS 300 mg nebulized twice daily for 28 days was shown to increase lung function, and subsequent cycles of therapy (i.e., month on/month off) reduced exacerbations when compared to placebo (Ramsey et al. 1999). Similar results have since been demonstrated with aztreonam inhalation solution (AIS) (McCoy et al. 2008; Retsch-Bogart et al. 2009), and it became the second inhaled antibiotic approved in the USA (http://www.accessdata.fda.gov/drugsatfda_docs/nda/2010/050814s000_cayston_toc.cfm). Both TIS and AIS have since been recommended for use as a chronic medication to maintain lung health independent of severity of lung impairment (Mogayzel et al. 2013). Since that time, a dry-powder formulation of tobramycin (tobramycin inhalation powder, TIP) has been developed and approved which has comparable efficacy to TIS but offers advantages of a shorter inhalation time and portability (VanDevanter and Geller 2011) (Table 9.1). The development and testing of inhalation-specific formulations of known antibiotics address a fundamental problem with the extemporaneous use of IV formulations, in that the latter often contain stabilizing excipients (e.g., EDTA, sulfites) that are considered safe for parenteral administration but that may be unsafe when chronically administered directly to the airway (Campbell and Saiman 1999).

Although the chronic suppression of *P. aeruginosa* with inhaled antibiotics has been shown to improve lung function and reduce exacerbations, there continues to be progressive loss of lung function over time (Moss 2001), and exacerbations among treated patients still occur with some frequency (McCoy et al. 2008; Ramsey et al. 1999). Some have suggested that the intermittent treatment regimen (i.e., month on/month off) is not optimal and allows for regrowth of bacteria and further injury to the airways (Lo et al. 2011), and there is an observed attenuation of the pulmonary function response to inhaled antibiotics with repeated administrations that is not entirely attributable to selection for antibiotic-resistant pathogens (Moss

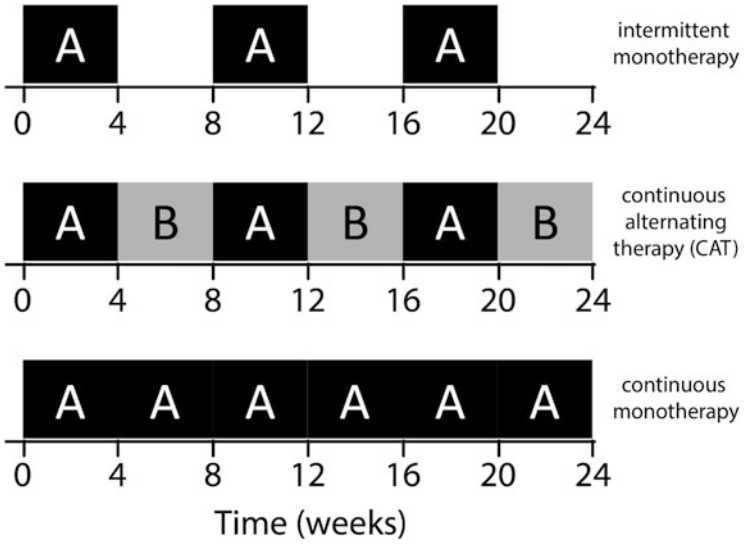

Fig. 9.3 Treatment regimens for chronic suppressive aerosol antibiotics

2001; Ramsey et al. 1999). Problems associated with intermittent treatment could be addressed by the use of continuous treatment (i.e., daily therapy with no time off). This treatment strategy has been used with colistimethate (Hodson et al. 2002; Schuster et al. 2013), whereas other clinicians have chosen to use an antibiotic of a different mechanistic class in a rotational strategy of continuous alternating therapy (CAT) (Dasenbrook et al. 2015; Flume et al. 2015) (Fig. 9.3). Registration of AIS in 2010 was followed by a substantial change in the prevalence of CAT therapy in the USA, with the proportion of patients who received any inhaled antibiotic therapy receiving CAT more than doubling in the population (Dasenbrook et al. 2015). Unfortunately, there are no objective data with which to advocate for a specific approach; enrollment in a randomized, placebo-controlled study comparing intermittent treatment of TIS with a continuous approach alternating TIS with AIS (clinicaltrials.gov NCT01641822) was halted due to an inability to enroll subjects (Flume et al. 2015). Investigators reported that many eligible subjects declined participation because they were already receiving CAT and did not want to be randomized to receive placebo (i.e., intermittent TIS treatment) for the 6-month study (Flume et al. 2015).

The observation of an apparent attenuated response to continued use of an inhaled antibiotic suggests the need for more antibiotic choices. There are currently two approved options in the USA (TIS and AIS), as well as recognized off-label use of IV antibiotic formulations (Table 9.1). Although it is understandable how clinicians begin to use antibiotics and regimens in an off-label manner, it is preferable to have data from controlled trials that demonstrate the efficacy and safety of an aerosolized antibiotics. In addition further development of new drugs is needed.

9.3.2 Eradication

Since chronic *P. aeruginosa* infection is associated with worse symptoms and progressive loss of lung function, it is desirable to try to avoid the development of chronic infection. Initial acquisition of *P. aeruginosa* appears to be largely limited to the upper airways and not widespread throughout the airways (Rosenfeld et al. 1999) and with a lower burden of bacterial pathogens that have not established a biofilm (Rosenfeld et al. 2003). Thus, there is the potential that antibiotic therapy could kill these bacteria leaving no residual infection (i.e., eradication). The evidence to support this theory would be the absence of *P. aeruginosa* in sputum or oropharyngeal swab cultures after treatment with antibiotics, but what can be stated with confidence is that airway cultures are negative for *P. aeruginosa*, which means *either* that the pathogen has been eradicated or reduced in numbers below the level of detection. The support for the former is the demonstration of a different bacterial genotype once infection is found to recur (Munck et al. 2001). The perceived benefit of eradication therapy is delay of chronic infection (Frederiksen et al. 1999), which may, in turn, reduce the inflammatory response and damage done to the lower airways, slowing the progression of disease.

There have been a number of studies of eradication protocols with varying choices of antibiotics, methods of drug delivery, durations of therapy, and number of subjects (Frederiksen et al. 1997; Munck et al. 2001; Ratjen et al. 2010, 2001; Tiddens et al. 2015; Treggiari et al. 2011; Valerius et al. 1991). What is common among them is the result: all of these eradication protocols are generally successful in converting patients' respiratory secretions to *P. aeruginosa* culture negativity. The largest of these were the ELITE (Ratjen et al. 2010) and EPIC (Treggiari et al. 2011) studies, both demonstrating that 1 month of an aerosolized antibiotic (in both cases, tobramycin) alone was sufficient to eradicate the pathogen in about 90 % of patients. The addition of an oral antibiotic (ciprofloxacin) did not improve on the result (Treggiari et al. 2011) nor did a longer course of inhaled antibiotic (Ratjen et al. 2010). The mean time to the next positive culture (e.g., *P. aeruginosa*) was approximately 2 years (Ratjen et al. 2010), and routine scheduled periodic therapy did not yield a different result than a strategy of treating based on positive culture only (Treggiari et al. 2011). A subsequent open-label study of aztreonam for inhalation solution also demonstrated effective eradication (i.e., culture negative) in newly infected pediatric patients (Tiddens et al. 2015).

There are encouraging data to suggest that this strategy does delay the time to chronic infection (Frederiksen et al. 1999), and this has been recommended in CF treatment guidelines (Doring et al. 2012). What is lacking is the evidence that this treatment strategy, by delaying the time to chronic infection, will result in improved lung function and longer survival, but as this approach is still fairly recent in the USA, it will take time for this question to be answered. Interestingly, a recent comparison of health outcomes 5 years after the attempted *P. aeruginosa* eradication among subjects enrolled in the EPIC study failed to identify significant differences in exacerbation rate or lung function decline between subjects who had experienced sustained eradication and those who had not (Mayer-Hamblett

et al. 2015). Although the clinical benefit of delaying chronic *P. aeruginosa* infection is not clear, what is clear is that the early prevalence of chronic *P. aeruginosa* infection in the US CF population is declining (Cystic Fibrosis Foundation Patient Registry 2013), and it is likely that the treatment of first *P. aeruginosa* infection is at least partially responsible for this trend.

9.3.3 Prophylaxis

There are a few small studies that have looked at the use of antibiotics to prevent the acquisition of *P. aeruginosa* infection (i.e., antibiotic prophylaxis) with somewhat conflicting results. A 2-year study of inhaled low-dose (20 mg) gentamicin in 29 children and adolescents with CF found no difference in the number developing *P. aeruginosa* in sputum (Kun et al. 1984). A prospective 3-year study compared the effect of prophylactic oral ciprofloxacin and inhaled colistin treatment with placebo in children with CF (Tramper-Stranders et al. 2012). Again, there was no difference in the rate of acquisition of *P. aeruginosa* observed between the groups, although *P. aeruginosa* antibodies emerged earlier in the control group. An open-label pilot study of inhaled gentamicin over 3 years in a small number of patients at high risk for acquisition showed that patients who remained on treatment did not have *P. aeruginosa* present in their airway cultures, while nearly 50 % of those patients who stopped treatment were found to have positive cultures (Heinzl et al. 2002). These results suggest that long-term prophylaxis with inhaled gentamicin could effectively delay acquisition of *P. aeruginosa* in children with CF. Also, it is not clear that prophylactic therapy yields better results than monitoring and treatment of the first infection with the purpose of eradication. Given the added cost and treatment burden, as well as the cumulative toxicity associated with many antipseudomonal antibiotics, most clinicians feel that prophylactic therapy against *P. aeruginosa* is not appropriate (Doring et al. 2012).

9.3.4 Treatment of Pulmonary Exacerbations

As noted earlier, patients may suffer an acute worsening of signs and symptoms of respiratory infection, often diagnosed as a CF pulmonary exacerbation. Although there is no accepted definition of a pulmonary exacerbation, it is often associated with an increase in cough and sputum production and a reduction in pulmonary function as measured by spirometry (Cystic Fibrosis Foundation 1997). There are multiple proposed causes of pulmonary exacerbations, but a discussion of these is beyond the scope of this chapter and the reader is referred elsewhere for more information (Ferkol et al. 2006; Flume and VanDevanter 2015). Typical treatment of a pulmonary exacerbation includes an increase in airway clearance therapies (Cystic Fibrosis Foundation 1997) and the use of systemic and/or inhaled

antibiotics (Wagener et al. 2013). Guidelines on the treatment of pulmonary exacerbations have been published (Flume et al. 2009) but there are no standardized approaches to treatment and many questions remain (Sanders 2015). The possibility that variability in exacerbation management, particularly with respect to antibiotic treatment, results in less than optimal health outcomes is currently being investigated in the USA (West 2015; Goss 2015).

9.3.4.1 Antibiotic Choices

Recommendations for antibiotic choices are usually based on what is known from susceptibility testing of previous sputum cultures (Cystic Fibrosis Foundation 1997). This would seem a reasonable recommendation; however, there is discordance between susceptibility test results and clinical outcomes (Hurley et al. 2012; McLaughlin et al. 1983; Smith et al. 1999, 2003). That is, it is common for the treated patient to respond positively, even when treated with antibiotics to which the strain identified by selective culture has been shown to be resistant. Explanations for this observation have been offered, including the possibility of synergistic effects of combination antibiotics; however, studies using synergy testing to guide antibiotic therapy have not demonstrated any additional benefit (Aaron et al. 2005; Moskowitz et al. 2011). It has also been recently recognized that the chronic infection of the CF airways is far more complex than revealed by traditional microbiological testing; when using culture-independent methods, we have learned that the CF airways can have several dozen taxonomically distinct bacterial components of infection (Rogers et al. 2004; Zhao et al. 2012). Further, there is good evidence that as patients are treated with antibiotics over extended periods, CF airway microbial communities become less diverse, often becoming dominated by a single species such as *P. aeruginosa* (Zhao et al. 2012). It has been recommended to use combination antibiotics to treat *P. aeruginosa* (Flume et al. 2009) although the data to support combination antibiotics as necessary to treat *P. aeruginosa* are poor; it may be that combination antibiotics may yet be best for the management of complex CF pulmonary infections. A common strategy is to choose antibiotics that have previously demonstrated success and to change antibiotics for other reasons, such as when there is insufficient improvement or the time between exacerbations is reduced.

9.3.4.2 Duration of Antibiotic Treatment

An optimal duration of antibiotic treatment has not been described (Flume et al. 2009), although previous guidelines have recommended at least 10 days of duration (Cystic Fibrosis Foundation 1997). Although 14 days is the most common duration of therapy, there is a broad variance in practice (Cystic Fibrosis Foundation Patient Registry 2013). One (of many) barrier in determining an optimal duration of treatment for exacerbation is a lack of understanding/consensus on the exact endpoint measure that should be employed to quantify "response." The changes in respiratory

signs and symptoms will vary among patients with an exacerbation diagnosis, and thus it follows that evaluation of what constitutes "response" also varies. Typical treatment outcomes include resolution of specific signs and symptoms (e.g., cough, increased sputum production) and recovery of lost lung function (VanDevanter 2015). Studies that have measured the time course for resolution of respiratory signs and symptoms suggest that little additional improvement occurs beyond 7–10 days after initiation of treatment, while almost all possible lung function recovery occurs in 10–14 days (VanDevanter et al. 2010). Given the broad distribution of treatment durations, it appears that clinicians dissatisfied with responses seen at 14 days commonly continue treatment (with apparently little benefit), leading to the counterintuitive observation that patients treated for longer periods tend to have worse lung function outcomes than those treated for shorter periods (Collaco et al 2010). Recent retrospective analyses have shown that a substantial majority of patients fail to recover to their pre-exacerbation lung function after treatment (Sanders et al. 2010).

9.4 What About Other Opportunists Identified by Selective Culture?

As noted earlier, the detailed discussion of *P. aeruginosa* above is the result of both early association of that organism with poor patient outcomes and also our dependence on selective bacterial culture to characterize CF airway infections. Using selective culture, other bacterial species have been recognized as commonly present in infections (Fig. 9.1), and data suggest some of these species also contribute to poor outcomes, including particular strains of *B. cepacia* complex (Jones et al. 2004), methicillin-resistant *Staphylococcus aureus* (MRSA) (Dasenbrook et al. 2010), and, most recently, nontuberculous mycobacteria (NTM) (Esther et al. 2010). It is reasonable, given such observations, to ask whether similar treatment strategies (i.e., prophylaxis, eradication, suppression) targeted at these other CF airway pathogens have the potential to improve patient outcomes.

9.4.1 Suppression

Interest in the potential benefits of chronic MRSA suppression has increased following completion of a phase 2 study of 28 days of treatment with a dry-powder form of vancomycin (clinicaltrials.gov NCT01746095), the results of which have yet to be published. However, in a recent survey of US CF care centers, the use of inhaled treatments for MRSA remains low (Zobell et al. 2015). A study of suppressive therapy using AIS in patients with chronic *Burkholderia* infection did not demonstrate a clinical benefit (Tullis et al. 2013). There have been no studies specifically evaluating suppression of *S. maltophilia* or NTM.

9.4.2 Eradication

Recent evidence suggesting chronic MRSA airway infection is associated with worse health outcomes in CF (Dasenbrook et al. 2010) has motivated two studies of the feasibility of MRSA eradication, one in patients with new MRSA isolation (clinicaltrials.gov NCT01349192; Goss et al. 2015) and another for patients with established MRSA infection (NCT01594827; Jennings et al 2014). MRSA eradication procedures are more complicated than *P. aeruginosa* eradication protocols, involving treatment of the nares with swabs, oral rinses, whole body washes, and specific laundering procedures in addition to antibiotic treatments (NCT01349192). As with *P. aeruginosa* eradication, there are as of yet no data to indicate that successful conversion of CF patients carrying MRSA to culture negativity will provide clinical dividends.

9.4.3 Prophylaxis

Chronic prophylaxis intended to prevent the acquisition of *S. aureus* has been reported in infants treated with oral flucloxacillin (Weaver et al. 1994), but this practice is used primarily in the UK (Ratjen et al. 2001; Smyth and Walters 2003; Weaver et al. 1994). A large and long US study of anti-*Staphylococcus* antibiotics did not demonstrate any benefit, and there was concern over a tendency for selection of *P. aeruginosa* (Stutman et al. 2002). Guidelines currently recommend against routine use of such prophylactic therapy (Mogayzel et al. 2013). There are currently no other recommended prophylactic strategies for any other pathogens (Doring et al. 2012). It should be noted that macrolide antibiotics are recommended for use as a chronic therapy to maintain lung health (Mogayzel et al. 2013) but their efficacy with respect to modest lung function benefit and exacerbation risk reduction is commonly attributed to their anti-inflammatory effects in the lungs (Southern et al. 2012) and not to a prophylactic antibiotic effect. Also, chronic macrolides are not recommended in this manner when NTM are present in sputum cultures, unless they are part of the treatment of active NTM disease (Mogayzel et al. 2013).

9.5 Bacterial Opportunists Identified by Culture-Free Surveillance

As noted above, it has become clear that a majority of the microbial load in a given CF airway can be composed of species *not* routinely identified by selective culture, including obligate and facultative anaerobes (Rogers et al. 2004; Zhao et al. 2012). Unless and until current efforts to characterize the natural history of disease

associated with the presence of these organisms, it is premature to consider how their presence might justify interventions.

9.6 Conclusion

Antibiotic therapy in all of its forms has become a cornerstone of CF patient management and is often cited as an important contributor to the improved predicted survival attained in recent decades (Cystic Fibrosis Foundation Patient Registry 2013). Prophylactic antibiotic treatment remains extremely uncommon and is not viewed broadly as beneficial; cost, treatment burden, and potential for toxicity associated are reasons that antibiotic prophylaxis is not recommended for CF bacterial opportunists. Treatment of new *P. aeruginosa* acquisition with antibiotics with the intent of conversion to culture negativity (i.e., eradication) has proven to be safe and largely successful and has become standard of care of CF patients. Although it has been shown to delay the time to chronic infection, it remains to be seen whether reducing the prevalence of chronic *P. aeruginosa* infection will translate to increased survival.

Suppressive antibiotic therapy has also become standard of care once chronic infection with *P. aeruginosa* has been established. There are two approved antibiotics for inhalation in the USA, and others are in development (Table 9.1); there is interest in more treatment options as today patients can be chronically infected with *P. aeruginosa* for decades, and although suppression is associated with lung function benefit and reduced exacerbations, lung disease remains progressive and exacerbations continue to occur. One major area that requires attention is determination of the optimum regimen(s) for chronic inhaled antibiotic therapy. Whereas the two approved products were developed using month on-month off treatment regimens, there is interest in testing whether continuous antibiotic therapy would improve outcomes. Continuous treatment could be achieved with a single antibiotic or by rotating antibiotics. Finally, there is increasing interest in understanding whether chronic antibiotic suppression targeted at other lung pathogens would also improve outcomes.

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Part III
Treatment of Other Rare Lung Diseases

Chapter 10
Diffuse Panbronchiolitis: Long-Term Low-Dose Macrolide Therapy

Mutsuo Yamaya, Arata Azuma, and Shoji Kudoh

Abstract Diffuse panbronchiolitis (DPB) is characterized by thickening and inflammation of the walls of respiratory bronchioles. Patients with DPB suffer from several severe symptoms, including dyspnea, wheezing, cough, and large amounts of purulent sputum; therefore, the development of precise therapy was required. Kudoh et al. first reported the clinical benefits of erythromycin therapy in a male patient with DPB in 1984, and they reported improved 5-year survival rates as a result of this intervention in 1998. Following the initial report by Kudoh et al., many studies confirmed the clinical benefits of long-term low-dose macrolide therapy for DPB and the effects that include the immunomodulatory and physiological properties of macrolides in addition to their antimicrobial effects. Here, we introduce the history of the development of long-term low-dose macrolide therapy for DPB, and the mechanisms by which this therapy exerts its clinical benefits.

Keywords Airway inflammation • Diffuse panbronchiolitis • Inflammatory cytokine • Macrolide • Mucin • Neutrophil

10.1 Introduction

DPB is characterized by thickening of the walls of respiratory bronchioles, as well as infiltration of lymphocytes, plasma cells, and histiocytes (Yamanaka et al. 1969; Homma 1975; Homma et al. 1983). These pathological changes produce disseminated small nodular shadows on chest radiographs. Patients with DPB suffer from severe symptoms, including severe dyspnea, wheezing, cough, and large amounts

M. Yamaya, M.D., Ph.D. (✉)
Department of Advanced Preventive Medicine for Infectious Disease, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan
e-mail: myamaya@med.tohoku.ac.jp

A. Azuma, M.D., Ph.D.
Department of Pulmonary Medicine and Oncology, Graduate School of Medicine, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan

S. Kudoh, M.D., Ph.D.
Japan Anti-Tuberculosis Association, 1-3-12 Misaki-cho, Chiyoda-ku, Tokyo 101-0061, Japan

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of purulent sputum (Kudoh et al. 1984, 1998; Azuma and Kudoh 2006); therefore, the development of precise therapy was required.

Kudoh et al. first reported the clinical benefits of erythromycin therapy in a 52-year-old male patient with DPB (1984), and they reported improved 5-year survival rates as a result of this intervention (Kudoh et al. 1998; Kudoh 2004). Macrolide therapy improved symptoms, pulmonary function tests, and the nodular shadows seen on chest radiographs (Kudoh et al. 1984); has a high quality of evidence level (1A); is strongly recommended (Friedlander and Albert 2010); and is an established therapy (Figueiredo Bde and Ibiapina Cda 2011), based on the review articles on long-term macrolide therapy (Crosble and Woodhead 2009; Friedlander and Albert 2010; Figueiredo Bde and Ibiapina Cda 2011). Many studies on the mechanisms of macrolides revealed that the effects of macrolide therapy on DPB are mediated by immunomodulatory and physiological properties separate from the medication's antimicrobial effects (Azuma and Kudoh 2006).

Here, we introduce the history of the development of long-term low-dose macrolide therapy for DPB, and the mechanisms underlying its clinical benefits.

10.2 Characteristics and Epidemiology of Diffuse Panbronchiolitis

10.2.1 Characteristics of Diffuse Panbronchiolitis

In the 1960s, Homma et al. reviewed the features of a disease process different from chronic obstructive pulmonary disease (COPD) (Yamanaka et al. 1969). They classified this disease as diffuse panbronchiolitis (DPB) (Homma 1975) and first reported the characteristics of DPB in Western countries in 1983 (Homma et al. 1983). In these reports, DPB was characterized by thickening of the walls of respiratory bronchioles and the infiltration of lymphocytes, plasma cells, and histiocytes (Yamanaka et al. 1969; Homma 1975; Homma et al. 1983). These pathological changes produce disseminated small nodular shadows on chest radiographs. The patients with DPB had several severe symptoms, including severe dyspnea caused by wheezing, cough due to obstructive functional impairment and airway inflammation, and large amounts of purulent sputum due to excessive mucus production and frequent bacterial infections (Kudoh et al. 1984,1998; Azuma and Kudoh 2006). Most patients with DPB have a long history of sinusitis, and chronic sinobronchial infection is a common feature of the disease (Kudoh et al. 1984, 1998; Azuma and Kudoh 2006).

10.2.2 Epidemiology of Diffuse Panbronchiolitis

Following the first report of DPB (Yamanaka et al. 1969), investigators conducted a nationwide survey and collected information regarding more than 1000 cases of probable DPB and 82 histologically confirmed cases between 1987 and 1980

(Homma 1981). Homma et al. reported that DPB is more prevalent in males (1983), whereas no remarkable differences in male-to-female ratio have also been reported (Azuma and Kudoh 2006) by another study. The age of onset of DPB varies, and most patients are older than 40 years (Homma et al. 1983), although DPB is evenly distributed throughout Japan and affects every generation (Homma et al. 1983).

DPB was also described in other East Asian populations, such as the Chinese and the Koreans, in the 1990s, and a number of case reports have been written in these countries (Kim et al. 1992; Chu et al. 1992; Zainudin et al. 1996; Tsang et al. 1998; Poh et al. 2001). DPB has been reported as being widely distributed in China (Chen et al. 2005), but the incidence of patients with DPB is low in Asian countries (Azuma and Kudoh 2006). Similarly, the number of reported cases in countries other than Asia is limited (Fitzgerald et al. 1996; Claxton and Markos 2000; Martinez et al. 2000). Of the cases reported in Western countries, approximately half have involved Asian immigrants (Brugiere et al. 1996). Therefore, DPB in Asian populations is often compared to cystic fibrosis in Westerners.

10.3 Development of Long-Term Low-Dose Macrolide Therapy

10.3.1 Treatment of DPB Prior to the Establishment of Macrolide Therapy

Patients with DPB were treated with steroids (glucocorticoids) and antibiotics prior to the establishment of macrolide therapy. These agents transiently improved its symptoms and reduced the volume of sputum, but these effects did not last (Kudoh et al. 1987). Medicines such as beta-lactams, including penicillin and cephem antibiotics, were the primary agents used, but these antibiotics frequently induced the microbial substitution of *Pseudomonas aeruginosa* in the sputum (Kudoh et al. 1987). Furthermore, the 5-year survival rate of patients treated with steroids or antibiotics was 75 % from the onset of the disease, 42 % from the first visit to the clinic, and only 8 % following the first detection of *Pseudomonas aeruginosa* in the sputum (Kudoh et al. 1987). Therefore, the development of an effective therapy for patients with DPB that would improve symptoms and survival rates was required.

