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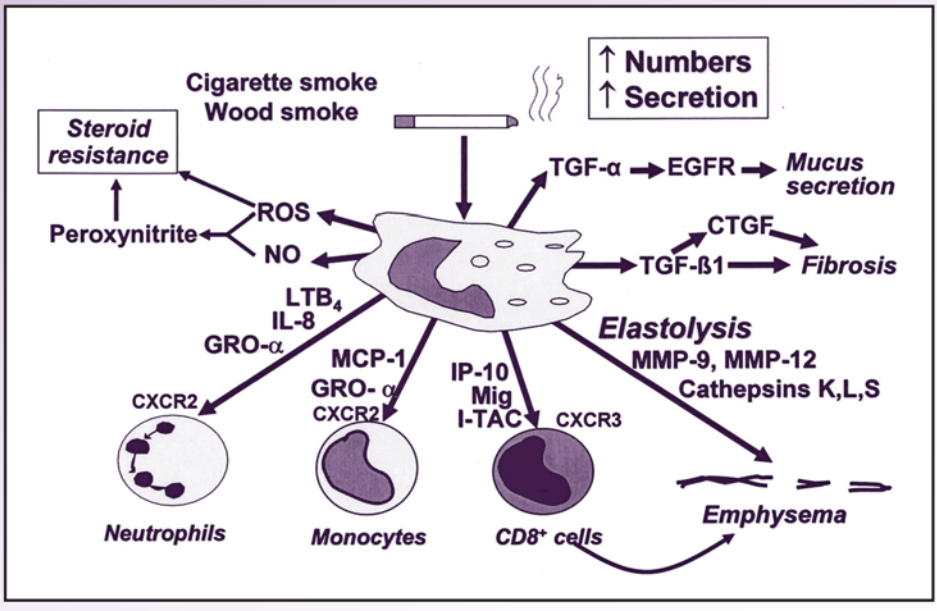
Chronic Obstructive Pulmonary Disease

Cellular and Molecular Mechanisms



edited by

Peter J. Barnes

CHRONIC OBSTRUCTIVE PULMONARY DISEASE

LUNG BIOLOGY IN HEALTH AND DISEASE

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CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Cellular and Molecular Mechanisms

Edited by

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Introduction

> Disease is very old, and nothing about it has changed. It is we who change as we learn to recognize what was formerly imperceptible.
>
> — Jean Marie Charcot (1825–1893)
> *De l'Expectation en Medicien*

In a way, this statement by Charcot, a pioneering neurologist of the nineteenth century, is applicable to chronic obstructive pulmonary disease (COPD). The definition and the name of the disease, or diseases (chronic bronchitis and emphysema), have changed over the years, but COPD today is what it was long before the name was introduced.

In 1978, the series of monographs *Lung Biology in Health and Disease* introduced a volume edited by Dr. Thomas L. Petty entitled *Chronic Obstructive Pulmonary Disease*. In the last chapter, "A Postscript with an Eye to the Future," the editor wrote:

> In COPD there are concerns that finding the disease early will increase anxiety and extend the time of knowledge of a progressive, probably fatal disorder. This anxiety, the warning goes, will only burden the patient and thus, paraphrasing the admonition, they should not be told (or found). What nonsense!

The second edition of this monograph, also edited by Dr. Petty and published in 1985, presented an entirely different outlook for COPD. Indeed, Dr. Petty asserted that

> research into basic mechanisms of lung damage via proteolytic and oxidative mechanisms will continue to elucidate the basis of inflammation and lung destruction ... drugs will be developed to help protect the lungs.

Clearly the page has been turned. As Charcot predicted, the scientific community is learning "to recognize what was formerly imperceptible." Serious attention is now being given to the underlying mechanisms of COPD, and research at the cellular and molecular level is intense and very productive. Already we have seen the development of medications correcting airways obstruction and anti-inflammatory medications have found a place in the therapeutic armamentarium of the clinician. Many additional medications are being developed and this would not have happened in the absence of the ever-increasing knowledge and understanding of the basic mechanisms of COPD. Undoubtedly, the more that is known about these mechanisms, the earlier in disease progression the cellular and molecular dysfunction will be identified. Hopefully, this will lead to better and more effective treatments that can be provided while the disease is still reversible.

This volume, edited by Peter J. Barnes, gives the reader a long view of the future and provides hope that COPD will soon become more fully treatable. Dr. Barnes, who has conducted pioneering research and applied creative thinking to the COPD field, has assembled peers and colleagues from several countries to develop this monograph. Altogether, the contributions from these authors will leave the readers with the strong conviction that today, in contrast to the last 1970s, physicians and other health workers must seek the full benefit of what is now known about treatment and prevention of COPD.

I want to thank Dr. Barnes and his colleagues, and express my gratitude for this important contribution to the series. This volume provides an important and monuemntal step toward a better and longer life for COPD patients.

Claude Lenfant, M.D.
Gaithersburg, Maryland

Preface

Chronic obstructive pulmonary disease (COPD) is now recognized to be a serious chronic disease of global proportions that appears to be increasing throughout the world as the world population ages and as developing countries smoke more cigarettes. The reduction in smoking (smoking being by far the most important cause of COPD) has been less than impressive despite government restrictions, taxes, and health warnings. COPD is now one of the most common causes of death and chronic disability in the world, and is the only common cause of death that is increasing in developed countries.

Yet COPD receives little attention from doctors, patients, the media, or the pharmaceutical companies as it is often seen as irreversible and self-inflicted. It is poorly diagnosed and undertreated, or managed like asthma, which is inappropriate. This means that there is far less funding devoted to research in COPD than other common inflammatory diseases, such as asthma and rheumatoid arthritis. This is now beginning to change as it is recognized that chronic lung inflammation is an important component of COPD, which may underlie the relentless progression of the disease in most patients. No currently available drug treatment has been shown to reduce the progression of the disease or the inflammatory process. In particular corticosteroids appear to be remarkably ineffective, compared to their striking efficacy in asthma, which is now easily controlled in most patients. This suggests that there is an urgent need to understand more about the underlying cellular and molecular mechanisms involved in COPD, particularly if we are to develop more effective therapies in the future. This will involve considerable injection of research money. The disease is prevalent

and the cause is usually known, giving important advantage to researchers in this field.

This volume brings together the world experts on the underlying mechanisms of COPD. This is a rapidly advancing field, but the current chapters provide an up-to-date perspective of the inflammatory cells, mediators, and molecular pathology of COPD in a single volume. I hope that the inclusion of this volume in the series will make up for some of the lack of information about the cellular and molecular mechanisms in COPD. This is an exciting time for COPD research as there have been major advances in understanding cellular mechanisms, cell signaling and gene expression relevant to COPD. Many novel types of drugs are now in development and it is very likely that some of the new approaches will bear fruit and help control and prevent the progression of this devastating disease.

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Peter Barnes
London
June 2004

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Why More Research into Molecular and Cellular Mechanisms of COPD Is Needed

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I. INTRODUCTION

COPD is a major and increasing global health problem, which is predicted to become the third commonest cause of death and the fifth commonest cause of disability in the world by 2020 (1). Indeed the World Health Organization has already demonstrated that COPD is now ranked as the fourth commonest cause of death worldwide (2). COPD is the only common cause of death in the United States of America that has increased over the last 20 years and in the UK it now causes over 30,000 deaths a year (3).

While there have been major advances in the understanding and management of asthma, COPD has been relatively neglected and there are no current therapies that reduce the inevitable progression of this disease. However, because of the enormous burden of disease and escalating health care costs, there is now renewed interest in the underlying cellular and molecular mechanisms (4,5) and a search for new therapies (6), resulting in a re-evaluation of the disease (7). Despite its enormous global importance, there has been relatively little research into COPD and it is the most underfunded of all diseases in relation to the global burden of disease (8). This is a clear indication that more research into the underlying cellular and molecular mechanisms of the disease are now urgently needed.

A. The Size of the Problem

The true prevalence of COPD is uncertain, particularly in developing countries and appears to vary considerably between countries. In European countries, recent survey has suggested that 4–6% of the adult population suffer from COPD, defined by largely irreversible airflow limitation. Even more importantly, COPD is an increasing cause of chronic disability and is predicted to become the fifth most common cause of disability in the world by 2020. The European Respiratory Society recently reported that clinically relevant COPD now affects 4–6% of adults in Europe (9). In the United States of America and Europe, COPD is a common and increasing cause of hospital admissions resulting in three major health care expenditure that now exceeds the costs of asthma by over threefold (10). COPD already accounts for over 20 million lost working days annually in the United Kingdom, placing an enormous and increasing burden on society.

B. The Neglect of COPD

Despite recognition as an increasingly important international health problem, COPD has suffered neglect from clinicians, researchers, and the pharmaceutical industry. This is largely because COPD is viewed as self-inflicted (by smoking) and also because the underlying disease process is perceived to be irreversible. Consequently, there is a fundamental lack of knowledge about the cellular, molecular, and genetic causes of COPD. Existing therapies for COPD are grossly inadequate and none has been shown to slow the relentless progression of the disease. Smoking cessation has a poor success rate. Anti-inflammatory drugs, which are used successfully to manage asthma, have few clear beneficial effects. Bronchodilators, which are the mainstay of current drug therapy, do not affect the underlying disease process and therefore do not slow disease progression towards respiratory failure and death. In terms of research funding, COPD despite its enormous global importance, there has been relatively little research into COPD and it is the most underfunded disease in relation to the global burden of disease (8). This suggests that there should now be a major investment in basic mechanisms research, in order to understand the pathophysiology of the disease and develop new therapeutic approaches in the future.

II. THE NATURE OF AIRWAY OBSTRUCTION IN COPD

COPD is characterized by slowly progressive development of airflow limitation that is poorly reversible, in sharp contrast to asthma where there is variable airflow obstruction that is usually reversible spontaneously or with treatment. A new definition of COPD has recently been adopted by the Global Initiative on Obstructive Lung Disease (GOLD): "a disease state

characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles and gases'' (11). For the first time, this definition encompasses the idea that COPD is a chronic inflammatory disease and much of the recent research has focused on the nature of this inflammatory response.

COPD includes chronic obstructive bronchiolitis with fibrosis and obstruction of small airways, and emphysema with enlargement of airspaces and destruction of lung parenchyma, loss of lung elasticity, and closure of small airways. Chronic bronchitis, by contrast, is defined by a productive cough of more than three months duration for more than two successive years; this reflects mucous hypersecretion and is not necessarily associated with airflow limitation. Most patients with COPD have all three pathological mechanisms (chronic obstructive bronchiolitis, emphysema, and mucus plugging) as all are induced by smoking, but may differ in the proportion of emphysema and obstructive bronchiolitis (4). In developed countries, cigarette smoking is by far the commonest cause of COPD accounting for over 95% of cases, but there are several other risk factors, including air pollution (particularly indoor air pollution from burning fuels), poor diet, and occupational exposure. COPD is characterized by acceleration in the normal decline of lung function seen with age. The slowly progressive airflow limitation leads to disability and premature death and is quite different from the variable airway obstruction and symptoms in asthma, which rarely progresses in severity. While COPD and asthma both involve inflammation in the respiratory tract, there are marked differences in the nature of the inflammatory process, with differences in inflammatory cells, mediators, response to inflammation, anatomical distribution, and response to anti-inflammatory therapy (7,12). Some patients appear to share the characteristics of COPD and asthma, however. Rather than this representing a graded spectrum of disease, it is more likely that these patients have both of these common diseases at the same time.

A. Differences from Asthma

Histopathological studies of COPD show a predominant involvement of peripheral airways (bronchioles) and lung parenchyma, whereas asthma involves inflammation in all airways but usually without involvement of the lung parenchyma (13). There is obstruction of bronchioles, with fibrosis and infiltration with macrophages and T-lymphocytes. There is destruction of lung parenchyma and an increased number of macrophages and T-lymphocytes, with a greater increase in $CD8^+$ (cytotoxic) than $CD4^+$ (helper) cells (14). Bronchial biopsies show similar changes with an infiltration of macrophages and $CD8^+$ cells and an increased number of neutrophils in patients with severe COPD (15). Bronchoalveolar lavage (BAL)

fluid and induced sputum demonstrate a marked increase in macrophages and neutrophils (16,17). In contrast to asthma, eosinophils are not prominent except during exacerbations or when patients have concomitant asthma (13,18).

Fixed narrowing of small airways, emphysema, and luminal obstruction with mucus secretions may all contribute to airflow limitation in COPD, but there is debate about which mechanism is most important. There are differences between patients and at different stages of disease progression in the contribution of each of these processes, but problems in making accurate measurements in patients have made it difficult to evaluate the importance of each mechanism in an individual patient.