10.3.2 Discovery of Macrolide Therapy for DPB

Kudoh et al. first reported the clinical benefits of erythromycin therapy in a 52-year-old male patient with DPB who had been treated with amoxicillin and prednisolone for 3 years (1984). Prior to the start of macrolide therapy, the patient received treatment with amoxicillin and prednisolone, but these therapies did not improve his symptoms, which included dyspnea and large amounts of purulent sputum

(200–300 mL/day). The patient visited Dr. Kudoh's clinic, and his therapy was subsequently discontinued. The patient returned to Dr. Kudoh's clinic 2 years later, at which time Dr. Kudoh found that the patient's dyspnea symptoms, lung function, and arterial blood gas analysis had improved, as follows: vital capacity (VC) increased from 51 to 69 %, residual volume (RV/TLC, residual volume/total lung capacity) decreased from 64 to 43 %, arterial partial oxygen pressure (PaO_2) increased from 65 to 85 Torr upon the inhalation of air, body weight increased from 56 to 65 kg, sputum volume decreased significantly, and dyspnea improved. The diffuse nodular shadows, which were seen in previous chest radiographs, were also improved. Because the patient's prescription records demonstrated that he had been treated with erythromycin (600 mg/day) and a steroid, dexamethasone (0.5 mg/day), by Dr. Miyazawa, it was determined that long-term therapy with low-dose erythromycin, administered every day for 2 years, had been effective (Kudoh et al. 1984).

10.3.3 Confirmation of the Clinical Benefits of Macrolide Therapy

The first open trial was initiated to confirm the clinical benefits of long-term low-dose macrolide therapy for patients with DPB, using erythromycin (Kudoh et al. 1987) (Table 10.1). After treatment with 600 mg of erythromycin (for the periods between 6 months and 3 years; mean, 19.8 months), symptoms and clinical parameters were markedly improved in all 18 patients. The improvements in exertional dyspnea, lung function, and arterial partial oxygen pressure (PaO_2, from 65.2 to 75.1 Torr) were detected within 3 months of initiation of therapy. Furthermore, improvements in lung function and the diffuse nodular shadows seen on chest radiographs, as well as elevations in body weight, were observed after

Table 10.1 Clinical benefits of long-term low-dose macrolide therapy for DPB

Clinical benefits	First author and publication year
Improved symptoms: dyspnea, sputum production	Kudoh et al. (1984), Sawaki et al. (1986a, b), Takeda et al. (1989), Yamamoto et al. (1990), Nagai et al. (1991), Ashitani et al. (1992), Mikasa et al. (1992), Fujii et al. (1995), Ichikawa et al. (1995), Tamaoki et al. (1995), Nakamura et al. (1999), Kadota et al. (2003)
Improved lung function	Kudoh et al. (1984), Sawaki et al. (1986a, b), Fujii et al. (1995), Ichikawa et al. (1995), Nakamura et al. (1999), Yamada et al. (2001), Kadota et al. (2003), Hui et al. (2013)
Improved findings on chest radiographs or chest CT	Kudoh et al. (1984), Nagai et al. (1991), Akira et al. (1993), Ichikawa et al. (1995), Yamada et al. (2001), Hui et al. (2013)
Increased body weight	Kudoh et al. (1984)
Improved survival rate	Kudoh et al. (1998)

CT computed tomography, *DPB* diffuse panbronchiolitis

20 months of therapy (Kudoh et al. 1987). The diffuse small nodular shadows seen on chest radiographs disappeared in more than 60 % of cases. In spite of the continued presence of bacteria in the sputum of 15 patients, general conditions improved. In two patients, persistent detection of *P. aeruginosa* was observed, but microbial substitutions involving other species of bacteria were not detected.

After the first report by Kudoh et al. (1984), the clinical benefits of macrolide therapy in treating patients with DPB were demonstrated by other researchers, benefits that included improvement in symptoms such as dyspnea and sputum production, as well as improved lung function, increased body weight, and improved nodular shadows (Sawaki et al. 1986a, b; Yamamoto et al. 1990; Nagai et al. 1991; Fujii et al. 1995; Ichikawa et al. 1995; Kadota et al. 2003).

Forced expiratory volume in one second (FEV_1), VC and PaO_2 increased, and RV/TLC decreased. Diffuse nodular shadows on chest radiographs improved along with lung function (Yamada et al. 2001; Ichikawa et al. 1995; Akira et al. 1993). Sputum bacteria were not replaced with *P. aeruginosa* during treatment. Even in cases of superinfection with *P. aeruginosa*, treatment with erythromycin improved airflow limitations and gas exchange abnormalities (Fujii et al. 1995; Sawaki et al. 1986b). In spite of the continued presence of bacteria in sputum, general conditions improved. Yamamoto et al. and Ichikawa et al. demonstrated in retrospective studies that the clinical efficacy of 3 months of erythromycin was superior to that of either fluoroquinolones (Yamamoto et al. 1990) or ampicillin (Ichikawa et al. 1992). A prospective randomized, placebo-controlled trial using erythromycin also confirmed these beneficial effects (Yamamoto 1991). Similar effects have also been reported in patients receiving new 14-membered ring macrolides other than erythromycin, including clarithromycin and roxithromycin (Takeda et al. 1989; Ashitani et al. 1992; Nakamura et al. 1999; Mikasa et al. 1992; Tamaoki et al. 1995). These new macrolides were sometimes effective even in cases where erythromycin was ineffective (Nakamura et al. 1999). Azithromycin, a 15-membered ring macrolide, had been in limited use in Japan until it was properly available in 2001. However, Hui et al. demonstrated in China that azithromycin therapy for DPB patients improves lung function and small nodular shadows on chest computed tomography (CT) (2013). Therefore, it appears to have similar effects on DPB, although researchers do not yet have sufficient experience to confirm the effects of azithromycin.

10.3.4 Improved Survival Rates by Macrolide Therapy

The effects of long-term low-dose macrolide therapy on survival rates were evaluated by Kudoh et al. in 498 patients with DPB (1998) (Fig. 10.1). The patients were divided into the following three groups according to the date of their first medication examination: group A, 1970–1979; group B, 1980–1984; and group C, 1985–1990. One hundred ninety patients in group A received conventional antibiotics, and 221 patients in group B had the opportunity to receive new anti-*Pseudomonas* antimicrobial agents, such as the new quinolone compounds. Eighty-seven

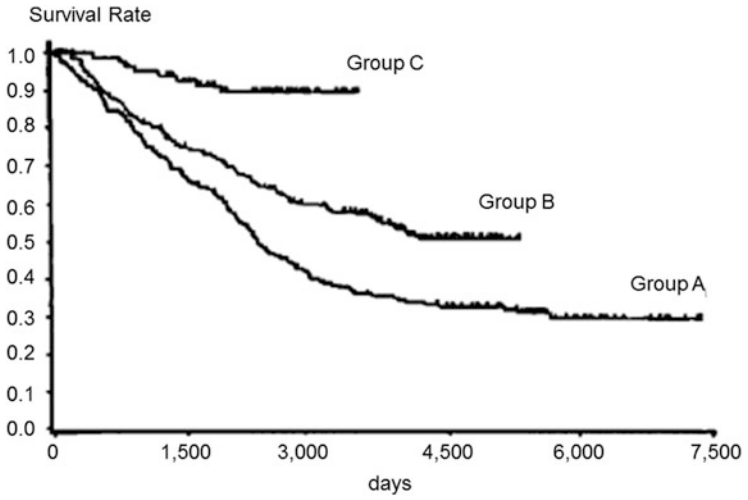

Fig. 10.1 Comparison of survival curves of DPB patients among the three groups (**a** 1970–1979, **b** 1980–1984, **c** 1985–1990). The survival rate of group C was significantly higher than that of the other groups. Significant differences were analyzed via the generalized Wilcoxon test (Reprinted with permission of the American Thoracic Society. Copyright © 2014 American Thoracic Society. Cite: Kudoh et al./1998/Improvement of survival in patients with diffuse panbronchiolitis/Am J Respir Crit Care Med/157/1829–1832. Official Journal of the American Thoracic Society)

patients in group C received erythromycin therapy (600 mg/day). In the 1970s, before the development of macrolide therapy, the overall 5-year survival rate was 63 %. Kudoh et al. demonstrated that the survival rate of group C (patients receiving erythromycin therapy) was higher than that of group A ($p < 0.0001$) (patients receiving conventional antibiotics) and group B ($p < 0.0001$) (patients receiving new quinolone compounds), and the 5-year survival rate in patients with DPB increased to 91 % (1998).

10.3.5 Worldwide Recommendation of Macrolide Therapy for DPB

Beneficial responses of macrolide therapy in patients with DPB, including improved 5-year survival rates (Kudoh et al. 1998; Kudoh 2004), improved symptoms, pulmonary function test, and diffuse nodular shadows on chest radiographs (Kudoh et al. 1984), have a high quality of evidence level (1A) (Friedlander and Albert 2010). Therefore, the macrolide therapy is a high-recommended, established therapy (Figueiredo Bde and Ibiapina Cda 2011) based on the review articles on long-term macrolide therapy for chronic inflammatory airway diseases (Crosble and Woodhead 2009; Friedlander and Albert 2010; Figueiredo Bde and Ibiapina Cda 2011).

10.4 Characteristics of Macrolides

Macrolides are macrocyclic lactones consisting of ≥8-membered rings. This large class (>2000 compounds) comprises both natural substances isolated from fungi and other organisms and synthetic molecules with similar structures (Jain and Danziger 2004). The most common agents used in clinics are semisynthetic 14-, 15-, or 16-membered ring antibiotics similar to erythromycin. These agents include erythromycin, clarithromycin, and roxithromycin, members of the 14-member class, and azithromycin, the prototypical 15-member compound (Labro 2004). Macrolide antibiotics bind to the 50*S* ribosomes of both prokaryotes and eukaryotes, inhibiting either the transpeptidation or translocation of nascent peptides. Anti-inflammatory effects of macrolides on airway epithelial cells and monocytes are mediated through the activation of intracellular signaling molecules, including nuclear factor-kappa B (NF-kB) and activator protein (AP)-1 (Desaki et al. 2000; Kikuchi et al. 2002; Suzuki et al. 2002; Asada et al. 2009; Yamaya et al. 2010). Macrolides accumulate in many tissues, such as the fluid of epithelial linings, and easily enter host defense cells, such as macrophages and polymorphonuclear leukocytes. Macrolide concentrations in both respiratory tract tissues and the extracellular fluid are higher than those in the serum (especially after the ingestion of clarithromycin), making these compounds useful for treatment of airway and alveolar infections (Jain and Danziger 2004).

10.5 Immunomodulatory and Physiological Activities of Macrolides

10.5.1 Anti-Inflammatory Effects of Macrolides on Airway Epithelial Cells

Macrolides have effects on bronchial epithelial cells in patients with inflammatory pulmonary diseases such as DPB, including the suppression of mRNA levels and the release of interleukin (IL)-8 (Takizawa et al. 1997; Desaki et al. 2000) through the activation of NF-kB and AP-1 (Desaki et al. 2000) (Table 10.2). A macrolide antibiotic, roxithromycin, reduces pro-inflammatory cytokine production in mouse lung extract induced by lipopolysaccharide (LPS) (Suzaki et al. 1999). Furthermore, clarithromycin and erythromycin reduce the production of inflammatory cytokines, including IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α, in primary cultures of human tracheal epithelial cells before and after infection with viruses, including rhinovirus (RV), respiratory syncytial (RS) virus, and seasonal influenza virus, through the inhibition of NF-kB (Suzuki et al. 2002; Asada et al. 2009; Yamaya et al. 2010). Pretreatment of TNF-alpha-stimulated A549 human airway epithelial cells with erythromycin, clarithromycin, and azithromycin decreases neutrophil survival-enhancing effects (Yamasawa et al. 2004).

Table 10.2 Immunomodulatory and physiological activities of macrolides

Mechanisms	First author
Decreases in the cell counts of neutrophils in BALF	Kadota; Oishi; Ichikawa; Tamaoki
Inhibition of neutrophil chemotaxis and survival	Kadota; Oda; Khair
Modulation of pro-inflammatory cytokine secretion	
From the airway and lung	Khair; Takizawa; Suzaki; Desaki; Suzuki; Tamaoki; Asada; Yamaya
From PMNs, monocytes, or macrophages	Ichikawa; Kadota; Oishi; Oda; Khair; Villagrasa; Kadota; Khan; Kikuchi; Reato; Tamaoki
Modulation of mucus secretion	Goswami; Tamaoki; Rubin; Tagaya; Shimizu; Inoue; Araki
Antibacterial effects	
Inhibition of	
Virulence factor production (pneumolysin)	Anderson
Mediator production (cytokine, etc.)	Khair; Takaki; Ishida; Araki
Pseudomonas twitching motility	Wozniak
Quorum-sensing mechanisms	Tateda
Biofilm formation	Kobayashi; Wozniak
Stimulation of defensin secretion	Ishizawa
Antiviral effects	
Inhibition of virus replication	Suzuki; Kido; Miyamoto; Asada; Yamaya; Gielen
Inhibition of virus infection-induced mediator production	Sato; Suzuki; Asada; Yamaya

BALF bronchoalveolar lavage fluid, *PMN* polymorphonuclear leukocyte

Erythromycin reduces *Haemophilus influenzae* endotoxin-induced production of intercellular adhesion molecule (ICAM)-1 and IL-8 by cultured human bronchial epithelial cells and also reduces the number of neutrophils that adhere to human bronchial epithelial cells (Khair et al. 1995).

10.5.2 Anti-Inflammatory Effects of Macrolides on Cells Other than Airway Epithelial Cells

The anti-inflammatory effects of macrolide antibiotics have also been demonstrated in cells other than airway epithelial cells. Clarithromycin and azithromycin affected the production of cytokines in human monocytes, including IL-1, IL-6, and TNF-α, to varying degrees. Clarithromycin treatment resulted in cell suppression in 71 % of subjects and cell increases in 29 % of subjects. Based on these results, Khan et al. concluded that clarithromycin and azithromycin alter cytokine production in

human monocytes and possess immunomodulatory properties (1999). Similarly, Kikuchi et al. demonstrated that clarithromycin suppresses LPS-induced IL-8 production by human monocytes through AP-1 and NF-kB transcription factors (2002). Oishi et al. demonstrated that treatment with erythromycin decreases the release of IL-8 from *Pseudomonas*-stimulated neutrophils (1994).

Kadota et al. (1993) and Oishi et al. (1994) demonstrated a high percentage of neutrophils in the bronchoalveolar lavage fluids (BALFs) of patients with DPB and other chronic airway diseases, and demonstrated that erythromycin treatment reduces the number of neutrophils. Treatment with erythromycin reduces number of neutrophils and neutrophil-derived elastolytic-like activity in BALF in patients with bronchiolitis (Ichikawa et al. 1992). Previously elevated neutrophil chemotactic activities (Kadota et al. 1993; Oda et al. 1994; Khair et al. 1995), as well as the production of the superoxide anion and leukotriene B_4 (LTB_4) (Kadota et al. 1998; Oda et al. 1995) by polymorphonuclear leukocytes, are reduced by erythromycin treatment in patients with DPB. Similarly, macrolides, including erythromycin and clarithromycin, reduce superoxide generation, as well as the release of elastase (Villagrasa et al. 1997) and IL-6 and TNF-α (Reato et al. 2004) from human blood polymorphonuclear leukocytes, which are induced by the chemotactic peptide *N*-formylmethionyl-leucyl-phenylalanine (Villagrasa et al. 1997) and LPS (Reato et al. 2004). By contrast, Villagrasa et al. demonstrated that treatment of neutrophils with erythromycin does not reduce the production of LTB_4 (1997). Several other macrolide-mediated anti-inflammatory effects have also been reported in BALF of DPB patients, including decreases in the cell counts of neutrophils and the levels of IL-8 (Tamaoki et al. 2004).

10.5.3 Anti-Inflammatory Effects of Macrolides on Airway Inflammation

The findings described above suggest that macrolides may modulate production of inflammatory mediators, including cytokines, reactive oxygen species (ROS), elastase, and leukotrienes, in airway epithelial cells, human peripheral blood monocytes, and polymorphonuclear neutrophils, which are stimulated by several agents and microorganisms, including bacteria and respiratory viruses. Because the elastase, ROS, and LTB_4 released from neutrophils augment airway inflammation (Yasuo et al. 2009; Yamaya et al. 2012; Tamaoki et al. 2004), these inhibitory effects of macrolides may modulate the adherence of polymorphonuclear neutrophils to airway epithelial cells and reduce the airway inflammation induced by activated neutrophils.

10.5.4 Inhibitory Effects on Mucus Secretion

Mucus hypersecretion is a primary symptom of DPB in affected patients. Airway inflammation-related goblet cell hyperplasia and the effects of chemical mediators, neutrophil elastase, and ROS are associated with mucus hypersecretion (Tamaoki et al. 2004; Azuma and Kudoh 2006; Yamaya et al. 2012). Several reports have demonstrated the inhibitory effects of macrolides on mucus hypersecretion. A parallel, double-blind, placebo-controlled study was conducted by Tamaoki et al. to determine the effects of long-term clarithromycin administration on sputum in patients with clinical conditions associated with excessive airway secretions, including DPB (1995). A total of 31 patients were divided into the following two groups: a clarithromycin group (100 mg, twice a day) and a placebo group, in which 16 patients had chronic bronchitis, 8 patients had DPB, and 7 patients had bronchiectasis. The authors reported that treatment with clarithromycin decreased sputum production (Tamaoki et al. 1995). Tagaya et al. also demonstrated that treatment with clarithromycin decreased sputum volumes in 16 patients with chronic bronchitis ($n = 5$) or bronchiectasis ($n = 11$) (2002). Furthermore, Rubin et al. demonstrated that clarithromycin decreases the volume of nasal secretions and increases mucociliary transportability in patients with purulent rhinitis (1997).

Shimizu et al. examined the effects of macrolide antibiotics on mucus hypersecretion in vivo, reporting that clarithromycin inhibits ovalbumin (OVA)- and LPS-induced mucus production in the rat nasal epithelium, effects induced by the intranasal instillation of OVA in OVA-sensitized rats, as well as intranasal LPS instillation (2003).

Goswami et al. demonstrated that erythromycin inhibits respiratory glycoconjugate secretion from human airway explants (1990). Similarly, in vitro studies have demonstrated that macrolide antibiotics such as erythromycin (Shimizu et al. 2003; Inoue et al. 2008), clarithromycin (Tamaoki et al. 1996; Shimizu et al. 2003), and azithromycin (Araki et al. 2010) have inhibitory effects on mucin and MUC5AC production and secretion following stimulation with IL-8 (Tamaoki et al. 1996), TNF-α (Shimizu et al. 2003), RV infection (Inoue et al. 2008), and extracts of *H. influenzae* (Araki et al. 2010) in airway epithelial cells and airway goblet cells.

10.5.5 Inhibitory Effects on Bacterial Virulence and Biofilms

Anderson et al. demonstrated that clarithromycin reduces the production of pneumolysin, a key virulence factor in *S. pneumoniae* infections (2007). Macrolides reduce the production of pro-inflammatory cytokines, soluble ICAM-1, and mucin in airway epithelial cells in response to endotoxins and extracts of *H. influenzae* (Khair et al. 1995; Araki et al. 2010; Takaki et al. 2003; Ishida et al. 2007). Azithromycin also maintains the integrity of airway epithelial cells during

P. aeruginosa infection (Halldorsson et al. 2010). These findings suggest that macrolides may inhibit virulence factor production and subsequent inflammation caused by bacteria. Furthermore, clarithromycin inhibits the twitching motility of *P. aeruginosa* (Wozniak and Keyser 2004), and azithromycin inhibits the quorum-sensing circuitry of *P. aeruginosa,* which is related to virulence factor production (Tateda et al. 2001). In addition, incubating *P. aeruginosa* with clarithromycin altered the structure and architecture of the biofilm (Kobayashi 1995; Wozniak and Keyser 2004). These findings suggest that macrolides may modulate the virulence of bacteria associated with DPB pathogenesis (Table 10.1).

10.5.6 Inhibitory Effects on Inflammation During Respiratory Virus Infections

Sato et al. (1998) and Tsurita et al. (2001) reported that erythromycin and clarithromycin reduce lung injury and the severity of pneumonia in mice infected with the influenza virus and suggested that these inhibitory effects are associated with the reduced production of nitric oxide, ROS, and interferon (IFN)-γ (Sato et al. 1998), as well as elevated IL-12 levels (Tsurita et al. 2001). Clarithromycin also suppresses the growth of the influenza virus and its release in mouse airways and epithelial cells (Kido et al. 2004; Miyamoto et al. 2008). Furthermore, Yamaya et al. (2010) reported that clarithromycin decreases the release of viruses and cytokines into supernatant fluids in human tracheal epithelial cells infected with seasonal type A influenza (H3N2) by reducing the expression of the viral receptor and inhibiting viral RNA entry.

Furthermore, macrolides reduce airway inflammation induced by respiratory viruses other than the influenza virus. Suzuki et al. (2002) showed that erythromycin inhibits RV infection by reducing the levels of ICAM-1, an RV receptor, by blocking RV RNA entry and that erythromycin reduces the production of pro-inflammatory cytokines in human tracheal epithelial cells. Gielen et al. demonstrated the inhibitory effects of azithromycin on RV replication associated with the induction of IFNs in human bronchial epithelial cells (2010). Asada et al. also reported that erythromycin and clarithromycin exert inhibitory effects on RS virus infections in human airway epithelial cells (2009), and they reduce the production of cytokines in these cells.

Although there is no evidence that macrolides reduce the frequency of DPB exacerbations induced by viral infections, viral infections are thought to be involved in the progression of DPB (Takeda et al. 1989). The antiviral effects and immunomodulatory effects of macrolides may be associated with the inhibition of viral infection-induced progression of DPB.

10.5.7 Summary of the Possible Mechanisms by Which Macrolides Exert Clinical Benefits in the Setting of DPB

As described above and in other reports, macrolides have several functions other than their antimicrobial properties (Tamaoki et al. 2004; Azuma and Kudoh 2006; Yamaya et al. 2012), including anti-inflammatory effects on airway epithelial cells and neutrophils, reduced mucus secretion, inhibitory effects on bacterial virulence and biofilm formation, enhanced production of antimicrobial peptides and human β-defensins (Ishizawa et al. 2005), and antiviral effects. The physiological functions of macrolides, including those reported by other authors, are shown in Table 10.2 (Yamaya et al. 2012). Based on these findings, the clinical effects of macrolides, such as erythromycin and clarithromycin, on DPB are thought to be mediated by immunomodulatory and physiological activities other than those related to antimicrobial effects (Azuma and Kudoh 2006).

10.6 Concluding Remarks

Long-term low-dose macrolide therapy improves 5-year survival rates (Kudoh et al. 1998; Kudoh 2004), symptoms such as dyspnea and sputum production, pulmonary function test, and diffuse nodular shadows on chest radiographs (Kudoh et al. 1984, 1987). The clinical benefits of macrolides are supported by high-quality evidence (Friedlander and Albert 2010); therefore, they are a high-recommended, established therapy (Figueiredo Bde and Ibiapina Cda 2011) based on the review articles on long-term macrolide therapy for chronic inflammatory airway diseases (Crosble and Woodhead 2009; Friedlander and Albert 2010; Figueiredo Bde and Ibiapina Cda 2011). Furthermore, the clinical benefits of macrolides in treating exacerbations of chronic inflammatory pulmonary disease, including bronchiectasis (Serisier and Martin 2011) and COPD (Suzuki et al. 2001; Yamaya et al. 2008; Seemungal et al. 2008; Albert et al. 2011), have been reported by multiple studies. Immunomodulatory and physiological properties of macrolides other than their antimicrobial effects are also thought to contribute to the clinical effects of these medicines against bronchiectasis and COPD. Macrolide therapy may also be effective in the treatment of chronic inflammatory pulmonary diseases other than DPB and cystic fibrosis.

Conflict of Interest Statement Mutsuo Yamaya is a professor in the Department of Advanced Preventive Medicine for Infectious Disease at Tohoku University Graduate School of Medicine. This department has received funding from eight pharmaceutical companies which are as follows: Abott Japan, Co., Ltd., Taisho Toyama Pharmaceutical Co., Ltd., Kyorin Pharmaceutical Co. Ltd., AstraZeneca Co. Ltd, Otsuka Pharmaceutical Co. Ltd., Teijin Pharma Co., Ltd., Toyama Chemical Co., Ltd., and Nippon Boehringer-Ingelheim Co., Ltd.

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Chapter 11
Idiopathic Pulmonary Fibrosis

Paolo Spagnolo

Abstract Idiopathic pulmonary fibrosis (IPF) is the most common fibrotic idiopathic interstitial pneumonia. The disease, which primarily occurs in older adults, is relentlessly progressive, with a 5-year survival of approximately 20 %. Improved understanding of disease pathobiology along with more precise disease definition has affected dramatically the approach to treatment. Indeed, originally thought of as a chronic inflammatory disorder, IPF is now believed to result from recurrent alveolar epithelial microinjury followed by an aberrant reparative response. This paradigm shift coupled with major improvements in disease definition and patient stratification has led to an exponential increase in the number of high-quality clinical trials of pharmacological interventions, most of which, however, have produced negative results, probably because of the multitude of cell types, growth factors, and signaling pathways involved in the fibrotic process. As such, until very recently, IPF has lacked effective therapies. Finally, in 2014, the US Food and Drug Administration (FDA) approved two drugs for IPF: pirfenidone, a compound with broad antifibrotic, anti-inflammatory, and antioxidant properties, and nintedanib, a tyrosine kinase inhibitor with selectivity for vascular endothelial growth factor, platelet-derived growth factor, and fibroblast growth factor receptors. Pirfenidone, however, had already been approved in Japan, Europe, India, and Canada. Both pirfenidone and nintedanib significantly slow functional decline and IPF disease progression with an acceptable safety profile. This is a major step forward. However, neither drug is a cure for IPF as the disease continues to progress in most patients despite treatment. A number of novel agents with high potential are currently being tested and many more are ready for clinical trials. Their completion is critical for achieving the ultimate goal of curing this devastating disease.

Keywords Diagnosis • Guidelines • Idiopathic pulmonary fibrosis • Nintedanib • Pirfenidone • Treatment

P. Spagnolo, M.D., Ph.D. (✉)
Respiratory Disease Unit, Department of Cardiothoracic and Vascular Sciences, University of Padova, Via Giustiniani 3, 35128 Padova, Italy
e-mail: paolo.spagnolo@unipd.it

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11.1 Disease Overview

Idiopathic pulmonary fibrosis (IPF) is the most common form of idiopathic fibrotic interstitial pneumonia (American Thoracic Society 2000). The disease, which occurs primarily in older adults, is limited to the lungs and is associated with the radiological and/or histopathologic pattern of usual interstitial pneumonia (UIP) (Raghu et al. 2011; Spagnolo et al. 2015). Radiological UIP is characterized on high-resolution computed tomography (HRCT) of the chest by the presence of reticular opacities, traction bronchiectasis, and honeycombing (i.e., clustered cystic airspaces, usually of comparable diameter on the order of 3–10 mm) typically in a basal, subpleural, and patchy distribution (Fig. 11.1) (Johkoh et al. 1999; Hansell et al. 2008). Histopathologic UIP consists of a combination of fibrotic areas with scarring and honeycomb change alternate with areas of less affected or even normal lung parenchyma (Fig. 11.2). The fibrotic zones are composed mainly of dense collagen, although scattered subepithelial foci of proliferating fibroblasts and myofibroblasts (so-called fibroblastic foci) are a consistent finding. Notably, a pattern of UIP can be found in a number of settings including, among others, connective tissue disease (CTD), chronic hypersensitivity pneumonitis (HP), and pneumoconiosis (mainly asbestosis), which makes it critical, a rigorous approach to the diagnosis of IPF (e.g., *idiopathic* UIP) (Wuyts et al. 2014). This point remains poorly appreciated by many physicians, and, as a result, patients are often incompletely evaluated and empirically treated as IPF.

Incidence and prevalence of IPF increase dramatically with age. Indeed, the disease is uncommon in patients younger than 50 years of age, but is present in as many as 0.2 % of those older than 75 years of age (Raghu et al. 2006a, 2014a). The underlying causes of the fibrotic process in IPF remain unknown. However, cigarette smoking, exposure to metal and wood dust, microbial agents, chronic microaspiration of gastric content (either acid or nonacid), and genetic factors have all been shown to increase the risk of developing the disease (Iwai et al. 1994; Olson and Swigris 2012; Spagnolo et al. 2014a, b; Bellaye and Kolb 2015; Behr et al. 2015). IPF is an almost invariably fatal condition, with a 5-year survival of approximately 20 % and a mortality burden higher than that of many cancers (Bjoraker et al. 1998). Yet, its clinical course and rate of progression are highly variable and unpredictable. In fact, periods of relative stability may be punctuated by episodes of accelerated deterioration (referred to as "acute exacerbations") often resulting in respiratory failure and death (Ley et al. 2011; Johannson et al. 2015; Selman et al. 2007).

Our understanding of IPF has improved substantially in the last two decades, and this has affected the approach to treatment. Indeed, original pathogenetic models of chronic inflammation leading to fibrosis have evolved to current models of recurrent alveolar epithelial cell microinjury and dysregulated reparative response characterized by uncontrolled proliferation of lung fibroblasts and their differentiation to myofibroblasts, which deposit an excessive amount of extracellular matrix (ECM) proteins in the interstitial space leading to progressive scarring of the

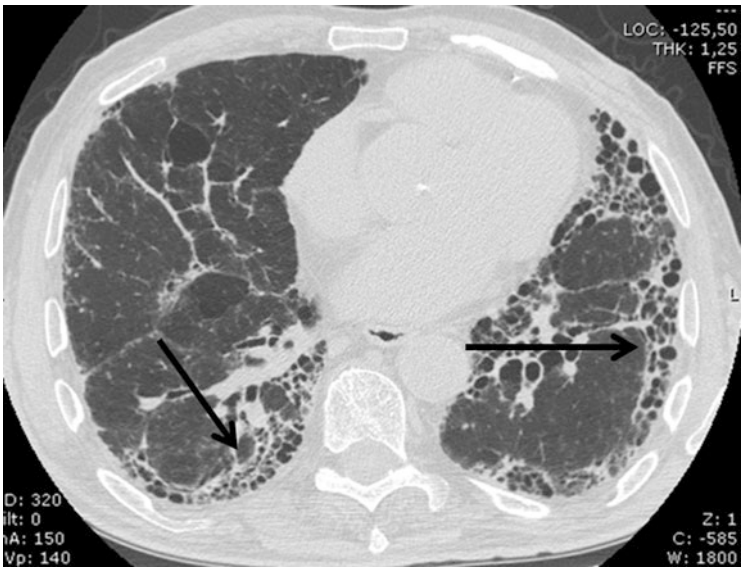

Fig. 11.1 Usual interstitial pneumonia pattern. Chest high-resolution computed tomography showing reticular abnormality in a typical subpleural distribution. Areas with honeycombing (*arrows*) are also seen

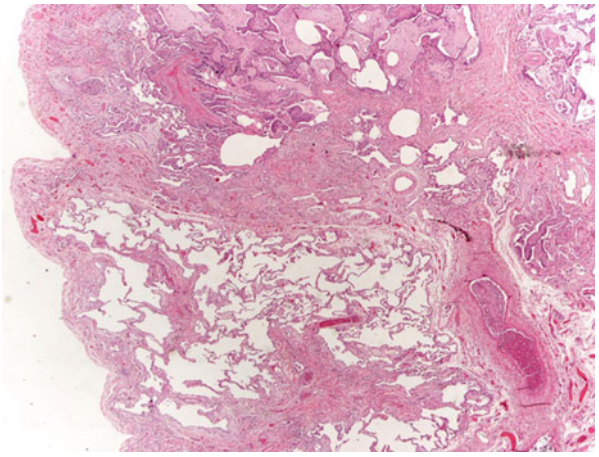

Fig. 11.2 Surgical lung biopsy specimen showing a pattern of usual interstitial pneumonia, characterized by the abrupt transition from dense fibrosis (*top right* and *bottom left*) to nearly normal lung (center). (Hematoxylin-eosin, 20×. Courtesy Giulio Rossi, MD, Modena, Italy)

lung, architectural distortion, and loss of function (du Bois 2010; Selman et al. 2001; Wolters et al. 2014; Spagnolo et al. 2014a, b). According to novel pathogenetic concepts, more recent clinical trials have evaluated the efficacy of molecules targeting the wound-healing cascade and fibrogenesis, although, overall, with disappointing results (Table 11.1), probably because of the multitude of

Table 11.1 Overview of the most recent randomized controlled trials performed in IPF

Study drug (author/trial's acronym)	Number of patients	Primary endpoint	Outcome/Comments	Reference
Pirfenidone (Azuma)	107	Change in the lowest oxygen saturation during a 6-minute exercise test	Primary endpoint not met. Positive treatment effect in VC and acute exacerbations	Azuma et al. (2005)
Pirfenidone (Taniguchi)	267	Change in VC (relative)	Primary endpoint met	Taniguchi et al. (2010)
Pirfenidone (CAPACITY 004)	435	Change in FVC (absolute)	Primary endpoint met	Noble et al. (2011)
Pirfenidone (CAPACITY 006)	344	Change in FVC (absolute)	Primary endpoint not met	Noble et al. (2011)
Pirfenidone (ASCEND)	555	Change in FVC (relative)	Primary endpoint met	King et al. (2014)
Imatinib (Daniels)	119	Time to disease progression (10 % decline in percent predicted FVC from baseline) or time to death	Primary endpoint not met	Daniels et al. (2010)
Nintedanib (TOMORROW)	432	Annual rate of decline in FVC (relative)	Primary endpoint not met. Nintedanib 150 mg twice daily was associated with a trend toward a reduction in FVC decline	Richeldi et al. (2011)
Nintedanib (INPULSIS-1)	513	Annual rate of decline in FVC (relative)	Primary endpoint met	Richeldi et al. (2014)
Nintedanib (INPULSIS-2)	548	Annual rate of decline in FVC (relative)	Primary endpoint met	Richeldi et al. (2014)
Anticoagulants (Kubo)	56	Overall survival; hospitalization-free time	Mortality associated with acute exacerbations of IPF was significantly reduced in the anticoagulant group	Kubo et al. (2005)
Warfarin (ACE)	145	Composite outcome of time to death, hospitalization, or a $\geq$10 % absolute decline in FVC	Primary endpoint not met. Trial terminated early	Noth et al. (2012)
NAC + AZA + CS versus AZA + CS (IFIGENIA)	182	Change in VC and DL_{CO} (relative)	Primary endpoint met	Demedts et al. (2005)

(continued)

Table 11.1 (continued)

Study drug (author/trial's acronym)	Number of patients	Primary endpoint	Outcome/Comments	Reference
NAC versus placebo versus NAC + AZA + CS (PANTHER)	236	Change in FVC (relative)	Primary endpoint not met. Trial terminated early	IPF Clinical Research Network (2012)
NAC versus placebo (PANTHER)	264	Change in FVC (relative)	Primary endpoint not met	IPF Clinical Research Network et al. (2014)
IFN γ-1b (Ziesche)	18	Change in FVC and TLC (absolute) and arterial blood gases	Primary endpoint met	Ziesche et al. (1999)
IFN γ-1b (Raghu)	330	Progression-free survival (time to disease progression or death)	Primary endpoint not met IFN γ-1b was associated with a trend toward enhanced survival in treatment-adherent patients	Raghu et al. (2004)
IFN γ-1b (INSPIRE)	826	Overall survival	Primary endpoint not met. Trail terminated early	King et al. (2009)
Etanercept (Raghu)	88	Change in FVC (absolute)	Primary endpoint not met	Raghu et al. (2008)
Bosentan (BUILD-1)	158	Change in 6MWD	Primary endpoint not met	King et al. (2008)
Bosentan (BUILD-3)	616	Time to IPF worsening (decline in FVC $\geq$10 % and decline in DL_{CO} $\geq$15 % or acute exacerbation) or death	Primary endpoint not met	King et al. (2011)
Macitentan (MUSIC)	178	Change in VC (relative)	Primary endpoint not met	Raghu et al. (2013a)
Ambrisentan (ARIES)	492	Time to disease progression (death, decline in FVC $\geq$10 %, decline in DL_{CO} $\geq$15 %, or acute exacerbation)	Primary endpoint not met. Trial terminated early	Raghu et al. (2013b)
Sildenafil (STEP)	180	Proportion of patients with an increase in the 6MWD of $\geq$20 %	Primary endpoint not met. Positive treatment effect in secondary endpoints	IPF Clinical Research Network (2010)

6MWD 6-minute walking distance, *AZA* azathioprine, *CS* corticosteroids, DL_{CO} diffusing capacity of the lung for carbon monoxide, *FVC* forced vital capacity, *IFN* interferon, *NAC* N-acetylcysteine, *TLC* total lung capacity, *VC* vital capacity

mediators, growth factors, and signaling pathways involved in the fibrotic process (Maher 2012). Finally, more recently, two compounds with pleiotropic mechanisms of action—pirfenidone and nintedanib—have proven effective in reducing functional decline and disease progression in patients with IPF (King et al. 2014; Richeldi et al. 2014). The evidence supporting the efficacy of pirfenidone and nintedanib in IPF along with an overview of clinical drug development is summarized in this chapter.

11.2 International Guidelines on Treatment of IPF

The management of patients with IPF is mainly based on the recommendations of the guidelines developed by the American Thoracic Society (ATS)/European Respiratory Society (ERS)/Japanese Respiratory Society (JRS)/Latin American Thoracic Association (ALAT) (Raghu et al. 2011). This evidence-based document, originally published in 2011, has recently been updated to incorporate reappraisal of previously evaluated treatment options and recommendations on novel agents (Raghu et al. 2015a). For each outcome of interest, a multidisciplinary panel assessed the overall certainty (e.g., the *confidence*) in effect estimate according to the Grading of Recommendations Assessment, Development and Evaluation (GRADE) methodology (Balshem et al. 2011), which is based on the following criteria: risk of bias, precision, consistency, directness of the evidence, risk for publication bias, presence of dose–effect relationship, magnitude of effect, and assessment of the effect of plausible residual confounding or bias. The confidence in effect estimates for each outcome was graded as *high*, *moderate*, *low*, or *very low* (Raghu et al. 2015a). In addition, for any given question, a multidisciplinary committee of experts made a recommendation *for* or *against* (Andrews et al. 2013). The recommendations were either "strong" or "conditional," based on the following factors: quality and strength of evidence, outcomes and associated importance to patients, desirable and undesirable consequences of treatment, costs, implications of treatment on health equity, feasibility of treatment, acceptability of treatment to stakeholders, and implementation issues. Current recommendations are summarized in Table 11.2.

In the following sections, only treatment interventions that received a *conditional* recommendation for use (e.g., pirfenidone, nintedanib, and antiacid medication) are discussed. However, the committee emphasized that recommendations with the same rating should not by default be considered equivalent.

Table 11.2 Key recommendations on pharmacological treatment of IPF according to current guideline

Agent/Intervention	2015 guidelines
Nintedanib	Conditional recommendation for use[a]
Pirfenidone	Conditional recommendation for use[a]
Antiacid therapy	Conditional recommendation for use[c]
Bosentan, macitentan	Conditional recommendation against use[b]
NAC monotherapy	Conditional recommendation against use[b]
Sildenafil	Conditional recommendation against use[a]
Ambrisentan	Strong recommendation against use[b]
Combination prednisone, azathioprine, and NAC	Strong recommendation against use[b]
Imatinib	Strong recommendation against use[a]
Warfarin	Strong recommendation against use[a]
Therapy for IPF-associated PH	Reassessment of previous recommendation was deferred
Lung transplantation: single versus bilateral	Formulation of a recommendation was deferred

Modified from Raghu et al. (2015a)
NAC N-acetylcysteine; *PH* pulmonary hypertension
[a]Moderate confidence in effect estimates
[b]Low confidence in effect estimates
[c]Very low confidence in effect estimates

11.3 Pirfenidone

Pirfenidone is an orally available, low molecular weight compound with broad antifibrotic, anti-inflammatory, and antioxidant activities (Kim and Keating 2015). Specifically, its biological properties include inhibition of the synthesis and activity of transforming growth factor (TGF)-β, inhibition of fibroblast proliferation and collagen synthesis, reduction of expression of profibrotic genes, and inhibition of proinflammatory cytokine, including tumor necrosis factor (TNF)-α production and release (Di Sario et al. 2002; Schaefer et al. 2011; Nakayama et al. 2008; Oku et al. 2002). Following promising results from a small open-label trial (Raghu et al. 1999), safety and efficacy of pirfenidone in patients with IPF were evaluated in a multicenter, double-blind, placebo-controlled, phase II study conducted in Japan by Azuma and coworkers (2005). This trial was stopped prematurely following an interim analysis showing that acute exacerbations of IPF (AE-IPF) had occurred exclusively in the placebo arm during the 9-month study period ($n = 5$; 14 versus 0 % in the pirfenidone arm; $p = 0.0031$). While change in the lowest oxygen saturation (SpO2) during a 6-minute exercise test (6MET), the primary endpoint, did not reach statistical significance ($p = 0.0722$), in a prespecified analysis of patients who maintained a SpO2 > 80 % during a 6MET at baseline, the lowest SpO2 during a 6MET improved in the pirfenidone group at both the 6- and 9-month time points ($p = 0.0069$ and $p = 0.0305$, respectively). Positive

treatment effects were also demonstrated in the change in vital capacity (VC) at 9 months ($p = 0.0366$). Subsequently, Taniguchi and coworkers conducted a multicenter, double-blind, placebo-controlled phase III study in which 275 Japanese patients were randomized in a 2:1:2 ratio to high-dose (1800 mg/day) or low-dose (1200 mg/day) pirfenidone or placebo (Taniguchi et al. 2010). The study met its primary endpoint. In fact, the rate of decline of VC from baseline to week 52 was lower in both the high-dose (−0.09 l) and low-dose pirfenidone arms (−0.08 l) as compared to placebo (−0.16 l; $p = 0.042$ and $p = 0.039$, respectively). Significant differences were also observed in terms of progression-free survival time (defined as time to death and/or ≥10 % decline in VC from baseline) between the high-dose and the placebo arms ($p = 0.028$) and change in total lung capacity (TLC) between the low-dose and the placebo arms ($p = 0.040$). Overall, pirfenidone was well tolerated. The most common drug-related adverse event was photosensitivity (observed in 51 % of patients in the high-dose group and 53 % in the low-dose group), which was mild in most cases and not a major cause of discontinuation of the study. Pirfenidone was approved in Japan for use in patients with IPF in 2008.

The Clinical Studies Assessing Pirfenidone in Idiopathic Pulmonary Fibrosis: Research on Efficacy and Safety Outcomes (CAPACITY) program consisted of two almost identical multinational, double-blind, randomized, placebo-controlled, phase III trials (PIPF-004 and PIPF-006) that involved 110 centers across Europe, North America, Mexico, and Australia (Noble et al. 2011). Both trials enrolled patients aged 40–80 years with a diagnosis of IPF made within the previous 48 months based on clinical, radiologic, and/or pathologic data, and according to the 2000 ATS/ERS guidelines (American Thoracic Society 2000). Inclusion criteria included also predicted forced vital capacity (FVC) of at least 50 %, predicted diffusing capacity of the lung for carbon monoxide (DL_{CO}) of at least 35 %, either predicted FVC or predicted DL_{CO} of 90 % or less, and 6-min walk test distance (6MWD) of at least 150 m. In study 004, 435 patients were assigned in a 2:1:2 dosing ratio to pirfenidone 2403 mg/day ($n = 174$), pirfenidone 1197 mg/day ($n = 87$), or placebo ($n = 174$), whereas study 006 had only two arms (e.g., pirfenidone 2403 mg/day, $n = 173$, and placebo, $n = 171$). The primary endpoint of both studies was change in percentage predicted FVC from baseline to week 72. In PIPF-004, mean FVC change at week 72 was −8.0 % in the pirfenidone 2403 mg/day group and −12.4 % in the placebo group ($p = 0.001$), whereas in the pirfenidone 1197 mg/day arm, the primary outcome was intermediate to that of the pirfenidone 2403 mg/day and placebo arms. In addition, 35 of 174 (20 %) patients in the pirfenidone arm and 60 of 174 (35 %) in the placebo arm had an FVC decline of at least 10 % ($p = 0.001$). Conversely, in study 006, the between-group difference in change in FVC at week 72 was not significant (−9.0 % in the pirfenidone arm and −9.6 % in the placebo arm; $p = 0.501$). Most common pirfenidone-related adverse events were nausea (36 versus 17 % in placebo), dyspepsia (19 versus 7 %), vomiting (14 versus 4 %), anorexia (11 versus 4 %), photosensitivity (12 versus 2 %), skin rash (32 versus 12 %), and dizziness (18 versus 10 %). However, they were generally of mild to moderate severity, reversible, and without clinically significant sequelae. These three trials had sufficient

methodological quality to be included in a Cochrane meta-analysis that showed that pirfenidone significantly reduces the rate of functional decline and risk of disease progression compared with placebo (Spagnolo et al. 2010). In 2011, pirfenidone was approved for the treatment of IPF patients with mild to moderate functional impairment in Europe but not by the US Food and Drug Administration (FDA), which requested an additional phase 3 study to confirm efficacy. In Assessment of Pirfenidone to Confirm Efficacy and Safety in Idiopathic Pulmonary Fibrosis (ASCEND), 555 IPF patients were randomly assigned to either pirfenidone 2403 mg/day ($n = 278$) or placebo ($n = 277$) for 52 weeks (King et al. 2014). Notably, in ASCEND, in order to enroll subjects at higher risk for disease progression, patients with major airflow limitation [ratio of the forced expiratory volume in one second (FEV1) to FVC <0.80] were excluded, and the minimum baseline DL_{CO} was reduced from 35 to 30 % of the predicted value. The study met its primary endpoint (i.e., change from baseline to week 52 in the percentage of predicted FVC). In addition, a relative reduction of 47.9 % in the proportion of patients who had an absolute decline of ≥10 % in percentage predicted FVC or who died [46 patients (16.5 %) versus 88 patients (31.8 %); $p < 0.001$] and a relative increase of 132.5 % in the proportion of patients with no decline in FVC [63 patients (22.7 %) versus 27 patients (9.7 %); $p < 0.001$] were seen in the pirfenidone arm compared to the placebo arm. A series of sensitivity analyses confirmed the robustness of these findings and the estimated magnitude of pirfenidone effect (e.g., an approximate 50 % reduction in FVC decline) in patients with IPF (Lederer et al. 2015). Pirfenidone treatment reduced also the decline in the 6MWD ($p = 0.04$) and improved progression-free survival (e.g., time to the first occurrence of any one of the following: a confirmed decrease of ≥10 % in the percentage of the predicted FVC, a confirmed decrease of 50 m or more in the 6MWD, or death) ($p < 0.001$). On the other hand, no significant differences between pirfenidone and placebo were found in dyspnea scores ($p = 0.16$) or in all-cause (4.0 versus 7.2 %; $p = 0.10$) or IPF-related mortality (1.1 versus 2.5 %; $p = 0.23$). However, a prespecified pooled analysis from the ASCEND and CAPACITY trials showed that pirfenidone significantly reduced both all-cause (3.5 versus 6.7 %; HR: 052; $p = 0.01$) and IPF-related (1.1 versus 3.5 %; HR: 0.32; $p = 0.006$) mortalities compared with placebo at week 52. As expected, gastrointestinal and skin-related events were more common in the pirfenidone arm than in the placebo arm, although they were generally of mild to moderate severity, reversible, and without clinically significant sequelae. Furthermore, the proportion of adverse events leading to discontinuation of study treatment did not differ between the pirfenidone (40 patients, 14.4 %) and the placebo (30 patients, 10.8 %) groups. Recommendations on management of pirfenidone-related adverse events based on existing guidelines, research evidence, and expert opinion have recently been published (Costabel et al. 2014a). In October 2014 the FDA approved pirfenidone use in patients with IPF irrespective of disease severity as assessed by functional impairment. Several reports have confirmed long-term favorable safety and efficacy profiles of pirfenidone (Oltmanns et al. 2014; Cottin and Maher 2015; Costabel et al. 2014b), including an interim analysis of RECAP, an ongoing open-label,

long-term, follow-up extension study that included patients who completed the CAPACITY or ASCEND trials, which showed that approximately half of the patients initially randomized to pirfenidone were still receiving therapy at 5-year follow-up (Cottin and Maher 2015).

11.4 Nintedanib

Nintedanib is an orally available inhibitor of the tyrosine kinase receptors, vascular endothelial growth factor receptor (VEGFR) 1–3, fibroblast growth factor receptor (FGFR) 1–3, and platelet-derived growth factor receptor (PDGFR) a and b (Hilberg et al. 2008). Specifically, nintedanib competitively and reversibly inhibits the adenosine triphosphate binding pocket of VEGFR 1–3, FGFR 1–3, and PDGFR a and b, thus blocking the intracellular signaling cascade needed for the proliferation, migration, and transformation of fibroblasts (Wollin et al. 2015; Keating 2015). The antifibrotic properties of nintedanib include also inhibition of TGF-β receptor (s) signaling, inhibition of fibronectin and collagen 1a1 mRNA expression independent of TGF-β signaling, and induction of noncanonical autophagy (Rangarajan et al. 2016).