B. Small Airways

It has long been recognized that there is narrowing of small airways in patients with COPD (19–22) (Fig. 1). There is an increase in the thickness of small airways with increased formation of lymphoid follicles and deposition of collagen in the outer airway wall that may restrict airway opening (23). The lumen of small airways is reduced by mucosal thickening containing an inflammatory exudate, which increases with the severity of disease. The mechanism for lymphoid follicle formation in more severe dis-

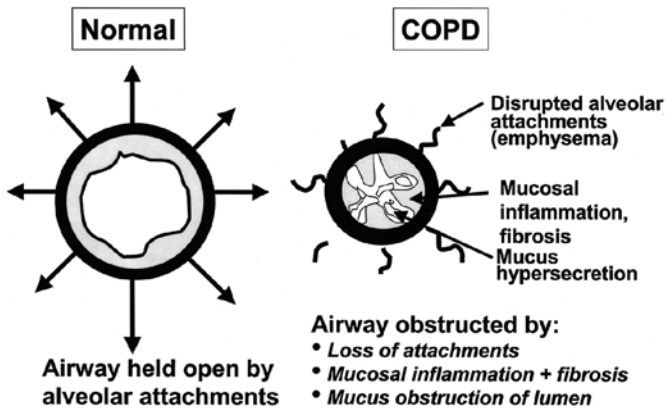

Figure 1 Mechanisms of airflow limitation in COPD. The airway in normal subjects is distended by alveolar attachments during expiration, allowing alveolar emptying and lung deflation. In COPD, these attachments are disrupted because of emphysema thus contributing to airway closure during expiration, trapping gas in the alveoli and resulting in hyperinflation (17). Peripheral airways are also obstructed and distorted by airways inflammation and fibrosis (chronic obstructive bronchiolitis) and by occlusion of the airway lumen by mucous secretions which may be trapped in the airways because of poor mucociliary clearance.

ease is unknown, but may reflect a response to chronic bacterial colonization and acute exacerbations of inflammation. The mechanisms of fibrosis around the airway are not yet understood, but are likely to represent an attempt to repair chronic inflammation. The role of specific growth factors, such as transforming growth factor-β (TGF-β) which shows increased expression in peripheral airways (24,25) and connective tissue growth factor (CTGF), are not yet known. TGF-β may induce fibrosis via the release of CTGF which may stimulate collagen deposition in the airways (26,27). A major barrier to understanding the contribution of small airway obstruction is the difficulty in quantifying small airway obstruction in patients using measurements of airflow due to the high variability and poor reproducibility of measurements (28).

C. Alveolar Destruction

Both panacinar and centrilobular emphysema may occur in smokers (29). The role of emphysema in causing airflow obstruction in COPD has been examined by measuring macroscopic emphysema in resected lung or on computerized tomography (CT) scans in relation to tests of lung function, or by measuring static transpulmonary pressure (P_L) as a measurement of alveolar disease. Many studies have shown significant, albeit weak, correlations between the grading of macroscopic emphysema and various tests of lung function (30,31). However, the assessment of macroscopic emphysema is dominated by destroyed or poorly functioning lung, whereas lung function tests reflect predominantly the function of the best surviving lung. In the extreme case of non-ventilated emphysematous bullae surrounded by normal lung, the two assessments are virtually independent, with lung function tests measuring only the reduced volume of surviving lung. In more common types of emphysema, a simple two-compartment model does not apply, but usually there will be greater heterogeneity of disease with more and more units becoming poorly functional as disease progresses, resulting in a rise in residual volume and a fall in vital capacity. Thus the strength of the correlation between assessment of gross emphysema and lung function will depend on the severity and homogeneity of "microscopic" disease in the less affected lung which is not often measured.

P_L (measured using an esophageal catheter) is plotted against airflow conductance or maximal expiratory flow at different lung volumes to indicate the contribution of alveolar disease (and by implication emphysema) to airflow limitation, with the assumption that the rest is due to intrinsic airway disease (32–34). However, it is not certain that the magnitude of decline in P_L accurately reflects the severity of emphysema and its effects on the airways. Reduction in P_L is likely to be largest with relatively uniform emphysema, as occurs in panacinar emphysema (e.g., α_1-antitrypsin

deficiency), whereas patchy centrilobular emphysema may have near normal P_L. Indeed, uniform "microscopic" emphysema might account for functional 'pseudoemphysema' without any CT change.

In practice, a reduction in conductance or maximum flow that is completely explained by a reduction in P_L is unusual, except in mild disease. Retrograde catheter studies in excised lungs from patients with severe airflow obstruction due to COPD have all found large increases in peripheral resistance at standard P_L (35–37). But there are many other possible changes in airway function due to emphysema which would result in an increased resistance at a given P_L, including abnormal angulation or compression of normal airways by surrounding overdistended lung, loss of parallel airways due to emphysematous destruction, or to functional loss of patent airways supplying poorly ventilated areas of lung. The effects of emphysema may not always reduce P_L, e.g., a short stenosis caused by local loss of alveolar attachments. Present analyses of airway morphology are not sufficient to reveal the anatomical basis of the consistent physiological finding of an increase in peripheral airflow resistance. Assuming that the increase is all due to "intrinsic" disease of the peripheral airways underestimates the role of emphysema. Emphysema may play a more prominent role in severe disease as the decline in lung function accelerates.

D. Mucus Hypersecretion

The contribution of mucus hypersecretion to airflow limitation in COPD is still uncertain. Although early studies supported the view that mucus hypersecretion was not associated with any physiological defect (38,39), more recent studies have demonstrated that mucus hypersecretion may be a potential risk factor for accelerated decline in lung function (40,41). The early studies examined the early stages of COPD and also included an occupational cohort. The most likely mechanism whereby chronic mucus hypersecretion contributes to progression of COPD may be due to the increased risk of exacerbations that appear to accelerate loss of FEV_1 (42). Chronic mucus hypersecretion may contribute little in the early phases of COPD when exacerbations are infrequent. It is possible that chronic mucus hypersecretion may reflect the inflammatory process around submucosal glands (43) and may reflect the intensity of inflammation in more peripheral airways. Increased numbers of neutrophils and mast cells have also been found around submucosal glands (43,44) and serine proteases and mast cell chymase are potent mucus secretagogues (45–47). In severe COPD chronic mucus hypersecretion is associated with mortality and this may also reflect an increased risk of terminal infection (48–50). Chronic cough and mucus production in smokers with normal lung function (GOLD Stage 0) do not appear to predict the later development of COPD (51).

III. INFLAMMATION IN COPD

It is apparent that there is a specific inflammatory response in the respiratory tract of COPD patients which differs form that seen in asthma. Recent studies show that the intensity of this inflammation increases with the severity of disease (23,52). This is in marked contrast to other chronic inflammatory diseases, including interstitial lung disease and rheumatoid arthritis where the inflammatory response appears to "burn out" with time. This offers considerable hope for the development of anti-inflammatory drugs in the future, even for patients with severe disease.

A. Innate vs. Acquired Immunity

Innate immunity represents a primitive defense response to noxious and infectious agents and can be rapidly mounted. In the respiratory tract macrophages and epithelial cells may be directly activated by particles and mucus is stimulated to protect the delicate mucosal surface. This acute inflammatory response can be mounted rapidly, has no specificity and no memory (53). It may be activated by pattern recognition receptors such as Toll-like receptor (TLR) and Nod receptors (54). By contrast the adaptive response in the lung involves both humoral and cellular immunity and has a memory for previous stimuli. The adaptive immune response involves T- and B-lymphocytes, which markedly increase in severe COPD (23). This acquired immunity may underlie the persistence of COPD, even when the causal mechanism smoking has been stopped. The link between innate and acquired immunity is the dendritic cells (55,56).

B. Persistence of Chronic Inflammation

An important question to address is why inflammation in the respiratory tract persists even in patients who have avoided smoking for several years. This is demonstrated by the intense inflammatory response in the airways of ex-smokers in COPD (17,57,58), although it is not known whether this is less pronounced than in active smokers. Longitudinal studies of smoking cessation are now urgently needed. It is possible that the inflammatory response to cigarette smoke may resolve in patients with mild disease, in keeping with the reduction in disease progression in patients with mild disease who quit (59). However, there is little improvement in progression of the disease in patients with more severe disease, possibly because the inflammatory process is driven by the acquired immune response with immunological memory.

C. Components of Inflammation

The specific cells and mediators of COPD are discussed in detail in the ensuing chapters. Although these cells and mediators are considered

separately, it is clear that there is a complex interaction between these inflammatory cells and mediators (Fig. 2). Indeed, it is likely that there is a cascade of inflammation, initiated by cigarette smoking indicating that innate immune cells of the airway, namely macrophages and epithelial cells, then release chemokines to recruit cells from the circulation into the lung, including monocytes (which differentiate into macrophages), neutrophils and T- and B-lymphocytes (Fig. 3). There is now much research identifying the specific chemotactic factors involved as this may represent a novel therapeutic approach. In addition to chemotactic mediators, other mediators induce inflammatory responses, whereas others induce structural changes

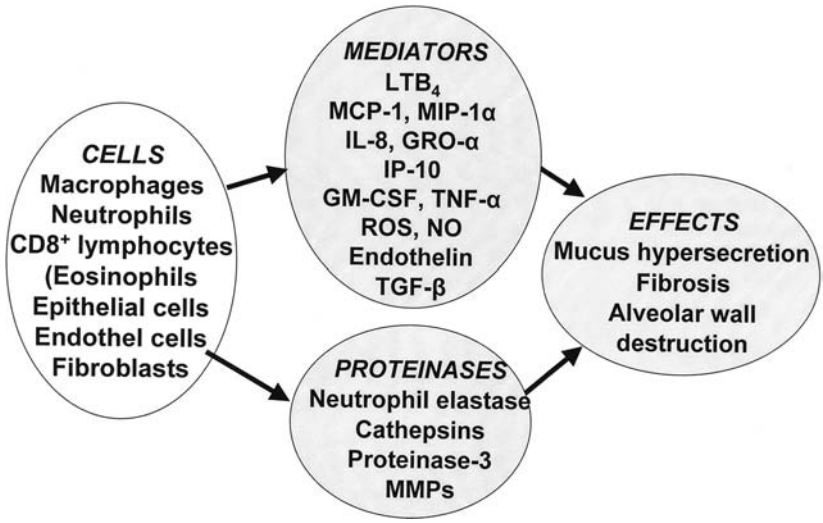

Figure 2 Inflammation in COPD is complex with may activated inflammatory and structural cells that release multiple mediators, including lipid mediators such as leukotriene B_4 (LTB_4) which is chemoattractant for neutrophils. Chemokines such as monocyte chemotactic protein (MCP)-l and macrophage inhibitory protein (MIP)-lα which attract monocytes, interleukin (IL)-8, and growth-related oncogene (GRO)-α which attract neutrophils and monocytes, interferon inducible protein (IP)-10 which attracts $CD8^+$ cells, reactive oxygen species (ROS), and nitric oxide (NO). Granulocyte-macrophage colony stimulating factor (GM-CSF) which prolongs neutrophils survival. Tumor necrosis factor (TNF)-α which amplifies inflammation by switching on multiple inflammatory genes and may also account for some of the systemic effects of the disease. Endothelin and transforming growth factor-β (TGF-β) which induce fibrosis. In addition multiple proteinases are releases that result in elastolysis, including the serine proteinases neutrophils elastase and proteinase C, cathepsins and matrix metalloproteinases (MMP). This combination of mediators that attract and activate inflammatory cells and proteinases which cause elastolysis and mucus hypersecretion result in the typical pathophysiology of COPD.

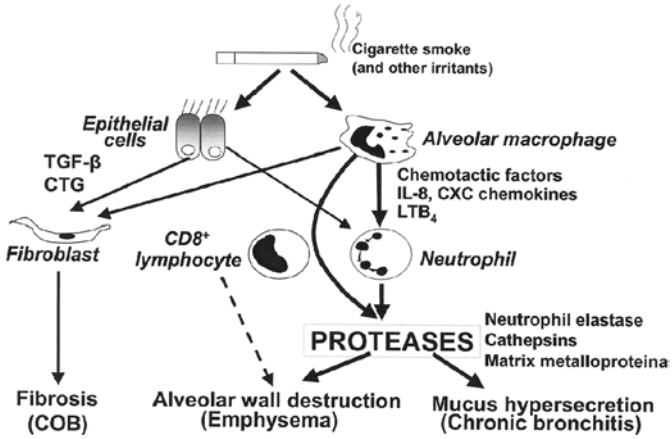

Figure 3 Inflammatory mechanisms in COPD. Cigarette smoke (and other irritants) activates macrophages in the respiratory tract that release neutrophil chemotactic factors, including interleukin-8 (IL-8) and leukotriene B_4 (LTB_4). These cells then release proteases that break down connective tissue in the lung parenchyma, resulting in emphysema, and also stimulate mucus hypersecretion. These enzymes are normally counteracted by protease inhibitors, including α_1-antitrypsin, secretory leukoprotease inhibitor (SLPI) and tissue inhibitor of matrix metalloproteinases (TIMP). Cytotoxic T cells ($CD8^+$) may also be recruited and may be involved in alveolar wall destruction. Fibroblasts may be activated by growth factors releases from macrophages and epithelial cells.

such as destruction of connective tissue (emphysema) and fibrosis (obstructive bronchiolitis). What is not yet understood is how these mechanisms change with time and the sequence of events.