The safety and efficacy of nintedanib in patients with IPF were initially evaluated in a dose-finding, 12-month, double-blind, randomized, placebo-controlled phase II trial [To Improve Pulmonary Fibrosis With BIBF 1120 (TOMORROW)] (Richeldi et al. 2011). Main inclusion criteria included age ≥40 years, IPF diagnosed within the previous 5 years, FVC of ≥50 % of predicted value, DL_{CO} of 30–79 % of predicted value, and HRCT of the chest performed within the previous 12 months. In TOMORROW, four different doses of nintedanib were tested [e.g., 50 mg once daily ($n = 86$), and 50 mg ($n = 86$), 100 mg ($n = 86$), or 150 mg ($n = 85$) all twice daily] against placebo ($n = 85$). The study nearly met its primary endpoint of annual rate of decline in FVC. Specifically, the adjusted annual rate of decline in FVC was 0.06 l/year in the group receiving nintedanib 150 mg twice daily and 0.19 l/year in the placebo group corresponding to a reduction of 68.4 % in the rate of loss of FVC [$p = 0.06$ using a closed testing procedure for multiplicity correction (primary analysis) and $p = 0.01$ using hierarchical testing, both prespecified] (Richeldi et al. 2011). In addition, compared with placebo, significantly fewer patients in the group receiving nintedanib 150 mg twice daily had a decline in mean FVC of ≥10 % or ≥200 ml (23.8 versus 44.0 %, respectively; $p = 0.004$). Furthermore, compared to placebo, the highest dose of nintedanib was associated with a lower incidence of AE-IPF (2.4 versus 15.7 per 100 patient-years, respectively; RR: 0.16; $p = 0.02$) and an improved quality of life as assessed by St. George's Respiratory Questionnaire (SGRQ) (−0.66 versus +5.46 points, respectively; $p = 0.007$). Overall, nintedanib showed an acceptable safety profile. The most frequent adverse events in the group receiving nintedanib 150 mg twice daily were diarrhea (55.3 versus 15.3 % in the placebo group), nausea (23.5 versus 9.4 % in the placebo group), and vomiting (12.9 versus 4.7 % in the placebo group).

Diarrhea, nausea, and vomiting were also the adverse events that most frequently led to study discontinuation, although the proportion of patients who discontinued the study medication due to adverse events did not differ between the nintedanib 150 mg twice-daily group and the placebo group. Clinically significant elevations in liver enzyme levels (e.g., at least three times the upper limit of the normal range for aspartate aminotransferase or alanine aminotransferase at any time after baseline) were observed in six patients in the group receiving 150 mg of nintedanib twice a day (7.1 %) and none in the placebo group; however, only two patients discontinued the study medication because of persistently elevated liver enzyme levels. The INPULSIS trials (INPULSIS-1 and INPULSIS-2) were two parallel 52-week, double-blind, randomized, placebo-controlled, phase III studies designed to confirm the efficacy and safety of nintedanib 150 mg twice daily in patients with IPF (Richeldi et al. 2014). Eligibility criteria for IPF patients were identical to those of the TOMORROW trial. A total of 1066 patients were randomized in a 3:2 ratio to receive either nintedanib 150 mg twice daily ($n = 309$ in INPULSIS-1 and $n = 329$ in INPULSIS-2) or placebo ($n = 204$ in INPULSIS-1 and $n = 219$ in INPULSIS-2). Both trials met the primary endpoint. In fact, nintedanib significantly reduced the rate of decline in FVC over the 52-week study period. The adjusted annual rate of decline in FVC was −114.7 ml in the nintedanib arm and −239.9 ml in the placebo arm in INPULSIS-1 (between-group difference: 125.3 ml; $p < 0.001$) and −113.6 and −207.3 ml in INPULSIS-2 (between difference: 93.7 ml; $p < 0.001$), respectively. A series of prespecified sensitivity analyses corroborated the robustness of the results of the primary analysis. In addition, in both trials, patients in the nintedanib arm were more likely to be stable at week 52 than those in the placebo arm (e.g., to have a decline in percent predicted FVC of no >5 %) (52.8 versus 38.2 % in INPULSIS-1, $p = 0.001$; and 53.2 versus 39.3 % in INPULSIS-2, $p = 0.001$, respectively). Furthermore, nintedanib, as compared to placebo, reduced the risk of disease progression (e.g., absolute decline in percent predicted FVC of ≥10 % or death) by 47 % in INPULSIS-1 (24.3 versus 40.7 % in the placebo group; HR: 0.53; $p = 0.0001$), by 33 % in INPULSIS-2 (29.8 versus 42.0 % in the placebo group; HR: 0.67; $p = 0.0054$), and by 40 % in the pooled analysis (27.1 versus 41.4 % in the placebo group; HR: 0.60; $p < 0.0001$). Subgroups analyses of pooled data from the INPULSIS trials showed that gender, age (<65, ≥65 years), ethnicity (White, Asian), baseline FVC % predicted (≤70, >70 %), baseline SGRQ total score (≤40, >40), smoking status (never, ex/current), systemic corticosteroid use (yes, no), and bronchodilator use (yes, no) did not influence the efficacy of nintedanib on the decline in FVC over 52 weeks (Costabel et al. 2016). As for the two key secondary endpoints (e.g., time to the first AE-IPF and change from baseline in the total score on the SGRQ), the two trials provided conflicting results. In fact, the time to the first AE-IPF was significantly increased in INPULSIS-2 (HR: 0.38, $p = 0.005$) but not in INPULSIS-1 (HR: 1.15, $p = 0.67$). However, a prespecified sensitivity analysis of pooled data from INPULSIS-1 and INPULSIS-2 showed that the time to first *adjudicated* AE-IPF (confirmed or suspected) was significantly increased with nintedanib compared to placebo (HR: 0.32, $p = 0.001$). Similarly, while in INPULSIS-2 there was a significantly smaller

increase in the total SGRQ score (consistent with less deterioration in health-related quality of life) in the nintedanib group than in the placebo group at week 52 (2.80 points in the nintedanib group versus 5.48 points in the placebo group; $p = 0.02$), in INPULSIS-1 the between-group difference in the adjusted mean change in the SGRQ total score from baseline to week 52 was not significant (4.34 points versus 4.39 points; $p = 0.97$). Finally, in a prespecified pooled analysis, there was no significant between-group difference in either all-cause (5.5 versus 7.8 %; HR: 0.70; $p = 0.14$) or respiratory-related (3.8 versus 5.0 %; HR, 0.74; $p = 0.34$) mortality. Similar to the TOMORROW trial, the most frequent adverse event in the nintedanib groups in both INPULSIS-1 and INPULSIS-2 was diarrhea (experienced by approximately 60 % of patients within the first 3 months of treatment), which led to premature study discontinuation in 4.4 % of patients in the nintedanib group (Corte et al. 2015). However, in both trials, the proportion of patients with serious adverse events did not differ between the nintedanib and placebo groups. In October 2014 nintedanib was approved by the FDA for use in patients with IPF irrespective of disease severity as assessed by functional impairment and has been licensed in Europe in early 2015.

11.5 Antiacid Therapy

Gastroesophageal reflux (GER), including clinically silent GER, is highly prevalent in patients with IPF (Raghu et al. 2006b, 2015b), and markers of aspiration (e.g., bile acids and pepsin) are significantly elevated in bronchoalveolar lavage fluid (BALF) from IPF patients compared to patients with non-IPF interstitial lung disease and healthy controls (Savarino et al. 2013). In addition, levels of inflammatory biomarkers such as lactate dehydrogenase, alkaline phosphatase, C-reactive protein, and TNF-α are higher in BALF of newly diagnosed IPF patients compared to newly diagnosed GER disease patients (Lozo Vukovac et al. 2014). Chronic microaspiration of gastric content is considered a risk factor for the development or worsening of the disease, suggesting that treatment of GER could play a role in the management of IPF. In an uncontrolled retrospective study of 204 IPF patients, antiacid treatment (AAT) was associated with reduced radiological fibrosis and was an independent predictor of longer survival time (Lee et al. 2011). Moreover, a post hoc analysis of data derived from patients randomized to placebo in three IPFnet-sponsored clinical trials showed that patients taking antiacid medications at baseline [either proton pump inhibitors or H_2 blockers; $n = 124/242$ (51 %)] had a smaller decrease in FVC at 30 weeks than those not taking AAT ($p = 0.05$) after adjusting for sex, baseline percentage predicted FVC, and baseline percentage predicted DL_{CO} (Lee et al. 2013). Conversely, a more recent post hoc analysis of patients assigned to placebo in three large randomized controlled trials [$n = 624$, 291 of whom (47 %) were taking AAT at baseline] showed that AAT did not improve progression-free survival (defined as FVC decrease $\geq$10 %, 6MWD decrease $\geq$50 m, or death), FVC decline, hospitalization, and all-cause and

Table 11.3 Selected compounds in development for IPF

Target	Putative role in IPF	Mechanism of action	Developmental phase/status	Drug code (ClinicalTrials.gov identifier)
NOX1/NOX4	Mediator of TGF-β1-induced fibroblast differentiation into myofibroblasts	NOX1/NOX4 inhibitor	Preclinical	GKT137831
Telomerase	Alveolar epithelial cell proliferation and epithelial repair	Telomerase activator	Preclinical	GRN510
SSAO	Profibrotic and proinflammatory cytokine production; extracellular matrix deposition	SSAO inhibitor	Preclinical	PXS4728A
TGF-β1	Major profibrotic cytokine	TGF-β1 inhibitor	Preclinical	PXS64; PXS25; Disitertide
SHIP1	Pluripotent regulator of hematopoietic cell function	SHIP1 activator	Preclinical	AQX-1125
IL-13	Myofibroblast differentiation and collagen deposition	IL-13 inhibitor	Phase I; completed	QAX-576 (NCT01266135)
Galectin-3	Mediator of TGF-β-induced lung fibrosis	Galectin-3 inhibitor	Phase I/II; ongoing	TD139; GR-MD-02 (NCT02257177)
PI3K-α and mTOR	Mediator of cell growth and survival	PI3K-α and mTOR inhibitor	Phase I; ongoing	GSK-2126458/omipalisib (NCT01725139)
Type V collagen	Autoimmune response to collagen V leading to fibrosis	Inductor of immune tolerance to collagen V	Phase I; completed	IW001 (NCT01199887)
TGF-β	Major profibrotic cytokine	Inhibition of all isoforms of TGF-β	Phase I; completed	Fresolimumab; GC-1008 (NCT00125385)
mTOR	Cell growth	mTOR inhibitor	Phase Ib; unknown	MLN0128_101 (Eudract number, 2013-003524-36)
mTOR	Cell growth	mTOR inhibitor	Phase II; ongoing	Sirolimus (NCT01462006)
Pentraxin-2	Modulator of monocyte differentiation	Recombinant human pentraxin-2	Phase Ib; completed	PRM-151 (NCT01254409)
LPA receptor	Epithelial cell apoptosis, endothelial cell leak and fibroblast accumulation	LPA receptor inhibitor	Phase II; ongoing	BMS-986020 (NCT01766817)

(continued)

Table 11.3 (continued)

Target	Putative role in IPF	Mechanism of action	Developmental phase/status	Drug code (ClinicalTrials.gov identifier)
B lymphocytes	Autoantibody-mediated lung injury	Pan-B-cell inhibitor	Phase II; ongoing	Rituximab (NCT01969409)
Integrin-α_v-β_6	TGF-β activation	α_v-β_6 inhibitor	Phase II; ongoing	STX-100 (NCT01371305)
CTGF	Major profibrotic cytokine	CTGF inhibitor	Phase II; ongoing	FG-3019 (NCT01890265)
IL-13	Myofibroblast differentiation and collagen deposition	IL-13 inhibitor	Phase II; ongoing	Lebrikizumab (NCT01872689)
IL-4 and IL-13	Myofibroblast differentiation and collagen deposition	IL-4 and IL-13 inhibitor	Phase II; completed	SAR156597 (NCT01529853)
LOXL2	Cross-linking of type-1 collagen molecules	LOXL2 inhibitor	Phase II; ongoing	Simtuzumab (NCT01769196)
IL-13	Myofibroblast differentiation and collagen deposition	IL-13 inhibitor	Phase II; active, not recruiting	Tralokinumab (NCT01629667)
IL-13	Myofibroblast differentiation and collagen deposition	IL-13 inhibitor	D2212C00002 J-Phase II; completed	Tralokinumab (NCT02036580)

CTGF connective tissue growth factor, *IL* interleukin, *JNK* c-Jun-N-terminal kinase, *LOXL2* lysyl oxidase-like 2, *LPA* lysophosphatidic acid, *mTOR* mammalian target of rapamycin, *NOX* NADPH oxidase, *PI3K-α* phosphatidylinositol 3-kinase-α, *SHIP1* SH2-containing inositol-5′-phosphatase 1, *SSAO* semicarbazide-sensitive amine oxidase, *TGF* transforming growth factor

IPF-related mortality. Furthermore, AAT was associated with a significantly higher rate of all-cause hospitalization (HR: 1.4, $p = 0.042$) (Kreuter et al. 2015). Despite the lack of evidence from prospective RCTs, the recently updated guidelines recommend AAT for patients with IPF based on the potential benefit and the favorable side effect profile of antiacid medications (Raghu et al. 2015a). However, further research focusing on efficacy and long-term safety of AAT in patients with IPF is clearly needed.

11.6 Most Developed Compounds for IPF

In recent years, improved understanding of disease pathogenesis has resulted in an exponentially increasing number of potential therapeutic targets in IPF (Table 11.3). Some of the compounds in most advanced development for IPF are listed below.

PRM-151 PRM-151 (Promedior, Inc., Lexington, MA, USA) is a recombinant form of human pentraxin-2 (PTX-2), a protein that is specifically active at sites of tissue injury. PRM-151 binds to Fc-gamma receptors on monocytes and induces

their differentiation into regulatory macrophages, thus promoting epithelial healing and scarring resolution. In a multiple ascending dose phase I study in patients with IPF, PRM-151 was safe and well tolerated and demonstrated a trend toward improvement in pulmonary function and circulating levels of surfactant protein D and VEGF at 8 weeks (Van Den Blink et al. 2013). PRM-151 is currently being tested in IPF in a phase II trial (clinicaltrials.gov identifier NCT02550873).

IW001 Ongoing adaptive immune response against autoantigens is thought to play an important role in disease progression in a sizeable proportion of patients with IPF (Kahloon et al. 2013). Type V collagen [col(V)] is a minor collagen normally confined within the lung interstitium and, therefore, hidden from the immune system. Lung injury may lead to col(V) exposure, making it available for activation of an autoimmune response (Sumpter and Wilkes 2004), which may result in abnormal lung remodeling and fibrotic changes (Vittal et al. 2013; Parra et al. 2006). In a phase I study (clinicaltrials.gov identifier NCT01199887), IW001 (ImmuneWorks, Inc., Indianapolis, IN, USA), an orally available compound that induces immune tolerance to col(V), was safe and well tolerated and showed a trend toward stabilization of FVC and metalloproteinase 7 levels in anti-col(V) Ab^+ IPF patients (Wilkes et al. 2015).

TD139 Galectin-3, a galactoside-binding lectin, has been shown to play a central role in fibrosis development and progression through the activation of macrophages and recruitment and activation of myofibroblasts (Nishi et al. 2007). TD139 (Galecto Biotech AB, Copenhagen, Denmark) is a specific inhibitor of the galactoside-binding pocket of galectin-3 formulated for inhalation. Safety and tolerability of TD139 in patients with IPF are currently being evaluated in a phase Ib/IIa trial (clinicaltrials.gov identifier NCT02257177).

BMS-986020 Lysophosphatidic acid (LPA) is a proinflammatory and profibrotic mediator released by platelets following epithelial damage (Watterson et al. 2007), and lysophosphatidic acid receptor 1 (LPA1) contributes to the development of IPF by inducing epithelial cell apoptosis, fibroblast recruitment and vascular leak (Tager et al. 2008; Xu et al. 2009). The safety and efficacy of BMS-986020 (Bristol-Myers Squibb, New York, NY, USA), a selective antagonist of LPA1, is currently being assessed in patients with IPF in a phase II trial (clinicaltrials.gov identifier NCT01766817).

FG-3019 It is a monoclonal antibody directed against connective tissue growth factor (CTGF), a key mediator of tissue fibrosis. Safety and tolerability of FG-3019 (FibroGen, Inc., San Francisco, CA, USA) in patients with IPF have been evaluated in a phase II dose escalation open-label study. Data of the first dose cohort (15 mg/kg IV every 3 weeks) have been published in the form of an abstract (Raghu et al. 2014b). Fifty-three subjects were enrolled and treated. Overall, FG-3019 was safe and well tolerated. The majority of patients [27 of the 38 (71 %) who had acceptable data at week 48] experienced improvement or <5 % decline in FVC % predicted at week 48. In addition, improved or stable lung fibrosis as measured by quantitative HRCT was observed in more than half of subjects. On average, patients with improved or stable fibrosis also had improved pulmonary function. A

randomized placebo-controlled phase II trial in patients with IPF is currently enrolling participants (clinicaltrials.gov identifier NCT01890265).

Lebrikizumab Lebrikizumab (Hoffmann-La Roche, Basel, Switzerland) is a humanized monoclonal antibody against interleukin (IL)-13. In vitro, IL-13 stimulates fibroblast proliferation and induces CCL6/C10, a chemotactic factor for mononuclear phagocytes, which in turn are an important source of mediators of extracellular matrix synthesis (Belperio et al. 2002). In vivo, IL-13 overexpression results in increased fibrosis in mice in response to bleomycin. In addition, elevated blood levels of Th2 inflammation, including IL-13, are associated with accelerated functional decline and mortality in patients with IPF (Herazo-Maya et al. 2013). Lebrikizumab (as both monotherapy and as combination therapy with pirfenidone background therapy) is currently being tested in patients with IPF in a phase IIb study (clinicaltrials.gov identifier NCT01872689).

Tralokinumab Tralokinumab (MedImmune LLC, Gaithersburg, MD, USA) is a human IL-13-neutralizing antibody. The IL-13 pathway is significantly enhanced in biopsy samples from IPF patients who exhibit a rapidly progressive disease course compared with patients with a slower rate of decline (Murray et al. 2014). Inhibition of human IL-13 by tralokinumab has been shown to attenuate established lung fibrosis and to promote alveolar epithelial repair in a humanized severe combined immunodeficiency (SCID) mouse model of IPF (Murray et al. 2014). Safety, tolerability, and effectiveness of multiple doses of tralokinumab are being evaluated in phase II studies (clinicaltrials.gov identifier NCT01629667 and NCT02036580, the latter only in Japanese patients), which are ongoing yet currently not recruiting patients.

SAR156597 IL-4, a cytokine structurally related to IL-13, has been implicated in the abnormal fibroblast proliferation in IPF (Jakubzick et al. 2004a), and targeting of IL-13 and IL-4 receptors modulates the proliferative properties of human lung fibroblasts (Jakubzick et al. 2004b). SAR156597 (Sanofi S.A., Paris, France) is a humanized bispecific antibody that neutralizes circulating IL-4 and IL-13. In a phase I study in patients with IPF, SAR156597 has been shown to be generally safe and well tolerated (Soubrane et al. 2014). The safety and efficacy of two dose levels of SAR156597 administered subcutaneously during 52 weeks in patients with IPF are currently being tested in a double-blind, randomized, placebo-controlled phase II study (clinicaltrials.gov identifier NCT02345070).

Simtuzumab A fibrotic matrix has been suggested to represent a self-perpetuating stimulus to collagen production by lung fibroblasts (Klingberg et al. 2014). Simtuzumab (Gilead Sciences, Foster City, CA, USA) is a humanized monoclonal antibody that allosterically inhibits lysyl oxidase-like 2 (LOXL2), a matrix-associated enzyme that cross-links collagen. LOXL2 protein expression is observed in the *fibroblastic foci* and collagenous regions of diseased IPF lung tissue (Barry-Hamilton et al. 2010), and serum LOXL2 levels are associated with increased risk for IPF disease progression (Chien et al. 2014). Moreover, in the bleomycin-induced mouse model of pulmonary fibrosis, inhibition of LOXL2 resulted in a

markedly decreased TGF-β pathway signaling, probably by decreasing matrix tension, a stimulus for TGF-β activation (Barry-Hamilton et al. 2010). The safety and efficacy of simtuzumab in patients with IPF are being evaluated in a phase II study (clinicaltrials.gov identifier NCT01769196), which is ongoing but currently not recruiting patients.

STX-100 STX-100 (Biogen Idec, Weston, MA, USA) is a monoclonal antibody against the integrin αv-β6, which plays an important role in promoting and maintaining fibrogenesis and epithelial injury by mediating TGF-β activation (Akhurst and Hata 2012). In murine bleomycin-induced pulmonary fibrosis, partial inhibition of αv-β6 effectively inhibits TGF-β activation, epithelial injury, and tissue fibrosis (Horan et al. 2008), whereas complete inhibition of TGF-β through gene deletion causes excessive inflammation in many organs, including the lungs (Kulkarni et al. 1993). The safety and tolerability of subcutaneously administered multiple, escalating doses of STX-100 in patients with IPF are being evaluated in a phase II study, which is currently recruiting participants.

Mesenchymal Stem Cells Mesenchymal stem cells (MSCs) are multipotent stromal cells capable of transdifferentiation, clonality, and self-renewal. MSC properties also include immunomodulation, epithelial repair, and secretion of growth factors (Huleihel et al. 2013). MSCs have been shown to ameliorate inflammation and mitigate parenchymal remodeling in bleomycin-induced pulmonary fibrosis (Weiss and Ortiz 2013), but the bleomycin model recapitulates only partially the complex pathobiology of IPF (Moeller et al. 2008). Therefore, the application of MSCs in patients with IPF is controversial and under studied (McNulty and Janes 2012). In a small cohort of patients with IPF ($n = 14$), Tzouvelekis and colleagues have shown that endobronchial infusion of autologous adipose-derived stem cells is not associated with serious adverse events (Tzouvelekis et al. 2013). However, despite their acceptable safety profile, there is only limited preclinical data to support the use of MSCs in IPF. In addition, because of their mesenchymal origin, MSCs may potentially differentiate into a fibroblast phenotype, thus promoting fibrosis (Mora and Rojas 2008). Other unanswered questions include the timing (early or advanced disease) and optimal route of administration (intravenous or endobronchial), source of MSCs (e.g., adipose tissue, bone marrow, or umbilical cord), frequency of infusions, and appropriate primary endpoints to show benefit. As of November 2015, there are three ongoing clinical trials evaluating the safety of either autologous (clinicaltrials.gov identifier NCT01919827 and NCT02135380) or allogenic (clinicaltrials.gov identifier NCT02013700) MSCs in patients with IPF.

11.7 Conclusions

Idiopathic pulmonary fibrosis is a progressive and almost invariably fatal disease. Over the last decade, our knowledge of the mechanisms involved in disease pathobiology has substantially improved, and this had allowed a number of clinical

trials of pharmacological interventions to be undertaken and completed. This massive effort of the medical and industry community has produced the approval of two drugs of comparable safety and efficacy profile, pirfenidone and nintedanib, which will soon become standard of care worldwide. Yet, this is only the beginning as neither pirfenidone nor nintedanib is a cure for IPF, neither drug improves lung function, and most patients continue to progress while on treatment. These limitations notwithstanding the concerted effort by the scientific, professional, and patient community and the pharmaceutical industry have the potential to finally develop a real cure for patients suffering from this devastating disease.

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Chapter 12
Treatment of Pulmonary Alveolar Proteinosis

Muhammad Muhye-ud-din Sheikh and Bruce C. Trapnell

Abstract Pulmonary alveolar proteinosis (PAP) is a rare syndrome caused by the disruption of pulmonary surfactant homeostasis that results in hypoxemic respiratory insufficiency (Trapnell et al. 2003). The syndrome occurs in a heterogeneous group of distinct diseases usefully divided into disorders of surfactant production and disorders of surfactant clearance. The former comprises disorders caused by mutations in genes required for normal surfactant production (e.g., *SFTPB*, *SFTPC*, *ABCA3*, *NKX2.1*). Disorders of surfactant clearance can be further divided into primary and secondary PAP. Primary PAP is caused by disruption of GM-CSF signaling, either due to neutralizing granulocyte-/macrophage-colony-stimulating factor (GM-CSF) autoantibodies as occurs in the disease autoimmune PAP or due to mutations in the genes encoding the GM-CSF receptor alpha or beta genes (*CSF2RA* or *CSF2RB*). Secondary PAP is caused by another disease or condition that reduces either alveolar macrophage numbers or surfactant clearance (and other) functions. These include hematologic diseases (most commonly myelodysplasia), immune deficiency/chronic inflammatory diseases, toxic inhalation disorders (e.g., respirable silica, aluminum, titanium), and drug-induced phospholipidoses (e.g., cationic amphiphilic drugs such as amiodarone, fluoxetine, gentamicin) (Trapnell and Luisetti 2015). Whole lung lavage is widely held to be therapeutically effective in patients with autoimmune PAP and hereditary PAP, and is also effective in some diseases associated with Secondary PAP but is not effective in patients surfactant production disorders. Off-label use of GM-CSF has been studied in autoimmune PAP and appears to be effective, however, further studies are needed to establish

M.M.-d. Sheikh, M.D.
Division of Pulmonary, Critical Care, and Sleep Medicine, University of Cincinnati Medical Center, Cincinnati, OH, USA

B.C. Trapnell, M.D. (✉)
Division of Pulmonary, Critical Care, and Sleep Medicine, University of Cincinnati Medical Center, Cincinnati, OH, USA

Division of Pulmonary Medicine, Cincinnati Children's Hospital Medical Center, Room 4029, CCRF, Cincinnati, OH 45229-3039, USA

Division of Pulmonary Biology, Perinatal Institute, Cincinnati Children's Hospital Medical Center, Room 4029, CCRF, Cincinnati, OH 45229-3039, USA
e-mail: Bruce.Trapnell@cchmc.org

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safety and to identify an optimal dose. These and other therapeutic approaches are reviewed in this chapter.