IV. WHAT ARE THE AMPLIFYING MECHANISMS?

The inflammatory changes and protease imbalance in COPD are also seen in cigarette smokers without COPD, but to a lesser extent (17,52,60–62), suggesting that the accelerated decline in lung function in COPD may be due to amplification of the normal pulmonary inflammatory response to irritants (Fig. 4). This may be due to increased production of inflammatory mediators and enzymes, or because of defective endogenous anti-inflammatory or antiprotease mechanisms. These differences might be explained by polymorphisms in the genes encoding cytokines, proteases, anti-inflammatory proteins, and antiproteases (63,64).

Another hypothesis is that these differences are due to latent virus infection (65). The latent adenovirus sequence E1A is more commonly detected in the lungs of patients with emphysema than in matched smoking control subjects and is correlated with an increased inflammatory response

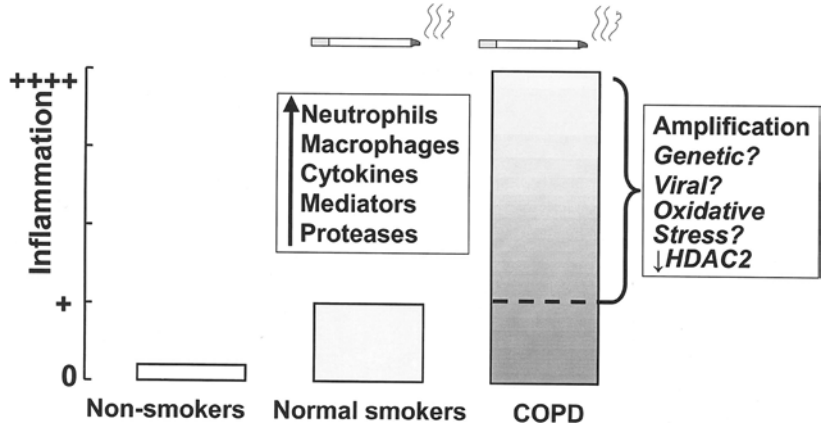

Figure 4 Amplification of lung inflammation in COPD. Normal smokers have an inflammatory response, which represents the normal response of the respiratory tract to inhaled irritants. In COPD, this same inflammatory response is markedly amplified. The molecular mechanisms of this amplification are currently unknown, but may be determined by genetic factors or latent viral infection. Oxidative stress is an important amplifying mechanism and may increase the expression of inflammatory genes through impairing the activity of histone deacetylase 2 (HDAC2) which is needed to switch off inflammatory genes.

(52). Adenovirus infection amplifies the inflammatory response to cigarette smoke in the airways of guinea pigs (66). Transfection of ElA into a human epithelial cell line results in increased activation of the transcription factor NF-κB with consequent increased release of IL-8 in response to cell activation and increased production of TGF-β1, providing a molecular mechanism for the amplification in inflammatory response (67,68).

Another molecular mechanism that may underlie the amplification of inflammation in COPD may involve impaired activity of histone deacetylases (HDAC) in alveolar macrophages (69). In macrophages from cigarette smokers, there is impaired activity of HDAC, which is involved in switching off the transcription of inflammatory genes by reversing the acetylation of core histones that is associated with their activation (70,71). In COPD, there is even more marked reduction in HDAC activity in peripheral lung of COPD patients than smokers without airway obstruction (72). This may lead to amplification of the expression of inflammatory genes, as is seen in alveolar macrophages from patients with COPD (62,73). There is also increased activation of NF-κB in these cells from patients with COPD (74,75). This reduction in HDAC2 expression and activity also appears to account for the resistance to corticosteroid seen in COPD patients, thus linking the two fundamental cellular abnormalities in this disease, namely amplification of inflammation and corticosteroid resistance.

Although smoking is the major causal mechanism in COPD, quitting smoking does not appear to result in resolution of the inflammatory response in the airways, particularly in advanced disease (52,57,58). This suggests that there are perpetuating mechanisms that maintain the chronic inflammatory process once it has become established. This may account for presentation of COPD in patients who stopped smoking many years before their first symptoms develop. The mechanisms of disease persistence are currently unknown.

V. THE IMPORTANCE OF TRANSLATIONAL RESEARCH

As discussed above, COPD has been very poorly investigated and our understanding of cellular and molecular mechanisms lags behind our understanding of asthma. Yet, in some ways, it is an easier disease to investigate, as the cause is known. The reason why only a proportion of smokers develop COPD is still not known, however. This might be due to genetic factors that amplify the inflammatory response to irritants, or reduce the endogenous anti-inflammatory mechanisms that would normally dampen down the inflammatory response.

It is clear that COPD is a heterogeneous disease and the reason why some patients have predominant emphysema, whereas others have predominant small airway disease, is not understood. The natural history of these different patterns of disease is also poorly understood. What is now needed is careful phenotyping and genotyping of patients to understand the cell and molecular mechanisms that determine the different patterns of pathology and progression, using a multidisciplinary approach to measure inflammation (including non-invasive markers of lung inflammation and high resolution imaging) (76).

It is only through careful phenotyping of patients and longitudinal studies to follow disease progression that we will begin to understand the role of specific genes, proteins, cells, and mediators. The techniques of molecular genomics, proteomics, and metabonomics clearly have important contributions to play in this understanding.

Ultimately better understanding of cell and molecular mechanisms of COPD should lead to more effective therapy, and in particular anti-inflammatory or disease modifying therapies that reduce disease progression that are currently lacking.

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2

Inflammation in Lung Parenchyma

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I. INTRODUCTION

Airflow obstruction is the functional consequence of pathological changes induced by cigarette smoking in peripheral airways and lung parenchyma. These include both inflammatory and structural changes, mainly airway remodeling and parenchymal destruction (which characterizes emphysema). Airway remodeling and inflammation will contribute to airflow obstruction by promoting airway narrowing and lumen occlusion, while parenchymal destruction will contribute to airflow obstruction by reducing the elastic recoil of the lung as well as by destroying alveolar attachments and thereby reducing the elastic load applied to the airways (1,2). Whether airflow obstruction is always caused by emphysema or whether a disease of the conducting airways without emphysema could also cause airflow obstruction is still uncertain. Furthermore, it has been reported that smokers may develop emphysema even in the presence of normal lung function (3,4). These facts highlight the complexity of Chronic Obstructive Pulmonary Disease (COPD) and particularly of the relationship among airway pathology, emphysema, and airflow obstruction.