Keywords PAP • Whole lung lavage • GM-CSF • Rituximab • Plasmapheresis

12.1 Introduction

Pulmonary alveolar proteinosis (PAP) is a rare syndrome caused by the disruption of pulmonary surfactant homeostasis that results in hypoxemic respiratory insufficiency (Trapnell et al. 2003). The syndrome occurs in a heterogeneous group of distinct diseases usefully divided into disorders of surfactant production and disorders of surfactant clearance. The former comprises disorders caused by mutations in genes required for normal surfactant production (e.g., *SFTPB*, *SFTPC*, *ABCA3*, *NKX2.1*). Disorders of surfactant clearance can be further divided into primary and secondary PAP. Primary PAP is caused by disruption of GM-CSF signaling, either due to neutralizing granulocyte-/macrophage-colony-stimulating factor (GM-CSF) autoantibodies as occurs in the disease autoimmune PAP or due to mutations in the genes encoding the GM-CSF receptor alpha or beta genes (*CSF2RA* or *CSF2RB*). Secondary PAP is caused by another disease or condition that reduces either alveolar macrophage numbers or surfactant clearance (and other) functions. These include hematologic diseases (most commonly myelodysplasia), immune deficiency/chronic inflammatory diseases, toxic inhalation disorders (e.g., respirable silica, aluminum, titanium), and drug-induced phospholipidoses (e.g., cationic amphiphilic drugs such as amiodarone, fluoxetine, gentamicin) (Trapnell and Luisetti 2015).

The nomenclature used to report on PAP in the medical literature is confusing at best, partly due to its early conceptualizion as a disease rather than a syndrome and partly due to the evolution of our understanding of the pathogenic mechanisms of PAP-causing diseases. For example, what is now referred to as autoimmune PAP has, in the past, been referred to variably and inconsistently as acquired PAP, idiopathic PAP, phospholipidosis, lipoproteinosis, phospholipoproteinosis, and other terms. Secondary PAP, while reported in association with a great number of underlying diseases, is usually referred to simply as PAP, thereby obfuscating the extreme mechanistic diversity in this group. Disorders of surfactant production have been most commonly referred to as congenital PAP (actually a misnomer based on the definition) and pulmonary surfactant metabolic dysfunction disorders. For readability, we will use the terms primary PAP, secondary PAP, and surfactant production disorders as defined above.

In considering therapies for PAP, it is important to recognize that PAP-causing diseases differ vastly with respect to pathogenic mechanism and therefore, in most cases, require different therapeutic approaches. For example, disorders of surfactant clearance can frequently (but not always) be treated by whole-lung lavage (WLL), a nonspecific procedure to physically remove PAP sediment by washing it out of the

lungs. In contrast, disorders of surfactant production generally don't respond to WLL and are currently treated by lung transplantation. Further, primary PAP caused by GM-CSF autoantibodies can be treated by administration of recombinant GM-CSF, but hereditary PAP caused by *CSF2RA*/*B* gene mutations cannot. Because autoimmune PAP accounts for approximately 85–90 % of cases and enormous strides have been made in our understanding disease pathogenesis, identifying molecular targets for pharmacotherapeutic development, in this chapter, we will focus on current and future therapies for autoimmune PAP.

12.2 Pathogenesis

12.2.1 Surfactant and Surfactant Homeostasis

In 1958, PAP was recognized as a syndrome of unknown etiology characterized by the accumulation of periodic Acid-Schiff (PAS)-positive, lipoproteinaceous material in alveoli based on a series of 27 patients (Rosen et al. 1958). A decade later, the lipoproteinaceous material was noted to include surfactant phospholipids that had also been identified only a few years earlier (Ramirez and Harlan 1968).

Ultrastructural and biochemical studies confirmed that the lipoproteinaceous material was primarily surfactant (Costello et al. 1975; Honda et al. 1993; Singh et al. 1983).

Surfactant is produced and secreted into alveoli by type-II alveolar epithelial cells and is comprised of ~85 % phospholipids (predominantly phosphatidylcholine), ~5 % neutral lipids (predominately cholesterol, but also containing triglycerides, free fatty acids), and ~10 % surfactant proteins (A–D) (Weaver and Whitsett 1991). It forms a multi-lamellar structure at the liquid–air-tissue interface within the alveolus and plays an essential role of stabilizing alveoli by reducing alveolar surface tension and preventing alveolar collapse (Bachofen and Schurch 2001). Surfactant homeostasis, which is also essential for alveolar stability and normal lung function, is maintained by the balanced secretion and clearance, by uptake and recycling and catabolism in type II alveolar epithelial cells, and by uptake and catabolism in alveolar macrophages (Wright and Dobbs 1991).

12.2.2 Role of GM-CSF

GM-CSF is a 23-kD dimeric glycoprotein produced by various cells including alveolar epithelial cells, macrophages, lymphocytes, mesothelial, and other cells (Francisco-Cruz et al. 2014). GM-CSF signals via cell-surface receptors comprised of an alpha chain (CD116) that mediates initial binding, a beta chain (CD131) that increases binding affinity, and the beta chain-associated kinase JAK2 (Gearing

et al. 1989; Hayashida et al. 1990). Ligand binding results in JAK2-mediated autophosphorylation as well as phosphorylation of alpha and beta chains and activation of multiple intracellular signaling pathways (D'Andrea and Gonda 2000; Matsuguchi et al. 1997; Watanabe et al. 1996).

An important albeit serendipitous first clue about PAP pathogenesis was the discovery, in 1994, that GM-CSF-deficient mice spontaneously develop a phenotype remarkably similar to human PAP (Dranoff et al. 1994; Stanley et al. 1994). Studies in these mice indicated PAP was due to reduced surfactant clearance, not increased surfactant production (Dranoff et al. 1994). Results in these mice also first identified the critical role of GM-CSF in surfactant homeostasis, alveolar stability, and lung function. Of importance to both pathogenesis and pharmacotherapeutics, PAP in GM-CSF-deficient mice was normalized by aerosol administration of murine GM-CSF directly to the lungs but not by systemic administration (Reed et al. 1999). Expression of GM-CSF in the airway epithelium by transgenic gene replacement or by adenoviral vector-mediated gene transfer also normalized surfactant homeostasis in these mice (Zsengeller et al. 1998).

Subsequent studies demonstrated that the presence of GM-CSF in the lungs was essential for the terminal differentiation of alveolar macrophages and that PU.1, a master macrophage transcription factor, mediated many of the effects of GM-CSF in alveolar macrophages including regulation of phagocytosis, adhesion, phagolysosome formation, microbicidal activity, and surfactant catabolism (Berclaz et al. 2002a; Shibata et al. 2001) (Berclaz et al. 2002b). Indeed, retroviral vector-mediated reconstitution of PU.1 expression in GM-CSF-deficient alveolar macrophage cell line normalized many of these functions (Fig. 12.1).

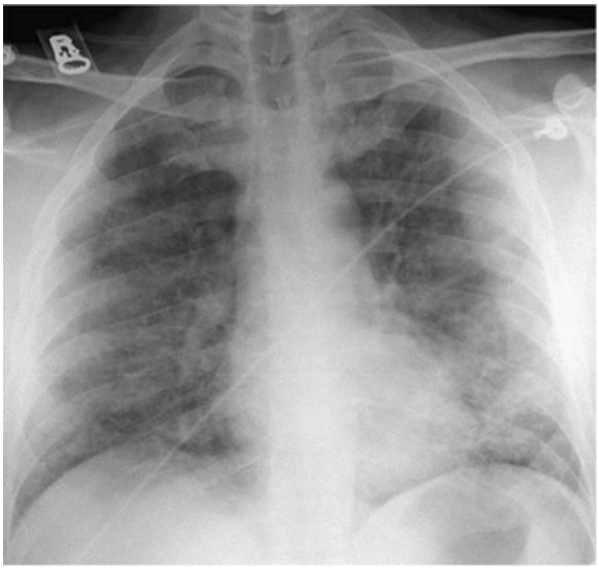

Fig. 12.1 Typical chest radiograph in autoimmune PAP. Reprinted from Murray & Nadel's Textbook of Respiratory, 6th edition, Authors: Trapnell, BC. And Luisetti, M., Pulmonary Alveolar Proteinosis Syndrome, Page No. 1274e2, Copyright 2016, with permission from Elsevier (Courtesy: Michael Gotway, MD.)

12.2.3 Role of GM-CSF Autoantibodies

A second important pathogenic clue was the observation by the Nakata group that GM-CSF autoantibodies were present in patients with idiopathic PAP (Kitamura et al. 1999). This finding was confirmed in several additional reports that also demonstrated a high level of such autoantibodies in idiopathic PAP but not in secondary PAP, surfactant production disorders, or other lung diseases (Bonfield et al. 2002b; Trapnell et al. 2003). Furthermore, at the high concentrations present in PAP patients, anti-GM-CSF antibodies were capable of eliminating GM-CSF bioactivity completely (Uchida et al. 2004). For example, the concentration of GM-CSF autoantibodies in 158 idiopathic PAP patients was 113 ± 7 μg/ml, while levels were <1 μg/ml in patients with secondary PAP, disorders of surfactant production, and other lung diseases (Uchida et al. 2004). Notwithstanding, their potential role in disease pathogenesis was uncertain because (1) GM-CSF autoantibody levels did not correlate with disease severity (Seymour et al. 2003), and (2) GM-CSF autoantibodies were also present in healthy people albeit at lower levels (Uchida et al. 2009). Subsequent studies answered this question. GM-CSF antibodies, isolated from patients with idiopathic PAP, were injected into healthy, nonhuman primates and resulted in replication of the key pathologic and biochemical features of disease including lung histopathology (surfactant-filled alveoli with preserved wall architecture, foamy alveolar macrophages) and molecular abnormalities (reduced PU.1, PPAR-gamma and ABCG1 transcription) (Sakagami et al. 2009, 2010). GM-CSF autoantibodies also impair neutrophil functions providing a mechanism to help explain the increased risk of opportunistic and non-opportunistic infections is PAP patients (Uchida et al. 2007). Thus, both serendipitous and directed observations led to our current understanding of idiopathic PAP as an autoimmune disease in which GM-CSF autoantibodies reduce or eliminate GM-CSF signaling in vivo causing a maturational arrest of alveolar macrophages and functional impairment of both macrophages and neutrophils (Fig. 12.2).

12.3 Epidemiology

The prevalence of autoimmune PAP has been estimated at 6–7 per in the general population (Inoue et al. 2008). The median age at diagnosis of autoimmune PAP varies from 39 to 51 in two reported series (Inoue et al. 2008; Seymour and Presneill 2002). A history of smoking is a risk factor for PAP. Analysis of all identified PAP patients reported in the medical literature between 1958 and 1979 (410 cases including all etiologies/diseases) revealed PAP was more common in men 71 % than women (*292 of 410 cases*), while no gender difference was present among nonsmokers (Seymour and Presneill 2002). In another report on 70 patients with autoimmune and secondary PAP in a single-center series from in Germany, 79 % of

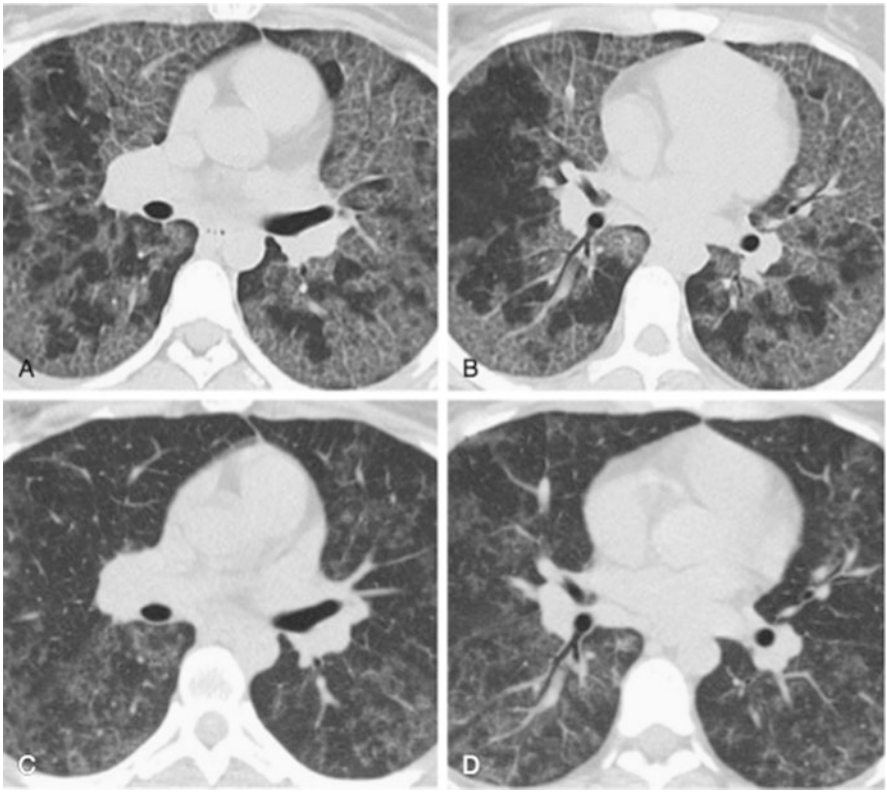

Fig. 12.2 Axial high-resolution CT images obtained at the initial time of diagnosis shows the typical appearance of "crazy paving" (**a** and **b**). Axial chest CT images obtained a year later showing reduction in ground-glass opacities. Patient had not undergone any therapy (spontaneous resolution). Reprinted from Murray & Nadel's Textbook of Respiratory, 6th edition, Authors: Trapnell, BC. And Luisetti, M., Pulmonary Alveolar Proteinosis Syndrome, Page No. 1274e8, Copyright 2016, with permission from Elsevier (Courtesy: Michael Gotway, MD.)

patients were either past or current smokers (Bonella et al. 2011). Conversely, other studies have not shown such a strong correlation. For example, among 223 patients autoimmune PAP in Japan, 43 % reported no history of exposure to tobacco smoke (Inoue et al. 2008). All together, smoking appears to be a risk factor for the development of autoimmune PAP. HLA antigen association studies have not been done in this population.

12.4 Clinical Presentation

Autoimmune PAP typically presents as progressive dyspnea of insidious onset although cough is a common finding (Inoue et al. 2008; Seymour and Presneill 2002). Physical exam commonly reveals inspiratory crackles and, in more protracted and severe cases, cyanosis (Goldstein et al. 1998). Since impaired alveolar macrophage activity is central to the pathogenesis of PAP, superimposed pulmonary infections are not uncommon. Weight loss, low-grade fever, chest pain, acute-onset dyspnea, and hemoptysis can be presenting signs and symptoms of PAP when a secondary infection is present (Seymour and Presneill 2002). Up to 30 % of cases can be asymptomatic at presentation with only radiographic abnormalities (Asamoto et al. 1995; Inoue et al. 2008). Although not studied prospectively, the clinical course appears to fall into three categories including spontaneous improvement in a small percentage of cases (~5–7 %), progressive decline, and stable or slowly progressive symptoms (most cases).

12.4.1 Radiographic Findings

Radiographic findings of PAP suggest the diagnosis but are not diagnostic (Mazzone et al. 2001). The chest radiograph typically shows symmetrical, bilateral infiltrates in a peri-hilar distribution with sparing the costophrenic angles simulating the appearance of pulmonary edema but without findings of congestive heart failure (Asamoto et al. 1995; Goldstein et al. 1998; Prakash et al. 1987). However, a number of other disorders present with a similar appearance (Wang et al. 1997).

Chest computed tomography (CT) typically reveals diffuse ground-glass opacification with superimposed interlobular septal thickening and interlobular lines—so-called crazy paving (Holbert et al. 2001; Rossi et al. 2003), a pattern that is characteristic of PAP but nondiagnostic. Crazy paving is seen in numerous other conditions including pulmonary edema, acute respiratory distress syndrome (ARDS), pulmonary hemorrhage syndromes, organizing pneumonia, nonspecific interstitial pneumonias, mucinous bronchoalveolar carcinoma, and atypical or *Pneumocystis* infection (Johkoh et al. 1999; Rossi et al. 2003).

Quantitative CT analyses (which rely on algorithms to calculate a score and airspace volume based on radiodensity measured in Hounsfield units) show good correlations with lung function parameters as measured by pulmonary function testing in patients with PAP (Guan et al. 2012; Lee et al. 1997). Quantitative CT may therefore become a useful tool for assessing response to therapeutic interventions such as whole-lung lavage and inhaled GM-CSF therapy (Guan et al. 2012). Three tesla (3 T) MRI detection of long T2 signals emanating from surfactant deposits within the alveoli is proving to be a very sensitive technique for assessing pre- and post-lung lavage changes in surfactant burden (Luo et al. 2013).

12.4.2 Pulmonary Function Tests

In many patients with autoimmune PAP, spirometry and lung volumes are normal. However, as the disease progresses, vital capacity and total lung capacity typically decrease resulting in a mild to moderate restrictive physiology (Selecky et al. 1977; Seymour and Presneill 2002). Importantly, the diffusion capacity (DLCO) is often severely and disproportionately decreased, reflecting the reduction in effective alveolar gas exchange interface. DLCO was $51 \pm 13\,\%$ predicted in one series (Briens et al. 2002) and $69 \pm 27\,\%$ in another (Inoue et al. 2008). Arterial blood gas analysis usually reveals a low PaO_2 and a widened alveolar–arterial oxygen gradient—indicating hypoxia, often without hypercarbia until very late in the course (Inoue et al. 2008; Seymour and Presneill 2002). A standard 6-min walk test often reveals near normal resting peripheral blood oxygen saturation and significant exercise-induced desaturation.

12.5 Diagnosis

The diagnosis of PAP is often delayed by many months from the onset of symptoms, in part by the insidious nature of the clinical presentation and in part because of the nonspecific clinical and radiographic findings. Further, it is often diagnosed as asthma or pneumonia until the failure of "appropriate" therapy prompts diagnostic reconsideration. Of importance, radiologic findings out of proportion (worse) compared to clinical symptoms is the norm and provide a useful clue PAP (Trapnell and Luisetti 2015). Flexible bronchoscopy and bronchoalveolar lavage (BAL) cytopathology and/or a transbronchial lung biopsy can establish the presence of the PAP syndrome. Grossly, BAL fluid is "milky" in appearance (Wang et al. 1997) and, microscopically, reveals large acellular eosinophilic bodies on a background of granular, PAS-staining sediment (Maygarden et al. 2001). Large "foamy" alveolar macrophages are a hallmark and occur due to their inability to clear surfactant (Martin et al. 1980). Pulmonary lymphocytosis is also commonly present (Milleron et al. 1991).

Historically, PAP has been identified based on a transbronchial or surgical lung biopsy—the latter has long been considered the "gold standard" for PAP diagnosis (Inoue et al. 2008; Seymour and Presneill 2002; Wang et al. 1997). However, a recent retrospective series of 25 patients revealed that a first transbronchial lung biopsy attempt identified PAP only about half the time while adding a second increased the yield to 80 % (Luo et al. 2015). Currently, the PAP syndrome is suspected based on typical history, physical exam, and chest CT findings and confirmed by BAL fluid analysis without the need for a lung biopsy (Bonella et al. 2011; Inoue et al. 2008; Xu et al. 2009). In a recent report from Germany on a series of 70 PAP patients, the primary method for PAP diagnosis was BAL fluid analysis (Bonella et al. 2011). Similarly, in a report from Italy on a series of

73 patients, only 56 % of patients had biopsy to confirm the diagnosis of PAP, while in the remainder, PAP was identified based on the chest CT, BAL cytology, and anti-GM-CSF antibody testing (see below) (Campo et al. 2013). Importantly, while a histopathologic examination of a lung biopsy and/or bronchoalveolar lavage cytology can identify the presence of the PAP syndrome, neither approach can diagnose the PAP-causing disease in any patient.

12.5.1 Anti-GM-CSF Antibodies: Role in Diagnosis

The availability of GM-CSF autoantibody testing—albeit as a clinical research test—has revolutionized the diagnosis of PAP (Trapnell and Luisetti 2015). In 2000, Nakata established a method for the serological diagnosis of PAP based on measurement of the GM-CSF autoantibody concentration in serum (Kitamura et al. 2000). One approach based on the use of an enzymatic immunosorbent assay (ELISA) was further developed and improved over a decade, and its sensitivity and specificity for a diagnosis of autoimmune PAP are both 100 % (Uchida et al. 2014). Using a PAP patient-derived, purified, polyclonal GM-CSF autoantibody reference standard, most healthy people and those with non-PAP lung diseases were found to have a serum GM-CSF autoantibody level of <3 mcg/ml, while more than 99 % of autoimmune PAP patients had a value of >9 mcg/m (Uchida et al. 2014). These results have been replicated using a serial dilution measurement approach, albeit without precise measurement of GM-CSF autoantibody concentration, which has shown that a serum GM-CSF autoantibody titer of $>1{:}1698$ was 100 % sensitive and 100 % specific for the diagnosis of PAP (Li et al. 2013). Thus, after establishing that the PAP syndrome is present as described above, it is now common practice to perform a serum GM-CSF autoantibody test to establish a "clinical research diagnosis" of autoimmune PAP. Algorithms for such an approach have been described in detail (Trapnell and Luisetti 2015).

12.5.2 Biomarkers

A number of biomarkers of PAP have been reported and include serum levels of GM-CSF, surfactant protein A (SP-A), SP-B, SP-D, carcinoembryonic antigen, Krebs von den Lungen (KL-6), lactate dehydrogenase, and CYFRA 21-1, all of which are increased in autoimmune PAP patients (Inoue et al. 2008; Seymour et al. 2003) [reviewed in (Trapnell and Luisetti 2015)]. However, most of these biomarkers are not specific for PAP, thus limiting their diagnostic utility (Brasch et al. 2004; Kuroki et al. 1998).

12.6 Current 'Standard' First-Line Therapy

12.6.1 Whole-Lung Lavage

Whole-lung lavage (WLL) has long been the standard first-line treatment for symptomatic pulmonary alveolar proteinosis (Ben-Dov and Segel 2014; Luisetti et al. 2010). The first whole-lung lavage was performed by Ramirez-Rivera in 1965 (Ramirez et al. 1965; Ramirez 1966). For decades, WLL was the only effective therapy for PAP, other than supportive measures like supplemental oxygen. Segmental flooding and lavage with fiberoptic bronchoscopes have also been reported frequently (Edis et al. 2007; Mclaughlin and Ramirez 1964; Rodrigo et al. 2015; Sadeghi 2013). Techniques vary among centers and have evolved over time to include more sophisticated patient monitoring and supportive measures (Rodi et al. 1995). To date, however, there are no randomized trials have been done to compare techniques for the purpose of optimizing and/or standardizing the procedure.

The indication for WLL is also not standardized among centers and a broad range of factors are used to trigger the initiation of WLL therapy including worsening dyspnea, functional limitation, and hypoxemia, e.g., indicated by arterial blood gas measurement of the PaO_2 or and alveolar–arterial (A-a) oxygen gradient (Michaud et al. 2009; Trapnell et al. 2003).

The WLL procedure is frequently performed in an operating room or intensive care unit under general anesthesia with hemodynamic monitoring of vital signs, oxygen saturation, blood gases, and end-tidal capnography (Michaud et al. 2009). An anesthesiologist is frequently present for double-lumen endotracheal intubation, airway management, general anesthesia, and management of ventilation of the untreated lung (Ben-Abraham et al. 2002). Operators vary in the placement of the treated lung in the dependent, nondependent, or decubitus position (Beccaria et al. 2004; Ernst and Michaud 2009; Michaud et al. 2009). Although not formally studied and reported, a general impression is that placing the lung to be treated in the nondependent position allows for both greater perfusion to the ventilated lung, thus improving the ventilation–perfusion ratio while optimizing the gravity-dependent drainage of emulsified surfactant from the treated lung. Fiberoptic bronchoscopy can be used during the WLL procedure to ensure ongoing maintenance of correct endotracheal tube positioning (Paquet and Karsli 2009). Single-lung ventilation studies can be done prior to initiating WLL and throughout procedure to ensure adequate ventilation and oxygenation before and throughout the procedure (Beccaria et al. 2004). In patients with severe hypoxemia, veno-venous extracorporeal membrane oxygenation can be effective and safe (Hasan et al. 2013; Krecmerova et al. 2015).

The WLL procedure is achieved by instillation of aliquots of sterile 0.9 % saline ranging from 0.5 to 2 l in volume, which are instilled through a circuit connected to the port of the endotracheal tube leading to the non-ventilated lung (Vymazal and Krecmerova 2015). The use of chest percussion while performing WLL, either

Fig. 12.3 Gross appearance of the lung lavage effluent obtained by therapeutic WLL. Saline (**a**), compared to the whole-lung lavage fluid from a patient with autoimmune PAP (**b**). The lavage fluid was allowed to settle and shows a "milky" appearance dense sediment. Reprinted from Murray & Nadel's Textbook of Respiratory, 6th edition, Authors: Trapnell, BC. and Luisetti, M., Pulmonary Alveolar Proteinosis Syndrome, Page No. 1269, Copyright 2016, with permission from Elsevier

manually to the hemithorax or to the entire chest using vibrating vest, is thought to increase the removal of the PAP sediment (Fig. 12.3). Manual percussion has been reported to be superior to mechanical percussion using handheld percussion devices or to WLL without chest percussion at all (Hammon et al. 1993). The use of repeated positional changes during WLL has also been reported to improve removal of sediment (Perez and Rogers 2004).

Other techniques to improve removal of surfactant-rich material focus on ventilating the lavaged lung with small volumes of air. One study reported delivering manual ventilation to the lavaged lung after instilling half the total volume for each cycle resulted in greater clearance of alveolar material and lower protein concentration in post-lavage BAL fluid and a longer time before relapse (Bonella et al. 2012).

The instilled lavage fluid is drained using gravity by lowering the head end of the bed. Strict account of volumes instilled and drained should be done to ensure maximal fluid removal after each cycle. WLL cycles are repeated until the effluent becomes significantly less opaque (Michaud et al. 2009).

Consensus has not been reached regarding the volume of instilled saline, number of instillation-drainage cycles, the length of lavage fluid dwell time, positioning of the treated lung, or use of chest percussion. An optimal strategy is one that leads to removal of the most PAP sediment, perhaps using the least number of cycles. Simulations using mathematical models have shown that the cumulative amount of eliminated protein is not affected by the duration of each cycle but dependent mostly on the total time of lavage and partially on the volume instilled (Akasaka et al. 2015). The amount of total protein removed from the lungs has been shown to correlate inversely with diffusing capacity of the lung for carbon monoxide (DLCO) and PaO_2 and directly with BAL KL-6 levels (Bonella et al. 2012). There are no correlations between quantity of proteinaceous material removed and serum GM-CSF antibody levels (Bonella et al. 2012).

Complications of lung lavage include adverse events related to general anesthesia, endotracheal intubation, and mechanical ventilation (Rebelo et al. 2012). The lavage itself poses risk of overspill of lavage fluid into the ventilated lung, barotrauma from infused saline resulting in hydropneumothorax, hypothermia, risk of refractory hypoxemia, and cardiac arrest during the procedure (Michaud et al. 2009). Notwithstanding, the procedure is generally well tolerated.

Although most patients improve after the first whole-lung lavage, many patients continue to require repeated lavages, months or years apart (Leth et al. 2013). In a series of 21 patients from Italy, only 3 patients required repeat lavage (Beccaria et al. 2004). In another series, also from Italy, 44 out of 73 patients required lung lavage, out of which 31 patients experienced durable benefit from a single lavage and did not require any further sessions. The Italian series reported no significant difference in baseline variables between patients who experienced sustained remission after a single lavage, compared to those who continued to require multiple lavages over time (Campo et al. 2013). Zhao et al. published one of the largest single-center series to date comprising 120 PAP patients, of whom 80 underwent WLL. Of these, 56 patients required only a single lavage, while 24 patients required repeated lavages (Zhao et al. 2015). Zhao and colleagues reported that the need for multiple lung lavages was significantly associated with pretreatment carcinoembryonic antigen (CEA) level, neuron-specific enolase (NSE) level, the 6-min walk distance, and percent-predicted diffusing capacity (DLCO).

There are no randomized trials or prospective studies comparing outcomes in patients who undergo WLL versus those who underwent placebo/sham treatment, as it would be unethical to withhold a treatment that is the standard of care. As such, the therapeutic impact of WLL may only be gauged from retrospective studies and case series. Seymour and Presneill (2002) published an analysis of 410 reported cases in literature over 44 years in which they reported that patients who underwent lavage at any time during the course of their disease had a superior survival. Patient, who underwent lavage, had a 5-year actuarial survival rate (from the time of diagnosis) of 94 (±2) % compared with 85 (±5) % for those not receiving such treatment. Since the data is retrospective, the comparison makes no allowance for the relative severity of the disease process itself, although it may be assumed that

those patients with more severe disease would have been more likely to undergo lavage.

In terms of functional outcomes, one of the largest series (Seymour and Presneill 2002) published to date with data on 410 patients and also reported statistically significant improvement in FEV1 (mean increase of 0.26 l), FVC (mean increase of 0.50 l), DLCO (mean increase of 4.4 ml/mmHg-min), arterial PO_2 (mean increase of 20.1 mmHg), and A-a gradient (mean decrease of 30.6). Another series of 80 patients who underwent lung lavage showed significant improvement in 6-min walk distance in addition to all of the above parameters, except for FEV1 (Zhao et al. 2015). In the Italian series from Pavia, more than 70 % of patients remained disease free at 7 years (Beccaria et al. 2004). In a retrospective analysis of data from more than 340 PAP cases over four decades, actuarial survival rates were estimated for cases after placing them in 10-year cohorts based on the year of initial publication. Five-year actuarial survival rates for patients reported in the years 1958–1967, 1968–1977, 1978–1987, and 1988–1997 were 52 %, 72 %, 93 %, and 100 %, respectively (Wang et al. 2012). The improvement in survival may be attributed, in part, to improved techniques and utilization of whole-lung lavage—the only effective treatment in that era.

12.7 Experimental Therapeutic Approaches

12.7.1 Inhaled GM-CSF Therapy

In 1999, Reed and colleagues showed that exposing GM-CSF-deficient mice to aerosolized GM-CSF could reverse the defects in macrophage function and surfactant clearance (Reed et al. 1999). This led to the study of the effects of inhaled GM-CSF in humans and its potential as a therapy for PAP.

Wylam and colleagues reported the first retrospective case series of 12 patients treated with aerosolized GM-CSF in lieu of whole-lung lavage (Wylam et al. 2006) (Tables 12.1). As whole-lung lavage was (and still remains) standard of care, the series essentially comprised patients who had refused to undergo WLL. Aerosolized GM-CSF was administered at a dose of 250 μg, twice a day—every other week. In one patient, the dose was increased to a maximum of 500 μg twice daily given every other week, because there was no response after at least 12 weeks of treatment. Eleven of the 12 patients showed response (response rate of 92 %). They reported improvement in mean arterial oxygen tensions—PaO_2 (improved by 17.1 mmHg), mean A-a gradient (reduced by 18.4 mmHg), mean diffusing capacity for carbon monoxide (DLCO) (improve by 16.6 % predicted), and mean FVC (improved by 13.5 % predicted) from the pretreatment values. Two patients experienced complete and sustained remissions at 2-year follow-up. However, it was notable that 5 patients (42 %) had recurrence of disease during a median follow-up period of 30.5 months.

Table 12.1 Clinical trials of inhaled GM-CSF therapy in autoimmune PAP

Study	Wylam et al. (2006)	Tazawa et al. (2010)	Ding et al. (2015)
Design	Case series—retrospective review	Prospective, multicenter, self-controlled, open-label, phase II clinical trial	Prospective, single-center, open-label clinical trial
Sample size	12	39	10
Dosing regimen	250 mcg twice daily (max: 500 mcg twice daily); every other week	Induction period: *Six cycles, 2 weeks each (12 weeks)* • Day 1–8: 125 mcg *twice* daily • Day 9–14: No drug therapy Maintenance period: *Six cycles, 2 weeks each (12 weeks)* • Day 1–4: 125 mcg *once* daily • Day 5–14: No drug therapy	Induction period: *Six cycles, 2 weeks each (12 weeks.)* • Day 1–7: 150 mcg *twice* daily • Day 8–14: No drug therapy Maintenance period: *Six cycles, 2 weeks each (12 weeks)* • Day 1–7: 150 mcg *once* daily • Day 8–14: No drug therapy
Duration of therapy	Variable	24 weeks	24 weeks
Response definition (or post treatment change)	↓ 12 mmHg in Oxygen A-a gradient ↑ 10 mmHg in PaO_2 ↑ 12 % in DLCO. ↑ 7 % in FVC *(mean change from baseline—retrospective study)*	↓ Oxygen A-a gradient by at least 10 mmHg at the end of 24 weeks of therapy	*After 6 months of therapy:* ↓ 16.1 mmHg in Oxygen A-a gradient (mean) ↑ 11.9 mmHg in PaO_2 (mean) ↑ in FVC % predicted ↑ in DLCO % predicted *($p < 0.01$ for each variable at 6 months compared to baseline)*
Response rate	92 %—(11 of 12 patients)	62 %—(24 of 39 patients)	N/A

Tazawa and colleagues published a multicenter, controlled, phase II trial done at nine centers in Japan (Tazawa et al. 2010). They enrolled 50 patients with a diagnosis of autoimmune PAP established by tissue biopsy/cytology and elevated anti-GM-CSF antibody levels (>3 μg/ml). Patients who had received lung lavage or prior GM-CSF therapy were excluded. Patients were treated with high-dose GM-CSF administration (125 mg twice daily on days 1–8, none on days 9–14) for six 2-week cycles and then low-dose administration (125 mg once daily on days 1–4, none on days 5–14) for six 2-week cycles. These two treatment periods were

intended to serve as induction and maintenance therapy, respectively. The authors reported an overall response rate of 62 % (24 out of the 39 patients who completed the high-dose treatment period) as defined by a decrease in A-a gradient of 10 mmHg or more from the pretreatment measurement. The mean improvement in A-a gradient among responders was 17.2 ± 2.1 mmHg. The treatment was safe, and the response was maintained in 83 % of the initial responders for 1 year, without the need for additional therapy. They also reported lower levels of KL-6 (a biomarker of disease severity) and no increase in the level of anti-GM-CSF antibodies (demonstrating that exogenous administration of GM-CSF does not seem to trigger a deleterious immune response) after treatment.

A more recent single-center trial from China (Ding et al. 2015) reported improvement in mean PaO_2, A-a gradient, vital capacity, and diffusing capacity of CO (DLCO) posttreatment in a group of ten patients who were treated with a different dosing schedule. Patients received high-dose therapy for 12 weeks (GM-CSF 150 μg twice a day on days 1–7, none for days 8–14—for 6 cycles) followed by low-dose therapy for 12 weeks (GM-CSF 150 μg inhaled once a day on days 1–7, none for days 8–14 (for 6 cycles)). Patients were followed-up for 1 year. No major adverse events were reported.

To assess the durability of GM-CSF treatment benefit, Tazawa et al. followed 35 out of the 39 patients in their original study who completed the high- and low-dose GM-CSF treatment phases for an additional 30 months of observation (Tazawa et al. 2014). They reported that 23 of the 35 patients (66 %) did not require any additional treatments during this follow-up period. Age, sex, baseline DLCO (diffusing capacity of carbon monoxide), baseline PaO_2, high-resolution CT scan scores, and serum markers (LDH, KL-6, CEA, SP-A, and SP-D) did not differ significantly between those who required additional treatment and those who did not. GM-CSF antibody titers do not seem to predict response to inhaled GM-CSF. The only variable that was significantly higher at baseline in the group that remained in remission was the forced vital capacity (FVC). Tazawa and colleagues suggested that a lower baseline FVC might predict the need for additional treatment after inhaled GM-CSF therapy. Specifically, a baseline FVC percent predicted of <80.5 % predicted the need for additional therapy with a sensitivity of 92 % and specificity of 74 % (as determined by receiver operating curve analysis). Interestingly, this is consistent with the findings of Seymour et al. in their study of subcutaneous GM-CSF in which a higher baseline vital capacity was predictive of better response to subcutaneous GM-CSF therapy (Seymour et al. 2001).

There are no head to head trials comparing the efficacy of GM-CSF therapy with whole-lung lavage or other more novel therapies such as rituximab or plasmapheresis. However, there is now enough prospective data on inhaled GM-CSF therapy that it is considered by most as a viable, safe, and noninvasive alternative to whole-lung lavage in mild to moderately symptomatic PAP patients (Papiris et al. 2015). Inhaled GM-CSF therapy results in radiologic (quantitative CT scores) and functional (symptoms, FVC, and DLCO) improvement as well as improvement in oxygenation (PaO_2, A-a gradient). The effects are sustained for up to 2.5 years in two-thirds of patients (Tazawa et al. 2014). The cumulative/pooled initial response

rate for inhaled GM-CSF therapy as determined by meta-analysis of pooled data from the 2010 study by Tazawa et al. and the earlier case series by Wylam and colleagues is 76.5 %, with a 15.2 % relapse rate (Khan et al. 2012).

The question as to why the response is heterogeneous and which subset of patients with autoimmune PAP respond best the inhaled GM-CSF therapy remains one that needs to be explored with further studies. The only consistent predictor of good response to therapy seems to be a higher (and in fact, normal) pretreatment vital capacity (Tazawa et al. 2014). It has been postulated that this may represent a subset of patients who have not developed pulmonary fibrosis (which has an association with PAP). This explanation was not supported by an analysis of chest computed tomography (CT) data, however, as no evidence of fibrosis was seen in most nonresponders in the same study (Tazawa et al. 2014). In another retrospective analysis (Arai et al. 2014), higher levels of the disease biomarker CYFRA 21-1 have been associated with better response to GM-CSF therapy. The underlying mechanism for this phenomenon is unclear. CYFRA 21-1 levels also correlate positively with disease severity (Arai et al. 2014), so it is conceivable that the response may be more pronounced and measurable in sicker patients. Commercial testing is not available and hence the utility at present is only relevant to research settings.

Inhalational GM-CSF therapy appears to be quite safe. Doses as high as 2500 mcg daily have been reported in the literature (Garber et al. 2015). No major adverse effects of inhaled GM-CSF therapy have been reported in prospective studies so far (Ding et al. 2015; Tazawa et al. 2010, 2014; Wylam et al. 2006). Adverse events that have been reported to occur during study period include fever, upper and lower respiratory tract infections, diarrhea, and tuberculous lymphadenitis—which may or may not be related to the treatment (Tazawa et al. 2010).

12.7.2 Subcutaneous GM-CSF Therapy

The first reported use of GM-CSF in the treatment of PAP was a single-patient case report from Australia in 1996 (Seymour et al. 1996). Subcutaneous administration of GM-CSF relieved symptoms and improved exercise capacity and oxygenation (A-a gradient). Their report sparked interest in studying the effects of subcutaneously administered GM-CSF in PAP patients via more robust (prospective) methodologies.

Kavuru and colleagues (2000) published the first prospective open-labeled observational study on the subject (Tables 12.2). Due to the rare nature of the condition, the study comprised of just four patients with a histopathologic diagnosis of PAP, all of whom were oxygen dependent. After 12 weeks of therapy with an escalating dosing algorithm of up to 9 μg/kg/day of subcutaneously administered GM-CSF, three of the four study participants no longer required supplemental oxygen. Among the three responders, there were statistically significant improvements in PaO_2, oxygen A-a gradient, lung volumes, diffusing capacity (DLCO), and

Table 12.2 Clinical trials of subcutaneous GM-CSF therapy in autoimmune PAP

Study	Kavuru et al. (2000)	Seymour et al. (2001)	Venkateshiah et al. (2006)
Design	Prospective, open-label observational study	Prospective, multicenter (multinational), open-label, observational study	Prospective, single-center, open-label, observational study
Sample size	4	14	25
Dosing regimen	Escalating, 3–9 mcg/kg once daily dosing for 12 weeks	3 mcg/kg/day dose for 5 days, increased to 5 mcg/kg/day dose on day 6. Dose increased (if no response) to a maximum of 30 mcg/kg/day	First month: 250 mcg once daily Second month: 5 mcg/kg once daily Third month: 9 mcg/kg once daily (increased to maximum of 18 mcg/kg once daily, if no response)
Duration of therapy	12 weeks	12 weeks	Up to 1 year
Response definition	*Not defined a priori*	Improvement of 10 mmHg in PaO_2	Improved of oxygen A-a gradient by 10 mmHg (on room air)
Post treatment change	PaO_2 ↑ by 18.5 mmHg Oxygen A-a gradient ↓ by 30 mmHg *(among the 3 responders)*	No significant increase in FVC and DLCO	Significant improvement in PaO_2, DLCO, total lung capacity, and 6-min walk distances in the group that responded
Response rate	75 % (3 of 4 patients)	43 % (6 of 14 patients)	48 % (12 of 25 patients)
Adverse effects (no. of patients)	Nausea and vomiting (1 of 4 patients)	Most common: local erythema and induration (6) Other: neutropenia, splenomegaly, drug-related fever, headache, and syndrome of fevers/chills/nausea	Most common: injection site reactions—redness (18), itching (11), swelling (12) Other: fatigue, fever, chills, cold-like symptoms, and headache

symptoms scores transitional dyspnea index (TDI). The fourth patient did not show any response to therapy and suffered progressive decline and death from respiratory failure. Despite the obvious limitations of an uncontrolled observational study with a very limited sample size, this study showed that the observed improvements were strongly indicative of therapeutic efficacy, as they were much greater than those expected from spontaneous resolution alone.

Seymour and colleagues (2001) published a multinational prospective study of 14 patients from the United States, Europe, and Australia who had a histopathologic diagnosis of PAP along with positive anti-GM-CSF antibodies. They administered 5–20 μg/kg/day doses of GM-CSF injected subcutaneously for a period of

12 weeks. Six out of the 14 patients (43 %) responded to therapy (which was defined as a 10 mmHg improvement in the A-a gradient) lasting a median of 39 weeks. The reported no statistically significant increase in spirometric values and diffusing capacity (DLCO). A partial response was seen in one patient after dose escalation, raising the possibility of a dose-dependent therapeutic effect.

A subsequent prospective, open-labeled study of twenty-five (25) patients conducted by Venkateshiah and colleagues (2006) showed a similar response rate using the same definition of clinical response as Seymour's study. Twelve out of the 25 patients in their study (48 %) had an improvement in oxygen A-a gradient of 10 mmHg of more, with a mean pre- versus posttreatment improvement of 20.8 mmHg in the group that responded. This group (i.e., roughly half of the study participants) had statistically significant improvements in PaO_2 (mean increase of 19.6 mmHg), DLCO (mean increase of 5.1 ml/min/mmHg), and total lung capacity (mean increase of 0.9 l). They also demonstrated improvements in 6-min walk test distance (mean increase of 432.5 ft) and improved radiographic opacity scores. Participants in this study reported improvement in symptoms as well as overall quality of life and functionality as measured by SF-36 scores.

The factors that predict therapeutic response to subcutaneous GM-CSF are not entirely clear. Seymour and colleagues (Seymour et al. 2001) measured biomarker levels before starting treatment and subsequently every 2 weeks during the study period and reported that normal serum LDH levels and higher plasma SP-B levels prior to starting therapy predicted a higher likelihood of response. SP-A and GM-CSF antibody titers did not predict treatment response. Whether GM-CSF antibody titer can be used as a predictor of response to GM-CSF therapy is unclear as the results are conflicting. Data reported by Bonfield and colleagues suggested that PAP patients with low titers of anti-GM-CSF antibodies have less active disease and respond to subcutaneous GM-CSF with a further decline in autoantibodies (Bonfield et al. 2002a). On the contrary, Seymour et al. did not find correlation between serum autoantibodies and PAP severity (Seymour et al. 2003). Similar to aerosolized (inhaled) GM-CSF therapy, a higher pretreatment vital capacity predicts response to subcutaneous GM-CSF (Seymour et al. 2001; Tazawa et al. 2014). In the trial by Seymour and colleagues, it was observed that patients that patients who responded to subcutaneous GM-CSF were more likely to have developed eosoinophilia during the course of therapy (Seymour et al. 2001).

In the study by Venkateshiah, subcutaneous administration of GM-CSF was associated with injection site reactions and other minor problems in 85 % of patients with PAP (Venkateshiah et al. 2006). A "first-dose" effect (fever, chills, nausea within 4 h of dosing) was reported in 4 out of 14 patients in the study by Seymour and colleagues, in addition to neutropenia, splenomegaly, headaches, and isolated drug fevers (Seymour et al. 2001).

12.7.3 Rituximab Immunomodulatory Therapy

Rituximab is a chimeric murine–human monoclonal antibody directed against CD-20: A B-lymphocyte-specific antigen (Grillo-Lopez et al. 1999). Rituximab has been shown to deplete human B cells in vivo and is FDA approved for the treatment of certain B-cell lymphomas (Harrison et al. 2014). The drug has shown success in treatment of autoimmune diseases such as ANCA-associated vasculitides (Geetha et al. 2015) and lupus nephritis (Iaccarino et al. 2015), and this led to hypotheses of its efficacy in autoimmune PAP.

Kavuru and colleagues performed a prospective, open-label, proof-of-concept, phase II clinical trial that enrolled ten adult, symptomatic, hypoxic patients with autoimmune PAP (Kavuru et al. 2011). All participants received two doses of rituximab in infusions of 1000 mg each, 15 days apart. The authors reported improved PaO_2, A-a gradient, TLC, and radiographic findings and improved symptoms by TDI. There were no significant improvements in FVC, DLCO, functional grade, or 6-min walk distance.

The mechanism of therapeutic response to rituximab is not entirely clear. The role of PPAR-gamma and ABCG1 in lipid homeostasis inside the alveolar macrophage is well established (Bonfield et al. 2003). Both have been found to be deficient in GM-CSF knockout mice as well as (human) patients with PAP (Bonfield et al. 2003; Thomassen et al. 2007). Furthermore, it has been shown that restoration of ABCG1 expression via various mechanisms (including PPAR-gamma-dependent expression) leads to reduced lipid accumulation and improved lung function in murine models of PAP (Malur et al. 2011a, b). Malur and Kavuru studied BAL samples from the patients enrolled in the open-labeled rituximab study and found that the expression of PPAR-gamma and ABCG1 mRNA expression increased 2.8- and 5.3-fold, respectively, after treatment with the drug (Malur et al. 2012). Additionally, they found alveolar macrophages from PAP patients were deficient in lysosomal phospholipase A2 (LPLA2), which is a key enzyme in surfactant degradation. Treatment with rituximab led to a 2.8-fold increase in LPLA2 expression (Malur et al. 2012). Hence, it is postulated that treatment with rituximab leads to improvement in lipid (and surfactant) homeostasis in the alveolar macrophage by increasing expression of PPAR-gamma, ABCG1, and LPLA2.

There are no head to head trials comparing efficacy of rituximab treatment with whole-lung lavage or GM-CSF replacement therapy. Nor are there any prospective studies on the effects of combination therapy. There is a single case report in which rituximab induced significant clinical improvement in a patient with autoimmune PAP and hypoxia, refractory to lung lavage, and GM-CSF replacement (Amital et al. 2010). The role of these "novel" therapies such as rituximab and GM-CSF replacement needs to be prospectively studied in combination with lung lavage.

12.7.4 Plasmapheresis

Plasmapheresis has been used in the treatment of antibody-mediated diseases such as myasthenia gravis (Zhang et al. 2014) and Goodpasture's syndrome (Cui et al. 2011). In theory, plasma exchange lowers circulating antibody levels and hence may improve pulmonary macrophage function and surfactant catabolism in autoimmune PAP.

To our knowledge, there are three reported cases (Bonfield et al. 2002a; Garber et al. 2015; Luisetti et al. 2009) in literature to date in which plasmapheresis was performed for PAP. Bonfield reported a case of a patient with PAP, refractory to repeated whole-lung lavages, who underwent multiple sessions of plasmapheresis and had improvement in resting PaO_2 and radiographic findings (Bonfield et al. 2002a). They found several-fold reduction in systemic anti-GM-CSF antibody levels and reported this in the context of their work on correlating antibody levels with response to treatment.

Luisetti and colleagues reported a case from Italy (Luisetti et al. 2009), of a patient with PAP not improving after multiple lung lavages over a year who underwent ten sessions of low-intensity plasma exchange (1.5 l in each session) without any clinical benefit. The authors showed a reduction in anti-GM-CSF antibody levels but noted that the posttreatment levels remained above the mean levels seen in their PAP population, and, hence, the reduction in antibody levels may not have been adequate. Whether more aggressive regimen of plasma exchange (more frequent and/or higher volume exchanges) would be more effective is unknown and requires further study.

More recently, Garber and colleagues reported a case of autoimmune PAP requiring very frequent lung lavages despite high doses of concomitant inhaled GM-CSF therapy and treatment with rituximab. The patient underwent whole-lung lavage, followed by five daily sessions of plasmapheresis, and immediately followed by a single dose of rituximab. The authors reported clinical improvement in dyspnea and improved DLCO. Moreover, the patient subsequently required less frequent lung lavages (Garber et al. 2015).

The efficacy and safety of plasmapheresis whether on its own or in combination with other treatment options remains uncertain at present and requires prospective (phase II and III) studies. The indications, optimal dose (volume of exchange), frequency, and number of exchanges remain a target of further research at this time. Outside of such research settings, the use of this semi-invasive treatment with potential adverse effects cannot be recommended.