The role of mucus hypersecretion in COPD is even more debated. Traditionally, mucus hypersecretion has been considered irrelevant to the development of chronic airflow obstruction in smokers (5,6). On the other hand, the observation that chronic sputum production was associated with both

an excess of FEV_1 decline and an increased risk of subsequent hospitalization because of COPD suggested a role for mucus hypersecretion in the development of chronic airflow obstruction (7). This hypothesis, however, appears to be counter to recent data showing that chronic sputum production in smokers with normal lung function (GOLD stage 0) (8) does not predict a subsequent establishment of airflow obstruction (9). Taken together, these observations suggest that mucus hypersecretion may play a role in the progression of COPD, but only in patients who have already developed a chronic airflow obstruction.

It is now widely accepted that inflammation plays a key role in the pathogenesis of COPD, so that recently this concept has been included for the first time in the definition of the disease. Indeed, according to the most recent guidelines, COPD is defined as a disease state characterized by not fully reversible airflow obstruction that is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases (8), particularly to cigarette smoking. The smoking habit has been identified as the most important risk factor for the establishment of COPD and several studies have shown that cigarette smoking is able to elicit an inflammatory process in the lung (10,11), which is thought to represent a normal nonspecific response to injury. However, among smokers, only a minority develop COPD, suggesting that the development of the disease requires both exposure to cigarette smoke and individual susceptibility. It has been hypothesized that, in susceptible individuals, an amplification of the nonspecific inflammatory response initiated by cigarette smoking is responsible for most of the structural changes associated with the progression of the disease (12,13). Indeed, the extent of lung inflammation appears to progressively increase from smokers with normal lung function to smokers with severe COPD (10,14,15). Although the hypothesis is attractive, convincing evidence that the development of structural changes is dependent on the prior development of chronic inflammation is still lacking (16). It seems equally plausible that structural changes could arise regardless of chronic inflammation.

Although the mechanisms of individual susceptibility to the development of COPD are not completely understood, a possible explanation for the amplification of the inflammatory response in susceptible smokers could be an altered production of inflammatory mediators. This impairment may be due to a genetic predisposition involving polymorphism in genes encoding proinflammatory cytokines (17–19). Environmental conditions including viral infections and pollutants may also play a role in triggering or maintaining the disease. Retamales et al. suggest that susceptible smokers may have an amplified inflammatory response in the lung parenchyma as a consequence of a latent viral infection (15). Interestingly, although smoking is the principal cause of COPD, quitting smoking does not appear to result in resolution of the lung inflammation (20,21), suggesting that there are

perpetuating mechanisms that maintain the chronic inflammatory process once it has become established (18,19).

In this chapter, we will focus on the inflammatory and destructive processes present in the lung parenchyma of smokers to highlight their relationship with airway pathology and pulmonary function, in an attempt to identify the possible mechanisms contributing to the development of chronic airflow obstruction in these subjects.

II. PARENCHYMAL INFLAMMATION

Only a few studies have attempted to quantify the alveolar inflammation in smokers by direct examination of surgical lung specimens. Eidelman et al. (22) found an increased number of inflammatory cells in the alveolar wall of smokers which was directly related to the degree of parenchymal destruction (Fig. 1). Subsequently, Finkelstein et al. (23) characterized this alveolar inflammatory process showing that the enhanced inflammatory response in smokers was due to an increase in macrophages and T-lymphocytes and that the number of these cells was directly correlated with the extent of emphysema. The phenotype of these T-lymphocytes has been recently identified, and it has been shown that it is the CD8+ T cell subtype that predominates in smokers with COPD (14). Indeed, CD8 T-lymphocytes are increased not only in the alveolar walls, but also in central and peripheral airways, suggesting that a consistent inflammatory process is present along the entire tracheobronchial tree in smokers with COPD (14,24,25). It is noteworthy that, in all these lung compartments, CD8-T-lymphocytes show a significant correlation with the degree of airflow obstruction, suggesting a role for these cells in the progression of the disease (14,24,25).

Interestingly, the neutrophilia reported in bronchoalveolar lavage (26,27) by several studies has not been observed in the alveolar walls of smokers with COPD (14). A possible explanation for the discrepancy between lavage and parenchymal tissue is the rapid migration of neutrophils through the alveolar tissue to the alveolar spaces, so that the effect of accumulation of these cells is undetectable by tissue analysis but becomes evident by lavage analysis. Even if the mechanism of neutrophil accumulation is not entirely clear, it probably involves neutrophil chemotactic factors, such as interleukin (IL)-8, which has been reported to be increased in both bronchoalveolar lavage and sputum of smokers with COPD (28–30).

Although an increased number of inflammatory cells, particularly neutrophils, macrophages, and CD8-T-lymphocytes has been clearly shown in COPD (Table 1), the role of these cell types in the pathogenesis of the disease is largely unknown (12,18,31,32).

Neutrophils have been implicated in the pathogenesis of emphysema since the appreciation of emphysema associated with deficiency of

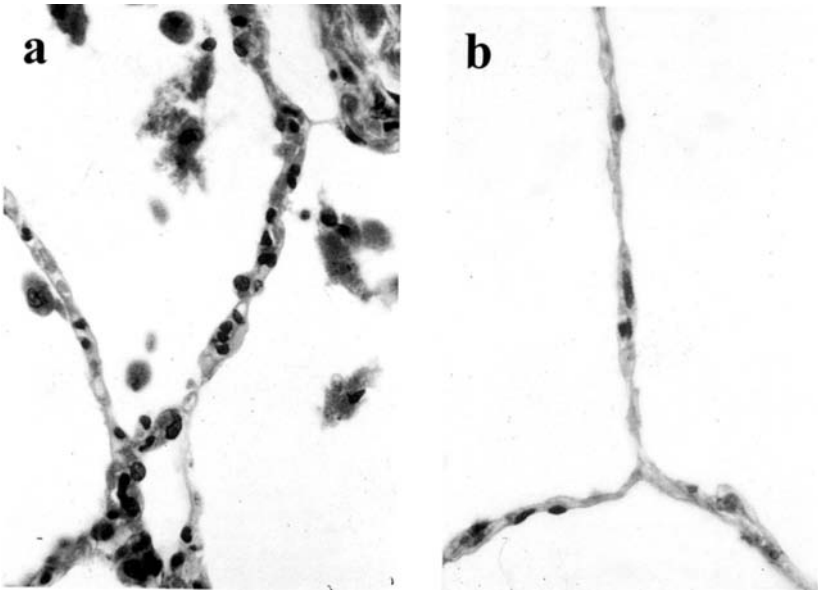

Figure 1 Alveolar walls from a smoker (a) showing an increased number of cells as compared to a nonsmoker (b). (Photomicrograps kindly provided by Manuel Cosio.)

αl-antitrypsin, an antiprotease that protects against the proteolytic effects of the neutrophil serine proteases (33). This observation led to the protease/antiprotease hypothesis which suggests that serine proteases, overwhelming local antiprotease activity, can degrade connective tissue components,

Table 1 Inflammatory Cells in COPD

Cell type	Location	Main hypothesized function
Neutrophils	Alveolar spaces	Elastolytic activity
	Alveolar walls (in severe disease)	
	Airway lumen	
Macrophages	Alveolar walls	Phagocytosis
	Central airway wall (83,84)	Elastolytic activity Inflammatory mediator
CD8-T-lymphocytes	Alveolar walls	Cytolysis and apoptosis of target cells
	Peripheral airway wall	
	Central airway wall	

destroying the lung parenchyma. Neutrophils release a large array of serine proteases including neutrophil elastase, proteinase 3, and cathepsin G, all of which are able to induce emphysema in animal models (34). The release of these serine proteases may be directly or indirectly encouraged by cigarette smoking.

There is now increasing evidence that metalloproteinases (MMP) derived from neutrophils and particularly from macrophages are likely to contribute to lung injury. Indeed, macrophages are capable of producing MMP-1 (collagenase), MMP-9 (gelatinase B), MMP-12 (macrophage elastase), and others (35). The activity of these MMPs may be counteracted by tissue inhibitors of matrix metalloproteinases (TIMP) including TIMP-1, TIMP-2, and TIMP-3 (19). Of interest, macrophages can also produce elastolytic cysteine proteinases including cathepsin K, L, and S which may damage the lung (19). As proposed by Shapiro (35), a disregulated expression of macrophage MMPs, induced either directly or indirectly by cigarette smoking exposure, could lead to the lung destruction characteristic of emphysema. The results of recent studies on chronic cigarette smoke exposure in mice support this hypothesis. In fact, when MMP-12 knocked out mice (MMP-12$-/-$) were exposed to chronic cigarette smoking, they failed to develop emphysema and failed to recruit macrophages to the lung. In contrast, the smoke-exposed wild type mice (MMP-12 $+/+$) develop both emphysema and macrophage recruitment (36). According to the authors, these data suggest that cigarette smoking induces constitutive macrophages to produce MMP-12, which in turn cleaves elastin, thus generating fragments chemotactic for monocytes. This positive feedback would perpetuate the accumulation of macrophages and lung tissue destruction (35). However, it has been recently shown that, in mice, smoke-induced tissue damage requires not only macrophage metalloelastase, but also neutrophils (37), and that TNF-α plays a central role in the process (38). All these observations suggest that, in mice, MMP-12 mediates smoke-induced inflammation by releasing TNF-α from macrophages, with subsequent endothelial activation, neutrophil influx, and matrix damage caused by neutrophil-derived proteases (39). As pointed out by Snider (40), in this model, macrophages and TNF-α are essential to the process, but neutrophils and their proteases are the final arbiters of matrix damage.

Whether neutrophil-mediated tissue destruction could explain smoking-induced emphysema in humans as well is still controversial. Although neutrophils have the potential to induce tissue damage, lung destruction is not a significant feature of other pulmonary diseases where neutrophilia is even more prominent, such as pneumonia, cystic fibrosis, bronchiectasis, and adult respiratory distress syndrome (ARDS) (19). Therefore, this mechanism may not fully explain the loss of lung tissue in cigarette smoking-induced human emphysema. A possible alternative mechanism of lung damage is a failure of the repair process. In vitro experiments have shown

that damage to elastic fibers initiates a repair process that rapidly restores the fibers to structural integrity (41). Although repair mechanisms in vivo are poorly understood, it is possible that an abnormal repair process could contribute to the development of smoking-induced emphysema (40).

In smokers with COPD, neutrophils and macrophages may be involved even in other features of the disease. Macrophages may be activated by cigarette smoking to release a large array of inflammatory mediators, including TNF-α, IL-8 and other CXC chemokines, monocyte chemotactic peptide (MCP-1), and reactive oxygen species (19), thus contributing to the development and the persistence of the inflammatory process. With regard to neutrophils, it has been proposed that they play a role in the mucus hypersecretion characteristic of chronic bronchitis (19). Indeed, neutrophil proteases, including neutrophil elastase, cathepsin G, and proteinase 3 are all potent stimulants of mucus secretion (42–44), and neutrophils are increased in both submucosal glands and bronchiolar epithelium of smokers (45,46). The location of neutrophils in these compartments may be crucial for the activation of the secretory function of glands and goblet cells, and therefore for the induction of chronic sputum production in smokers with chronic bronchitis. The observation that, in the bronchiolar epithelium of smokers, the neutrophilia is paralleled by an increased of goblet cells supports this hypothesis (46).

Along with macrophages and neutrophils, a crucial cell type in COPD is the CD8 T-lymphocyte. The major activity of $CD8^+$ T-lymphocytes has been considered the rapid resolution of acute viral infections, and there is increased evidence that viral infections may play a role in the acute exacerbations that punctuate the progression of COPD (47,48). Moreover, the observation that people with frequent respiratory infections in childhood are more prone to develop COPD supports the role of viral infections in this disease (49). It is conceivable that, in response to repeated viral infections, an excessive recruitment of $CD8^+$ T-lymphocytes may occur and damage the lung through the release of TNF-α and perforins which are involved in lung destruction and emphysema (24,50,51). Frequent viral exacerbations may thus contribute to the development of emphysema and therefore to the worsening of lung function decline. This hypothesis is supported by the recent observation that patients with COPD who suffered frequent exacerbations experienced a significantly greater decline in FEV_1 than the patients who had infrequent exacerbations (52).

$CD8^+$ T-lymphocytes could be able to damage the lung even in the absence of a stimulus such as viral infection, as shown by Enelow et al. (53) who clearly demonstrated that recognition of a lung "autoantigen" by T cell may directly produce a marked lung injury. Taking into account these findings, it can be hypothesized that the $CD8^+$ T cell accumulation observed in COPD could be a response to an "autoantigenic" stimulus originating in the lung and induced by cigarette smoking (13).

An important effect of $CD8^+$ T-lymphocytes is the apoptosis of target cells, and it would be not surprising that apoptosis plays a role in the destruction of lung tissue in patients with emphysema. Majo et al. (54) have reported that, in smokers with emphysema, both the degree of apoptosis and the number of $CD8^+$ T cells in the alveolar walls increased in parallel with the amount of cigarette smoke inhaled. Moreover, Kasahara et al. (55) demonstrated that the destruction of lung tissue in emphysema may involve accelerated apoptosis of endothelial and epithelial cells through a mechanism dependent on vascular endothelial growth factor (VEGF). It can be therefore hypothesized that $CD8^+$ T-lymphocytes may participate in lung destruction by inducing apoptosis of structural cells.