12.8 Knowledge Gaps and Future Research

12.8.1 What Are the Optimum Dose, Dosing Schedule, and Duration of Treatment for Inhaled GM-CSF?

Studies on GM-CSF have used complicated dosing regimens derived from the use of GM-CSF in oncology literature. In their study, Wylam et al. reported using dose of 250–500 mcg twice daily on alternating weeks and reported a response rate of 92 % (Wylam et al. 2006). In comparison, the study by Tazawa et al. used lower doses of 125 mcg twice daily for 8 of 14 days in 12 repeating 2-week cycles followed by 125 mcg once daily for 4 of 14 days in 12 repeating 2-week cyles and reported a lower response rate of 62 % (Tazawa et al. 2010). The difference in response rates may indicate a potential dose–response relationship that needs to be studied further. Further studies are required to determine the optimal duration of treatment as well dosing schedule, which may enhance response rates.

12.8.2 What Characteristics Predict Good Response to GM-CSF Therapy?

As mentioned in the previous sections of this chapter, predictive biomarkers are needed to guide GM-CSF treatment. A higher pretreatment vital capacity seems to predict a favorable response to both subcutaneous and inhaled GM-CSF therapy (Seymour et al. 2001; Tazawa et al. 2014). Some studies have suggested that lower anti-GM-CSF antibody levels predict better response to subcutaneously administered GM-CSF; however, results are conflicting at best (Bonfield et al. 2002a; Seymour et al. 2003). There is no data to suggest correlation between anti-GM-CSF antibody levels and response to inhaled GM-CSF. Few biomarkers have been shown to *consistently* predict response to treatment (see section on GM-CSF therapy). Thus, there is need for further studies to explore the demographic, clinical, and biochemical characteristics that predict favorable response to GM-CSF therapy.

12.8.3 Is Combination of Lavage and GM-CSF Superior to Either Treatment Alone?

There are case reports in the literature that suggest administration of inhaled GM-CSF after lung lavage (combination therapy) can induce remission in patients who have not responded to either therapy alone (Yamamoto et al. 2008; Yu et al. 2014). Theoretically, it seems possible that reducing the burden of

neutralizing antibody and proteinaceous debris in the alveolar environment may enhance delivery of inhaled GM-CSF to its cell-surface receptors. At the time of writing of this chapter, there is an ongoing phase II/III randomized parallel group clinical trial in Italy being conducted by Luisetti and colleagues comparing WLL to a combination of WLL and GM-CSF therapy (Luisetti 2009) (NCT00901511).

12.8.4 What Is the Role of Novel Therapies: Rituximab, Plasmapheresis, and IV Immunoglobulin?

Though they are not novel, in the sense that they have been used for years in the treatment of other autoimmune diseases, therapies such as rituximab, plasmapheresis and IV immunoglobulin need to be further studied in the context of potential efficacy in autoimmune PAP. So far, data has been limited to case reports (see sections on rituximab and plasmapheresis).

12.8.5 Evidence-Based Practice Guidelines Are Needed

As more prospective studies and clinical trials are undertaken and some of the above questions are answered, the next logical step seems to be a consensus statement or practice guidelines to provide some standardization in the management of PAP patients.

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Chapter 13
Treatment of Lymphangioleiomyomatosis (LAM)

Mariam Anis and Francis X. McCormack

Abstract Treatment strategies for lymphangioleiomyomatosis (LAM) have historically been based on the antagonism of estrogen action, driven by the remarkable predilection of the disease for patients of the female gender, expression of estrogen and progesterone receptors on LAM cells, and anecdotes of accelerated progression caused by estrogen-containing agents, pregnancy, premenopausal status, and the menstrual cycle. Multiple small, uncontrolled antiestrogen trials have produced conflicting results, and the jury is still out on the role of GnRh agonists, estrogen receptor antagonists, and selective estrogen response modifiers in the treatment of LAM. Advances in the understanding of the molecular pathogenesis of LAM, especially the key role of dysregulation of the mTOR pathway, have identified multiple additional therapeutic targets. A recent double-blind randomized trial called MILES has revealed that the mTOR inhibitor, sirolimus, stabilizes lung function and improves measure of functional performance and quality of life in patients with moderately severe LAM. Menopausal status had a dramatic effect on disease progression in the placebo group of MILES, with a fivefold increase in the rate of lung function decline in the premenopausal cohort, stimulating interest in a future controlled trial of estrogen antagonism. This section provides a comprehensive review of the therapeutic strategies available for LAM.

Keywords Lymphangioleimyomatosis (LAM) • Mammalian Target of rapamycin (mTOR) • Vascular endothelial growth factor D (VEGF-D) • Sirolimus • Angiomyolipoma (AML)

13.1 Introduction

Lymphangioleiomyomatosis (LAM) is a rare, progressive, cystic lung disease that occurs much more frequently in women than in men. The prevalence of LAM ranges from 3.4 to 7.8 (Harknett et al. 2011) per million women in the general

M. Anis (✉) • F.X. McCormack
Division of Pulmonary, Department of Internal Medicine, Critical Care and Sleep Medicine, University of Cincinnati, Cincinnati, OH, USA
e-mail: anismm@ucmail.uc.edu; frank.mccormack@uc.edu

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population. Cystic changes consistent with LAM are present in about 26–47 % (Kitaichi et al. 1995; Cudzilo et al. 2013; Urban et al. 1999; Taylor et al. 1990) of women and 10–15 % of men with tuberous sclerosis complex, a neurocutaneous syndrome that affects about one million people worldwide. LAM also occurs in a sporadic form in patients who do not have TSC (S-LAM) and who are exclusive female, with one reported exception (Schiavina et al. 2007). Although at least fivefold less common than tuberous sclerosis-associated LAM (TSC-LAM), sporadic LAM (S-LAM) patients constitute approximately 75–85 % of those who present to pulmonary clinics and for enrolment in registries and trials. The most common clinical presentations of LAM include dyspnea on exertion and recurrent pneumothorax (McCormack et al. 2002; Costello et al. 2000; Moss et al. 2001; Ryu et al. 2006). Other manifestations include chylous pleural effusions or ascites and abdominal tumors, including renal angiomyolipomas and lymphangiomyomas (Henske and McCormack 2012).

13.2 Diagnosis

It is important to establish a confident diagnosis of LAM before embarking on a potentially protracted course of treatment. The diagnosis of LAM can be made on clinical grounds if a combination of typical features is present (Johnson et al. 2010). High-resolution CT (HRCT) scanning is a useful modality for LAM diagnosis, revealing round, uniform, thin-walled cysts diffusely distributed throughout the lungs. A confident clinical diagnosis of LAM requires both the presence of characteristic findings on CT scan and at least one clinical feature from the following list: an angiomyolipoma (kidney), thoracic or abdominal chylous effusion, lymphangioleiomyoma or lymph node involved by LAM, or definite or probable TSC (Johnson 2010; Johnson et al. 2010). A confident diagnosis can also be validated by a serum VEGF-D of >800 pg/ml in a patient with typical findings on CT of the chest (Young et al. 2010). Transbronchial biopsy has a yield of >50 % and appears to be safe in several small-case series (Ye et al. 2010; Zavala 1975; Meraj et al. 2012a), but interpretation of the small samples obtained requires consultation with expert pulmonary pathologists who are familiar with LAM (Fig. 13.1). Pulmonary thoracoscopic biopsy remains the gold standard for the diagnosis of LAM but, given the risk and morbidities (such as chronic thoracic pain), should only be pursued when the more conservative approaches above are nondiagnostic or when atypical historical or clinical features (such as a substantial smoking history, elevated Sjogren's antibodies, or a family history of emphysema) introduce diagnostic uncertainty. Occasionally, cytological findings on thin needle aspirates of retroperitoneal masses, abdominal or thoracic nodes, or chylous fluids can be used to confirm the diagnosis (Mitani et al. 2009; Meraj et al. 2012b). Typical histological features of lung biopsy specimens include smooth muscle cell infiltration of lymphatics, airways, vessels, and alveolar septa and cystic remodeling of the pulmonary parenchyma. Cells of both spindle-shaped and epithelioid morphologies are present. Positive immunohistochemical staining with

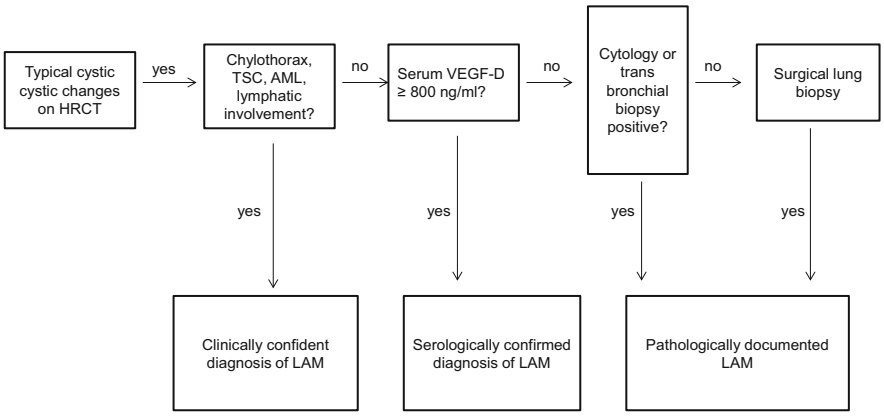

Fig. 13.1 Algorithm for diagnosis of LAM. In a patient with characteristic cystic changes on HRCT and a history of TSC, AML, or chylous effusions, a clinical diagnosis can be made, based on ERS guidelines (reference). If these characteristics are not present, a VEGF-D level >800 pg/ml can be used to establish a serological diagnosis. An attempt at cytological diagnosis can be made if chylous effusions or lymph nodes are available for transcutaneous needle aspirate or biopsy. Transbronchial biopsy has a yield of >50 %. Video-assisted thoracoscopy remains the gold standard, although risks of general anesthesia and chronic thoracic pain apply (modified from Meraj et al. 2012a, b)

antibodies to alpha smooth muscle actin, desmin, vimentin, and the human melanoma black 45 (HMB 45) supports the diagnosis of LAM. HMB-45 staining is especially useful when attempting to establish the diagnosis of LAM using small specimens such as transbronchial biopsies (Johnson 2006; Meraj et al. 2012b).

13.3 Molecular Pathogenesis of LAM

Advances in the understanding of the molecular pathogenesis of LAM have identified multiple potential targets for therapy (Fig. 13.2). Mutations in one of the TSC loci, TSC1 or TSC2, are found in cells within LAM involved areas in most cases, but typically in only a fraction of cells that comprise the lesion (Badri et al. 2013). In patients with TSC-LAM, but not in those with S-LAM, TSC mutations are also present in non-lesional cells, such as circulating blood leukocytes or histologically normal sections of lung or kidney. Thus, patients with TSC-LAM have germ-line mutations in TSC genes that are inherited or which occur during embryogenesis, whereas mutations in patients with S-LAM are thought to be due to two acquired somatic TSC mutations. The current metastatic paradigm for LAM holds that mutations in TSC genes result in unregulated cellular proliferation in an unknown peripheral source, local invasion and extravasation into the lymphatic tree facilitated by lymphangiogenic remodeling, spread via the lymphatic-venous circulatory axis to the pulmonary microvasculature, intravazation and invasion of the pulmonary parenchyma, and destructive remodeling driven by lymphangiogenic stimuli

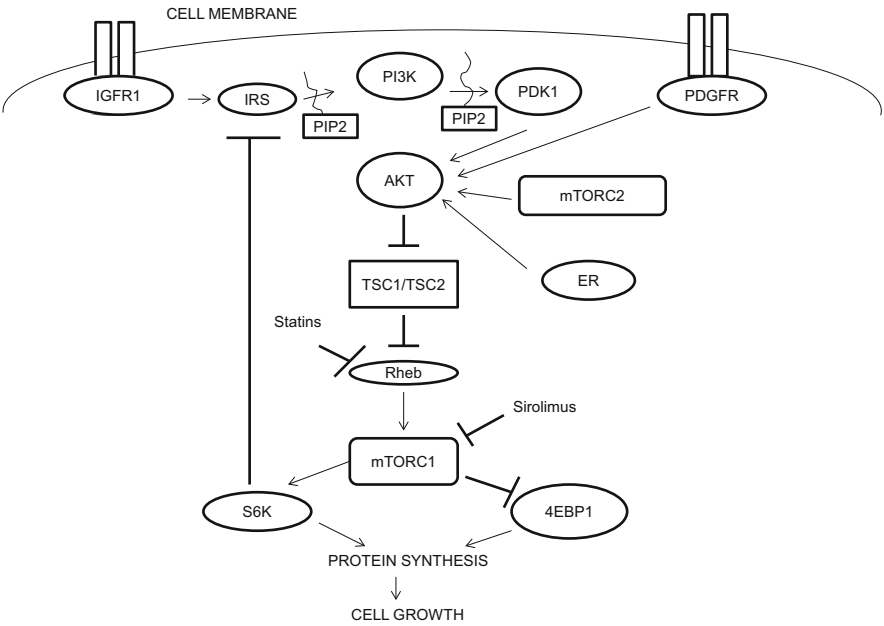

Fig. 13.2 Schematic overview of the TSC1/TSC2 signaling. The *solid arrows* denote activation and T-shaped bars denote inhibition. Growth factor receptors such as insulin (IGFR-1) and platelet-derived growth factor (PDGFR) upon phosphorylation lead to downstream activation of insulin receptor tyrosine kinase (IRS and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) followed by Akt. Akt can also be activated by estrogen receptor pathways (ER). This leads to phosphorylation of TSC2 blocking its ability to maintain Rheb in an inactivated state. Therefore, mutations in TSC1 and TSC2 genes lead to activated Rheb. Rheb in turn activates mTORC1 (mTOR-RAPTOR). Phosphorylation of S6K (pS6K) phosphorylates S6 and 4E-BP1, releasing elF4E, which together lead to cell growth. Activated S6 also inhibits the activation of IRS and its downstream effector Akt, providing an inhibitor feedback loop. Adapted from http://www.pathwaytx.com/technology.html

(Seyama et al. 2010). The molecular basis of these activities has become much clearer in the past decade. The TSC gene products, hamartin and tuberin, form a complex that negatively regulates the activity of the mechanistic target of rapamycin (mTOR), a critical node in a key cellular pathway that controls cell growth, survival, and replication. Heteroligomers composed of mTOR and raptor and other signaling molecules drive protein translation through S6 and 4EBP1 effectors, while complex formation between mTOR and rictor regulates cytoskeletal organization and motility through effectors, Rock and Rho. When mutations in TSC1 genes render tuberin or hamartin defective or deficient, mTOR is constitutively activated, leading to activation of downstream effectors that regulate protein translation, angiogenesis, lymphangiogenesis, survival, proliferation, and movement. The mTOR inhibitor sirolimus binds to FKBP12, forming a complex which blocks mTORC1 activity, but not mTORC2 activity (Goncharova and Krymskaya 2008). Estrogen appears to play an important role in mTORC1-mediated protein

synthesis by increasing the translation of a key transcription factor, Fra1 (El-Hashemite et al. 2005; Gu et al. 2013). The mTOR pathway is known to be regulated by multiple other signals including those arising from the sensing of cellular levels of amino acids, insulin, ATP, and mitogens (Krymskaya 2003; Nobukini and Thomas 2004). Lymphangiogenesis plays a key role in the progression and spread of LAM, and matrix-degrading enzymes such as MMPs are known to contribute to the extensive cystic remodeling in the lung (Finlay 2004; Kumasaka et al. 2004). Insights into the roles of mTOR activation, estrogen signaling, lymphangiogenesis, and remodeling of the cellular matrix have revealed a wealth of potential targets for the treatment of LAM (Fig. 13.2). Strategic approaches are based on each of the potential steps in neoplastic life cycle of LAM, including mutation, local invasion, migration/extravazation, invasion/intravazation, and tissue destruction (Fig. 13.3). For instance, for TSC patients who have biopsy-documented LAM in the uterus but only a few cysts in the lung, hysterectomy might be a reasonable future consideration for trials that attempt to prevent seeding of the lung, whereas trial strategies based on inhibition of tissue remodeling (such as MMP inhibitor therapy) may be most appropriate for patients who already have a heavy burden of LAM cells in the lung.

13.4 Overview of Hormonal Therapies

Although LAM occurs in both women and men, advanced, symptomatic LAM occurs almost exclusively in women. In most cases, the disease becomes apparent before menopause. Pregnancy and estrogen-containing medications have been reported to result in exacerbation of disease (Brunelli et al. 1996). LAM cells possess estrogen receptors (ER) and progesterone receptors (PR) (Berger et al. 1990). There are various case reports and case series of antihormonal approaches, including nonpharmacologic (oophorectomy) and pharmacologic therapies including progestins (medroxyprogesterone and progesterone), GnRh agonists (leuprolide, goserelin, triptorelin, etc.), and serum estrogen response modulators (SERMs) (tamoxifen) (Table 13.1). The data from these uncontrolled and retrospective studies are conflicting and inconclusive. The largest study (Taveira-DaSilva et al. 2004) of progesterone treatment to date did not reveal any evidence of a reduction in the rate of decline in forced expiratory volume in one second (FEV1) in the treatment group versus the untreated cohort. Although the rate of decline in diffusing capacity for carbon monoxide (DLCO) appeared to be greater in the treatment group, the study design suffers from the systematic bias termed confounding by indication, and it is not possible to conclude that progesterone accelerates decline in DLCO.

The recent ERS guidelines state that IM progesterone can be trialed if there is a rapid decline in lung function and should only be used for 12 months, but this approach is controversial (Johnson et al. 2010). Some authors argue that GNRH analogues may provide benefit when an adequate dose is used at an earlier stage

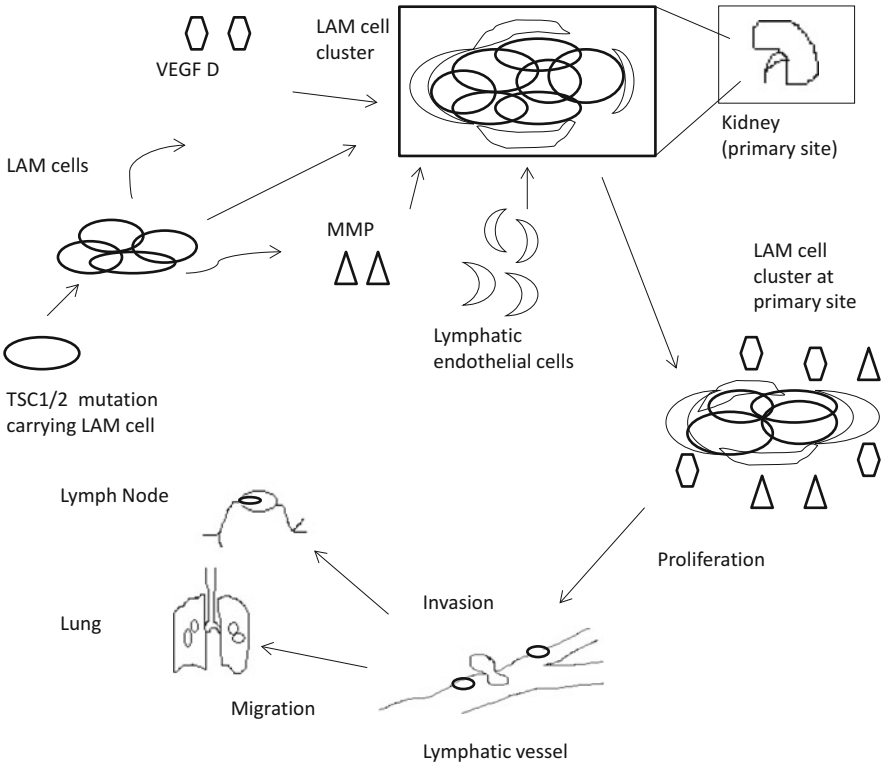

Fig. 13.3 Model of LAM pathogenesis. LAM cells originate from TSC1-/TSC2-deficient cells which secrete matrix metalloproteinases (MMP) and VEGF-D. VEGF-D recruits lymphatic endothelial cells to form LAM cell clusters. Estrogen inhibits apoptosis of LAM and induces metalloproteinases (MMPs), which result in breakdown of extracellular matrix facilitating invasion. LAM cells covered by lymphatic endothelial cells enter lymphatic vessels and migrate to other organs such as lungs and lymph nodes, where they induce remodeling (Yu and Henske 2010)

(Harari et al. 2008; Desurmont et al. 1996; Baldi et al. 2011). Tamoxifen is unproven and has partial agonist properties that have been linked to exacerbation in some patients (Yu et al. 2004). Combination treatments comprised of GNRH analogues and tamoxifen, or surgical oophorectomy with or without progesterone, have also not yielded conclusive results (Urban et al. 1992).

The rate of decline in lung function has been reported to slow after menopause in patients with LAM, suggesting a possible role for estrogen in disease progression (Johnson and Tattersfield 1999). In the placebo group of MILES, FEV1 fell by an average of about 40 cc in the first year in the postmenopausal group, compared to 200 cc in the premenopausal group (unpublished results, FXM). Trial strategies based on antagonism of estrogen action remain a high priority for the LAM community but are difficult studies to fund and enroll. A multicenter trial of estrogen suppression with the aromatase inhibitor letrozole in postmenopausal patients with LAM is underway in the United States.

Table 13.1 Hormonal therapies in LAM

Author	Year	Country	#pts	Median f/u	Study design	Therapy	Outcome	Meno (pre/post)
Eliasson et al. (1989)	1989	USA	30 cases 12 evaluable	All case reports	Meta	Prog; prog and ooph; prog and tamox; ooph and prog and tamox combined	Progesterone or oophorectomy or both are the most effective treatments, leading to improvement or stabilization of the disease in the majority of cases	Unk
Taylor et al. (1990)	1990	USA	32 pts, 19 prog	32 mos	Retro	Prog	In 2 pts, dyspnea improved (both with chylous effusions), 6 were unchanged and 11 declined	2 post 30 pre
Poh and Wang (1991)	1991	Singapore	3	11 yr	Prosp	Prog	1 pt improved, 2 were unchanged	Pre
Radermecker et al. (1992)	1992	USA	2 pts	13 and 5 mos	Prosp	Prog	No benefit	Pre 2
Kitaichi et al. (1995)	1995	Korea Taiwan Japan	60 patients with 40 evaluable	5 years	Prosp	Prog	5 deteriorated, 2 unchanged, and 5 nonevaluable	Unk
						Prog and GnRh agonist	1 deteriorated, 1 unchanged	Unk
						Prog and tamoxifen	3 deteriorated, 3 Nonevaluable	Unk
						GnRh agonist	4 deteriorated and 1 nonevaluable	Unk
						Ooph and GnRh agonist	1 deteriorated	Unk
Desurmont et al. (1996)	1996	France	2 pts	20 and 40 mos	Prosp	GnRh agonist	Stabilization	Pre 2

(continued)

Table 13.1 (continued)

Author	Year	Country	#pts	Median f/u	Study design	Therapy	Outcome	Meno (pre/post)
Urban et al. (1999)	1999	France	69 pts/34 evaluable. 57 dx by bx	8.2 ± 6.3 yrs (disease onset to death or closing date) years Range of f/u (0.8–27 yrs)	Retro, multictr	Prog ($n = 46$); tamox and prog ($n = 22$), GnRh agonists (14); ooph ($n = 5$) and somat ($n = 6$)	≥15 % FEV1 increase in 4 pts, (tamox and prog =2, prog =1; ooph = 1) 19 stabilized, 11 declined	7/57 diagnosed on biopsy were post
Johnson and Tattersfield (1999)	1999	UK	50 pts, 43 evaluable	Up to 3 yrs	Retro	21 pts prog (12 IM, 8PO, 1 by both routes), 5 ooph, 6 tamox and 5 lung pt	For both pre- and postmenopausal pts, there was a trend toward a slower decline in FEV1 in those receiving treatment ($p =$ n. s.)	Post 16
Seyama et al. (2001)	2001	Japan	11 pts 6 rx	7.4 yrs	Retro	Prog, tamoxifen, ooph, GnRh agonist	6/6 declined	
Taveira-DaSilva et al. (2004)	2004	USA	275	4 years	Retro	Prog either IM ($n = 72$) or PO ($n = 67$) versus untreated ($n = 136$)	FEV1 decline was similar in rx and unrx patients DLCO decline greater in tx than untx	Pre 161 Post 114
Harari et al. (2008)	2008	USA	11 pts	3 yrs	Prosp	GnRh agonist	All declined, except 1 pt with stabilization of FEV1	Post 11 Post 1
Park et al. (2010)	2010	Korea	63 pts 35 rx	17 yrs	Retro, multictr	Prog ($n = 17$); prog and tamox ($n = 8$), GnRh agonist ($n = 1$) and rapa ($n = 2$)	Inconclusive	Unk

Baldi et al. (2011)	2011	Brazil	9 pts	1 yr	Retro	GnRh agonist	FEV1 increased 80 ml and FVC increased 130 ml over 1 yr (p = n.s.). 5/9 increased FEV1, 3 unchanged, 1 declined	Unk

FEV 1 forced expiratory volume in 1 second, *FVC* forced vital capacity, total volume of air expired after a full inspiration, *Pts* patients, *Mos* months, *Dx* diagnosis, *BX* biopsy, *Unrx* untreated, *Rx* treated, *Pre* premenopausal, *Post* postmenopausal, *Unk* unknown menopausal status, *Meta* meta-analysis, *Retro* retrospective, *Prosp* prospective, *Multictr* multicenter, *Ooph* oophorectomy, *Prog* progesterone, *Tamox* tamoxifen, *Somat* somatostatin, *PO* by mouth, *IM* intramuscular, *yr* year

Table 13.2 Mtor inhibitors, prospective trials

Author	Year published	Country	Study design	# pts	Therapy	Median f/u	Outcome
Bissler et al. (2013) CAST	2008	USA	Open-label nonrandomized trial	25 pts with AML including 18 with LAM 11 with pulmonary outcome data	Target 10–15 ng/ml	24 mos total. 12 mos rx, 12 mos f/u	Significant reduction (53.2 % ± 26.6 %) ($p < 0.001$) in the AML volume 12 mos. FEV_1 and FVC increased by 10–15 %, respectively. Significant reduction in % pred RV. No sig. change in DLCO or 6MWD
Davies et al. (2011) TESSTAL	2011	USA	Prosp, multictr Phase 2 nonrandomized open-label trial	16 patients with TSC or sporadic LAM with renal AML	Steady-state blood levels of sirolimus 3–10 ng/ml	2 years	Reduction in summated AML diameters in all 16 pts and by 30 % or more in 8. FEV1, FVC and DLCO all declined in pts with available data
Dabora et al. (2011) Multicenter Phase 2 trial	2011	USA	Phase 2 multictr trial, open-label, single arm	36 adults with TSC or TSC/LAM 28 evaluable	6.7 mg/d, target serum level of 9–15 ng/ml	52 weeks	The mean decrease in kidney tumor size was 29.9 %. FVC increased by more than 5 % in 5/15 (33 %) TSC/LAM subjects and FEV1 increased by more than 5 % in 4/15 (27 %) TSC/LAM subjects
McCormack et al. (2011) MILES	2011	USA	Randomized double-blind comparison of sirolimus with placebo	89 pts	Sirolimus levels in the active-treatment group were maintained between 5 and 15 ng/ml	12 mo rx followed by 12 mo observation period	Improvement in FEV1, FVC, FRC, serum VEGF-D, QOL, and functional performance. No sig between-group difference in the change in 6MWD or DLCO

Franz et al. (2013) EXIST −1	2013	USA	Prospective, double-blind, randomized, placebo-controlled, Phase 3 study	117 patients, 44 with AML	Median-dose intensity (everolimus—5.5 mg/m^2/day; placebo: 5.7 mg/m^2/day)	9.7 months	>50 % reduction in SEGA size in 35 % of pts, compared to no SEGA shrinkage in the placebo group. No data on lung function was reported
Bissler et al. (2013) EXIST- 2	2013	USA	Double -blind, placebo-controlled, phase 3 trial	118 pts	Mean dose intensity was 8.6 mg/day in the everolimus group and 9.6 mg/day in placebo group	24 weeks	At week 24, 55 % of everolimus pts had at least a 50 % reduction from baseline in sum of volumes of target AML lesions compared with 0 % of placebo pts. Slightly lesser decline in DLCO and FEV1 in everolimus group compared to placebo

mTOR mammalian target of rapamycin, *mos* months, *FEV1* forced expiratory volume in 1 second, *FVC* forced vital capacity, *TLC* total lung capacity, *DLCO* diffusing capacity of lung for carbon monoxide, *6MWD* 6-minute walk distance, *Prosp* prospective, *Multictr* multicenter, *Retrosp* retrospective, *AML* angiomyolipoma

13.5 mTOR Inhibitor Therapies

Insight into the molecular pathogenesis of LAM has led to the recognition that over activation of the mTOR pathway is a key molecular target. Therefore, recent clinical trials have been focused on the use of mTOR inhibitors such as sirolimus and everolimus for treatment of LAM (Table 13.2). Sirolimus has been shown to reduce neoplastic proliferation in rodents with TSC (El-Hashemite et al. 2004; Kenerson et al. 2005; Lee et al. 2005).

There have been four recent notable trials testing sirolimus use for improvement in lung function and treatment of angiomyolipoma in patients with LAM and TSC. Below we discuss the salient features of these trials.

13.5.1 Cincinnati Angiomyolipoma Sirolimus Trial

Cincinnati Angiomyolipoma Sirolimus Trial (CAST) was an open-label, nonrandomized trial to determine if sirolimus therapy reduces the size of angiomyolipomas in patients with sporadic LAM or TSC (Bissler et al. 2008). 25 patients were enrolled. Sirolimus was administered for the first 12 months followed by an observation period of 12 months. The initial sirolimus dose was 0.25 mg/m^2 of body-surface area, targeted to achieve a serum level of 1–5 ng/ml. Further adjustments at 2 and 4 months were made to target serum levels of 6–10 ng/ml and 11–15 ng/ml, respectively, unless a 10 % reduction in angiomyolipoma diameter was achieved, in which case dose escalation was aborted. The dose was then continued through the end of the 12-month treatment period. The primary endpoint was change in angiomyolipoma volume at the end of trial. Serial MRIs of abdominal angiomyolipomas, brain lesions, and CT scans of chest were performed. Pulmonary function monitoring including FEV1, FVC, TLC, lung volumes, diffusing capacity, and 6-min walk distance was performed at baseline, 6, 9, 12, and 24 months. Sirolimus therapy resulted in nearly a 50 % reduction in angiomyolipoma volume and, surprisingly, an increase in FEV1 and FVC of 10 % and 15 %, respectively. However, after stopping sirolimus the angiomyolipoma volume and FEV1 returned toward the baseline. The increase in FVC and reduction residual volume was more durable and remained substantially improved from baseline at the end of 24 months. There was no change noted in DLCO or 6MWD during the treatment period. The most common adverse events during therapy were aphthous ulcers, which occurred in the majority of patients and resolved with topical treatment and sirolimus dose adjustment. About 50 % of patients had an increase in lipid levels. Serious adverse effects included diarrhea, pyelonephritis, respiratory infections, and stomatitis, several of which required hospitalization.

13.5.2 Trial of the Efficacy and Safety of Sirolimus in Tuberous Sclerosis and LAM (TESSTAL)

This multicenter Phase 2 nonrandomized open-label trial conducted in the United Kingdom included 16 patients with tuberous sclerosis or sporadic LAM and renal angiomyolipomas who were treated with oral sirolimus for up to 2 years (Davies et al. 2011). Sirolimus levels were maintained at 3–10 ng/ml. The primary outcome was reduction in size of renal angiomyolipomas measured by MRI. Secondary outcomes included safety, neurocognitive function, and pulmonary function. Pulmonary lung function testing including FEV1, forced vital capacity (FVC), and diffusing capacity of the lung for carbon monoxide (DLco) were done at baseline and at 4, 6, 12, and 24 months in all patients with LAM. Abdominal MRI without contrast was used to measure angiomyolipomas at baseline, 2, 6, 12, and 24 months. Sirolimus dose was adjusted to reach goal reduction of 10 % in angiomyolipoma diameter. This trial was similar to the CAST trial in terms of endpoints and design except patients received sirolimus for 24 months rather than 12 months. An overall response rate of 50 % was observed with 8 of 16 patients showing reduction in size of at least 10 % and about 80 % in per protocol group with 8 out of 10 showing reduction in angiomyolipoma size. Angiomyolipomas became smaller in all patients. Of 23 target angiomyolipomas evaluated at 24 months, 21 were smaller and 2 were unchanged. Compared to the CAST trial, the angiomyolipomas volume reduction was greater (about 60 %). Serum levels of sirolimus of <10 ng/ml appeared to be required for angiomyolipoma volume reduction, and most of the tumor shrinkage occurred in the first year of the trial. Tumor response to sirolimus was maintained due to the longer course of therapy compared to the CAST trial. The most common adverse effect was oral mucositis, followed by respiratory infection, including one death due to pneumonia. Although no improvement in lung function measurements were observed, there were only six patients who had pulmonary data available at the 12-month point.

13.5.3 Multicenter Phase 2 Trial of Sirolimus for Angiomyolipomas

In a Phase 2 study in patients with TSC or LAM, Dabora et al. demonstrated that rapamycin treatment for 1 year induced regression of kidney and liver angiomyolipomas and subependymal giant cell astrocytomas (SEGAs) (Dabora et al. 2011). Thirty-six patients (18–65 years of age) received rapamycin at a loading dose of 6 mg followed by 2 mg/day. Doses were subsequently adjusted to maintain plasma concentrations of 3–9 ng/ml (up to 16 weeks) and 9–15 ng/ml if there was no progression of tumor shrinkage. Adherence to the dosage regimen was not accomplished for most patients. Common adverse events (AEs) included stomatitis, hypertriglyceridemia, hypercholesterolemia, and bone marrow

suppression. Rare Grade 3 AEs included lymphopenia, headache, and weight gain; however, none of these events required intervention. At the end of the treatment period, there was a decrease in brain tumor and liver angiomyolipoma diameters, as well as improvement in skin lesions including plaques, facial angiofibromas, hypomelanotic macules, shagreen patches, and ungual fibromas. In patients with TSC/LAM, lung function stabilized in 15 of 24 female patients and in a subset (5/24) with features of moderate LAM, there was mild improvement in FEV1 and FVC. Other endpoints including seizure frequency and size of subependymal nodules and tubers did not show any changes after treatment with sirolimus. Serum VEGF-D levels were decreased by sirolimus and correlated with reduction in angiomyolipoma volume.

13.5.4 Multicenter International LAM Efficacy of Sirolimus Trial

Multicenter International LAM Efficacy of Sirolimus (*MILES*) was a two-stage, double-blind, randomized control, intention to treat trial designed to determine if treatment with sirolimus led to an improvement in lung function (FEV1) (McCormack et al. 2011). MILES enrolled 89 patients with LAM who had moderate lung impairment and were administered sirolimus for 12 months followed by a 12-month observation period. The primary endpoint was the difference between the placebo and sirolimus groups in the rate of change (slope) in FEV1. Secondary measures included between-group differences in mean changes from baseline to 12 months in FVC, DLCO, residual volume (RV), 6MWD, and serum VEGF levels. Sirolimus dose was adjusted to achieve a trough level of 5–15 ng/ml. All endpoints were evaluated on follow-up visits every 3 months in the treatment year and every 6 months in the observation year. The treatment year results demonstrated that the rate of change in FEV1 was 1 ± 2 ml/month in the sirolimus group and -12 ± 2 ml/month in the placebo group ($p < 0.001$). The absolute between-group difference in mean change in FEV1 during the treatment period was 153 ml or approximately 11 % of the mean FEV1 at the time of enrolment. At the 12 month visit, FEV1 values remained at or above baseline in 46 % of the patients in the sirolimus group compared to 12 % of patients from the placebo group ($p < 0.001$). Improvement in FVC and FRC in the treatment group was also significant compared to the placebo group. There were no significant differences between groups in DLCO, 6MWD, or RV. In the observation year when the drug was held, lung function decline resumed in the sirolimus group and paralleled that in the placebo group. Adverse events were more common with sirolimus, including oral mucositis and hypercholesteremia, but serious adverse events were balanced between the groups. These results indicate that sirolimus may be useful in treating patients with moderately severe LAM. The mean FEV1 for patients in MILES (48 % of the predicted value) was lower than the population-based mean among patients in the

NIH LAM registry (mean 70 % predicted), making it difficult to generalize the results from the patients in MILES to patients with milder or more severe lung disease due to LAM. Since the stabilization of lung function appears to require continuous exposure to sirolimus, additional trials are needed to determine which patients will benefit from treatment and the optimal dose and duration of treatment.

13.6 Other mTOR Studies

Subependymal giant cell astrocytomas (SEGAS) are brain tumors that cause significant morbidity in patients with tuberous sclerosis, including hydrocephalus. The Exist-1 study (Franz et al. 2013) was a double-blind, placebo-controlled trial which demonstrated that the mTOR inhibitor everolimus resulted in >50 % reduction in SEGA size in 35 % of patients, compared to no SEGA shrinkage in the placebo group. Pulmonary function was not reported. Everolimus received FDA approval for the treatment of SEGAs in patients with tuberous sclerosis in the form of an oral suspension in August 2012.

The Exist-2 trial was a double-blind, randomized-controlled trial that demonstrated that everolimus shrinks angiomyolipomas in patients with tuberous sclerosis or LAM (Bissler et al. 2013). Randomization was 2:1, everolimus-placebo. The response rate, defined as a 50 % or greater reduction in angiomyolipoma size, was 42 % in the treatment group and 0 % in the placebo group. LAM was present in 29/188 patients (22 in the everolimus group and 7 in the placebo group), including 18 patients with TSC-LAM and 11 patients with sporadic LAM. There appeared to be a small salutary effect on lung function, but interpretation of this exploratory endpoint was limited because of the short duration of treatment exposure (approx. 38 weeks) and the low number of patients. Everolimus received FDA approval for the treatment of AMLs in patients with tuberous sclerosis in 2012.

The RAD001x2201 trial is a recently completed, open-label study of everolimus in 24 patients with LAM. The results of the study have been presented in abstract form.

Data from a number of recent retrospective, observational studies, and case reports suggested that sirolimus has promise in improving lung function, resolving chylous effusions and reducing the size of renal angiomyolipomas (Taveira-DaSilva et al. 2011; McCormack et al. 2011; Neurohr et al. 2011) (Table 13.3).

Table 13.3 Mtor inhibitors

Author	Year published	Country	Study design	# pts	Therapy	Median f/u	Outcome
Egan et al. (2008)	2008	UK	Case report	1 pt	Started at 2 mg sirolimus qd/mean trough level of 2.6 ng/ml	At 9 mo f/u pt doing well and on 3 mg of sirolimus per day	FEV1 returned to baseline, exercise capacity improved HRCT = progressive radiologic improvement
Ohara et al. (2008)	2008	Japan	Case report	1 pt	Sirolimus at 1 mg/day was initiated 66 days after lung transplantation	Until chylous drainage resolved (35 days)	Reduction in chylous lung and peritoneal drainage
Neurohr et al. (2011)	2011	Germany	Observational, non randomized study w/o control group	10	Oral sirolimus target trough level of 5–10 ng/ml	12.1 ± 2.81 mos (range 6.1–28.1 mos)	No sig change in ABGs and mean TLC. FEV1, FVC, DLCO showed sig improvement from baseline. Modest increase in 6MWD in 5 pts
Casanova et al. (2011)	2011	Spain	Case series	3 pts	Sirolimus 2 mg/day	Case 1 = 35 mos Case 2 = 24 mos Case 3 = 28 mos	Case 1 had significant improvement in FEV1 = 870 ml, Case 2 showed mild improvement in FEV1 = 30 ml, Case 3 had delayed decline in lung function
Piha-Paul et al. (2011)	2011	USA	Case report	1 pt	Bevacizumab 2.5 mg/kg IV on the first day, along with temsirolimus 20 mg IV on d 1, 8, and 15 of a 21 d cycle	18 weeks	At week 18 (end of cycle 6), a sustained response was seen with a 68 % decrease in target lesion volume. Treatment is ongoing
Taveira-DaSilva et al. (2011)	2011	USA	Observational study	19 pts	Mean dose of sirolimus was 2.6 ± 0.9 mg/d (range, 1–5 mg/d),	Average duration of therapy = 2.6 ± 1.2 years (range, 0.7–5.4 y).	All lung measures sig increased including FVC (3.2 % ± 0.5 % predicted), FEV_1 (1.8 % ± 0.5 %

							predicted), and DLCO (0.8 % ± 0.5 % predicted) per year Complete resolution of effusions was noted after 410 ± 111 days of therapy
Moua et al. (2012)	2012	USA	Case report	1 pt	Maintenance serum level between 5 and 15 ng/ml	7 mos	FVC improved from 52 % pred to 75 %, FEV1 from 32 to 55 % pred. FEV1/FVC ratio and DLCO also improved. Chylothorax nearly resolved
Ando et al. (2013)	2013	Japan	Retrosp, observational	15 pts	Blood trough levels of sirolimus lower than 5 ng/ml	17.5 mos (SD, 5.9 mos)	Improvement in the annual rates of change in FVC and FEV1 in 9 pts without chylous effusions. In remaining 6/7 patients with chylous effusions the chylothorax resolved completely within 1–5 months of treatment
Cai et al. (2014)	2014	USA	Prosp	23 pts	Sirolimus dose adjusted to maintain serum levels between 5 and 15 ng/ml	2.2 + 0.4 yrs of sirolimus therapy	Detection rates of LAM cells were significantly decreased to 25 % in blood ($p < 0.001$) and 8 % in urine ($p = 0.003$). A greater loss of circulating LAM cells was seen in postmenopausal patients ($p = 0.025$)

mTOR mammalian target of rapamycin, *mos* months, *pts* patients, *pt* patient, *sig* significant, *FEV1* forced expiratory volume in 1 second, *FVC* forced vital capacity, *TLC* total lung capacity, *DLCO* diffusing capacity of lung for carbon monoxide, *6MWD* 6-minute walk distance, *Prosp* prospective, *Multictr* multicentric, *Retrosp* retrospective, *AML* angiomyolipoma, *HRCT* high-resolution CT scan, *pred* predicted

13.7 Role of VEGF-D as a Biomarker and Therapeutic Target

Serum vascular endothelial growth factor D (VEGF-D) levels are expressed by LAM cells and known to be elevated in patients with LAM. The combination of a serum VEGF-D level >800 ng/ml and cystic change on a chest CT scan is diagnostically specific for patients with sporadic LAM (Young et al. 2010). Patients requiring supplemental oxygen have higher baseline VEGF-D levels than who do not requiring oxygen, as do patients with a bronchodilator response, a low DLCO, and hyperinflation (Young et al. 2013). In addition, the serum VEGF-D level is associated with chylous effusions, lymphatic involvement, and a CT grade of severity (Xu et al. 2013; Glasgow et al. 2009). Serum VEGF-D levels are associated with disease severity and can be used clinically as a biomarker for disease progression and treatment response with sirolimus (Young et al. 2013; Chang et al. 2012). Patients with elevated VEGF-D levels are more likely to progress without therapy and to respond to therapy. VEGF-D levels decline with treatment on sirolimus and are positively correlated with improvement in FEV1 and FVC levels at 12 months (Young et al. 2013). It is not yet clear whether early VEGF-D responses (such as at 1 month) correlate with a beneficial effect on lung function at a later time point (such as at 12 months or later).

LAM cells also express VEGF-C in varying intensities, and expression correlates with degree of lymphangiogenesis and disease progression (Kumasaka et al. 2004). VEGF-C and VEGF-D bind to vascular endothelial growth factor receptor 3 (VEGFR-3) leading to lymphangiogenesis and parenchymal destruction.

13.8 Recommendations for Treatment in LAM

To date, sirolimus is currently the only proven therapy for LAM. LAM typically progresses slowly, and given the risks of sirolimus therapy, treatment decisions should be made on an individual basis. In most cases, for patients with disease on the milder end of the spectrum, a period of observation is warranted to assess the rate of disease progression. Treatment is currently recommended for patients who meet MILES enrolment criteria and who have no contraindications. These include patients with an FEV1 after bronchodilation of 70 % of the predicted value or less. Taken together, the data from case reports and small-case series and observational trials also suggests that patients with problematic chylous effusions and lymphangiomyomas should also be considered for therapy (Taveira-DaSilva et al. 2011). Treatment may also reasonable for patients with FEV1 > 70 % predicted but moderate reduction in DLCO, marked hyperinflation or dynamic hyperinflation, or rapidly progressive lung function decline, although definitive recommendations for this subset of patients await prospective trials. In the latter group, rather than observing lung function decline as a prerequisite for assessing

advisability of treatment, elevation in a biomarker such as VEGF-D might be incorporated into future diagnostic algorithms (Young et al. 2013). Given that postmenopausal patients tend to progress more slowly, special consideration should be given to monitoring rate of progression before embarking on a course of therapy with mTOR inhibitors in this subset of patients. A recent retrospective trial revealed that low-dose (1 mg/day) sirolimus was also effective for LAM, based on improvement in lung function and resolution of chylous effusions. Many LAM experts favor treatment with lower doses of sirolimus, such as 1 mg/day, with target serum levels between 2 and 7 ng/ml. Future studies to determine if VEGF-D can be used as a barometer of optimal mTOR pathway suppression and sirolimus dosing, if early therapy can prevent progression and protect the lung from further damage, and whether long-term, low-dose therapy is safe and effective, are high priorities for LAM research.

13.9 Practical Considerations for the Use of Sirolimus for Treatment in LAM

Consultation with an expert center should be considered (see LAM Clinic Network, www.thelamfoundation.org) prior to initiating treatment with mTOR inhibitors. Patients should be screened for contraindications to therapy, such as hypersensitivity, impending surgery, concurrent malignancies, and frequent infections. Baseline studies that should be obtained include complete blood count, renal profile, pregnancy test (for women of childbearing age) urinalysis, hepatic profile, and lipid profile. Sirolimus is usually started at 1 mg/day. A trough serum level can be drawn at 2–3 weeks, ideally as a trough level 24 ± 4 h following the last dose, with no dose interruption. It is good practice to see the patient at the 2–3-week point and then at 3-month intervals to determine if the drug is well tolerated and the sirolimus level remains in an acceptable range (generally <10 ng/ml, and ideally 5 ± 3 ng/ml). A dose of 1 mg will typically produce a serum level of about 2–3 ng/ml, but many drugs affect the metabolism of sirolimus, and patients should be provided with a list of interacting agents (Table 13.4) and instructed to call when new medications are added. Attempts should be made to choose compatible drugs so that the sirolimus therapy can continue uninterrupted. CBC, renal profile, sirolimus level, and fasting lipid profile should be repeated every 3 months. It is useful to plot FEV1 over time to determine if sirolimus results in a durable plateau in the rate of decline in lung function. Dose escalation can be considered if lung function stabilization does not occur or if chylous complications persist. Full pulmonary function tests and 6MWT should be obtained at baseline and every 6–12 months, with simple spirometry in between at 3- or 6-month intervals for patients who need closer monitoring, respectively.

Table 13.4 Partial list of interacting drugs and agents with sirolimus

Interacting drugs/agents
Live vaccines
St. John's Wort
Ketoconazole, itraconazole, fluconazole, voriconazole
Clarithromycin, erythromycin
Carbamazepine
Diltiazem, verapamil
Cyclosporine
Rifampin
Phenytoin

13.10 Precautions and Side Effects

The drug should be held for a period of about 2 weeks (1 week before and 1 week after) in settings where optimal wound healing is required, such as surgery or trauma. Good oral hygiene is recommended to prevent stomatitis. Mouth ulcers are usually self-limited and can be managed supportively with magic mouthwash containing lidocaine or Orabase (Agricola et al. 2013). Antibacterial mouth rinses such as chlorhexidine can be considered but may result in discoloration of teeth. Elevations in cholesterol should first be approached through dietary modifications, but pharmacologic therapy with statins may be required based on degree of elevation, family history, and personal risk factors (Stone et al. 2013). Lower extremity edema responds to physiotherapy and compressive stockings. Sirolimus pneumonitis is uncommon when used as monotherapy at the doses that are commonly employed in LAM, but it is a potentially serious development. There have been reports of fatalities due to sirolimus pneumonitis in other disorders where the drug is used for immunosuppression (Table 13.5). Sirolimus pneumonitis typically regresses when the drug is held. Latent malignancy is a theoretical risk with sirolimus and all immunosuppressive therapies and, unlike other side effects, persists after the drug is withdrawn. Sirolimus currently has a black box warning for lung transplant patients due to bronchial dehiscence in patients who were started on the drug postoperatively for primary immunosuppression. Most of these events occurred at 6–8-week posttransplant, most likely due to anti-angiogenic effects of sirolimus and failure of revascularization of the graft. It is therefore unclear whether mTOR inhibitors pose a wound healing risk when stopped on the day of transplant, as the drug washes out within a week, and several LAM patients who were treated with sirolimus while listed for transplant have fared well when the drug therapy was interrupted in this manner.

Table 13.5 Typical side effects experienced in patients taking sirolimus as monotherapy in low doses for LAM

More common	Less common	Uncommon but severe
Aphthous ulcers, usually self-limited Acne, usually self-limited, minor scattered lesions	Anemia	Pneumonitis
Lower extremity edema, usually after prolonged use, and persistent while drug therapy continues	Leukopenia	Hypersensitivity reaction
Elevated cholesterol	Elevation in creatinine	Hypertriglyceridemia
Impaired wound healing	Proteinuria	Latent malignancy
Dyspepsia	Hepatotoxicity	
Diarrhea/loose stools	Cough, fever	
	Headache	
	Hypertension	

13.11 Future Directions

There are many ideas for future therapies based on a rapidly expanding understanding of the molecular pathogenesis of LAM. Targetable steps include prevention of metastasis, implantation, infiltration, proliferation, and tissue destruction. Prominent among these include combination approaches with mTOR inhibitors and agents with potential to trigger apoptosis such as sirolimus + hydroxychloroquine (SAIL Trial, Henske-PI), or sirolimus + simvastatin (SOS Trial, Krymskaya-PI), anti-lymphangiogenesis therapies with pazopanib, soluble VEGFR-3 or anti-VEGFR-3, and antiestrogen strategies with GnRh agonists + aromatase inhibitors. The LAM Foundation Clinical Research Network, now comprised of over 45 clinics around the world, will facilitate the coordination and conduct of trials going forward.

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