Although $CD8^+$ T-lymphocytes may play an important role in the pathophysiology of COPD, the cytokine profile of these cells and their chemokine receptor expression have not been fully investigated. A current paradigm in immunology is that the nature of an immune response to an antigenic stimulus is determined largely by the pattern of cytokines produced by activated T cells (56). Type-1 T cells express cytokines, such as interferon γ (IFNγ), crucial in the activation of macrophages and in the response to viral and bacterial infections, whereas type-2 T cells express cytokines, such as interleukin (IL)-4 and -5, involved in Ig-E-mediated responses and eosinophilia characteristic of allergic diseases. It has recently been shown that the $CD8^+$ T cells infiltrating the peripheral airways in COPD produce IFNγ and express CXCR3 (57), a chemokine receptor that is known to be preferentially expressed on type-1 cells (58). Moreover, CXCR3 expression is paralleled by a strong epithelial expression of its ligand CXCL10, suggesting that the CXCR3/CXCL10 axis may be involved in the recruitment of type-1 cells in peripheral airways of smokers with COPD (57).

In smokers peripheral airway inflammation is likely to play a role in alveolar destruction, particularly in the selective destruction of alveolar walls directly attached to bronchioles (alveolar attachments). This hypothesis is supported by observation that the destruction of alveolar attachments in smokers is correlated with the degree of inflammation in peripheral airways (59). It is possible that mediators released by inflammatory cells may weaken the alveolar tissue and facilitate its rupture, particularly at the point where the attachments join the airway wall, where the mechanical stress is maximal. The destruction of alveolar attachments allows the airway wall to deform, thus narrowing the airway lumen and therefore contributing to airflow obstruction.

COPD is a progressive disease that in a minority of subjects may worsen toward a very severe stage. Investigating these patients may be of interest because a better characterization of their lung pathology may help to clarify why, among patients with a similar smoking history, only a minority develop a severe disease. Furthermore, even if patients with severe COPD

are only a small percentage of smokers, they represent a significant health and economic burden through hospital admission and absenteeism from work. Lung specimens from patients undergoing lung volume reduction surgery (LVRS) or lung transplantation for severe emphysema represent a unique opportunity to examine lung pathology in living patients who develop a severe stage of the disease. Moreover, the fact that these patients perform pulmonary function tests before surgery allows investigation of the relationship between lung pathology and measurements of pulmonary function. In COPD patients, as airflow obstruction progressively worsens, the parenchymal inflammation enhances, as shown by the pioneering study by Retamales et al. (15). The authors demonstrated that there is an increase in the intensity of the inflammatory response in the alveolar walls and alveolar spaces of these patients, and that this inflammatory process is characterized by an increased number of all the inflammatory cell types, including neutrophils, macrophages, CD4 and CD8 T-lymphocytes, and eosinophils. Moreover, a strong correlation was observed between the numbers of all these inflammatory cells, with exclusion of eosinophils, and the amount of emphysematous destruction as determined by computerized tomography scan. A recent study extended these findings showing that, when the disease progresses, there is an amplification of the inflammatory response even in the peripheral airways and that this enhanced airway inflammatory process is correlated with the degrees of airflow obstruction, lung hyperinflation, CO diffusion impairment, and radiologic emphysema (60). Taken as a whole, all these findings suggest a role for this enhanced inflammatory response in the clinical progression of the disease. The observation that all the inflammatory cell types contribute to the amplification of the inflammatory response in the lung parenchyma of severe emphysema suggests that, as pointed out by Shapiro (61), our challenge now is more to find out how these inflammatory cells interact and contribute to the disease than to detect the specific role of a single cell type.

III. PARENCHYMAL DESTRUCTION

Although the pathogenesis of parenchymal destruction in smokers remains enigmatic, increasing evidence suggests a role for alveolar wall inflammation in the destructive process. The most common type of parenchymal destruction in smokers is that of centriacinar (or centrilobular) emphysema, which is characterized by focal destruction restricted to respiratory bronchioli and to the central portions of the acinus, surrounded by areas of grossly normal lung parenchyma (62–64). These lesions occur more frequently in the upper lobes of the lung. Microscopically, in the early classical lesion, the enlarged destroyed respiratory bronchioles coalesce to produce sharply demarcated emphysematous lesions separated from the acinar periphery by intact

alveolar ducts and sacs of normal size. When the lung is severely involved in centriacinar emphysema, most of the acinus might be destroyed and the diagnosis of emphysema type might be very difficult or almost impossible. Furthermore, in severe disease, classical example of centriacinar emphysema is uncommon, atypical examples predominate and their interpretation may be difficult (64).

Interestingly, smokers can develop also panacinar emphysema, a type of emphysema not usually linked with smoking. In fact, panacinar emphysema is characteristic of patients who develop emphysema relatively early in life, and is usually associated with deficiency of α1-antitrypsin, which normally protects the respiratory region by forming a highly effective antielastase screen. This form of emphysema, in contrast to the centriacinar form, occurs more frequently in the lower lobes of the lung and involves destruction of the alveolar walls in a fairly uniform manner, i.e., all alveolar ducts and alveolar sacs beyond the terminal bronchiole are involved. The mildest form of panacinar emphysema can be clearly identified using a microscope. In fact, in normal lung, the multifaceted alveoli form a contrast to the larger cylindrical conducting structures (alveolar ducts and respiratory bronchioles), whereas in panacinar emphysema the distinction between alveoli and ducts becomes lost as alveoli enlarge, losing their sharp angles and their contrast in size and shape with the ducts. As panacinar emphysema worsens, there is progressive effacement of tissue and loss of the orderly arrangement of the lung until all that remains are strands of tissue crossing a large emphysematous space (64).

Recently, Vlahovic et al. (65) reported an increased septal wall thickness in smokers with COPD, which was correlated with the degree of lung destruction. The fact that the septal thickness increase was due to deposition of both elastin and collagen confirms previous observations by Lang et al. who demonstrated an increase in collagen mass in emphysematous lungs (66). The results of these studies are suggestive of the presence of active alveolar wall fibrosis in the emphysematous lungs, despite the fact that emphysema is defined as "permanent, abnormal enlargement of the respiratory airspaces, accompanied by destruction of their walls, without obvious fibrosis" (67).

The two major morphological forms of emphysema are thought to have distinct functional properties and distinct peripheral airway involvement. In particular, the lung compliance is greater in panlobular than in centrilobular emphysema, whereas the extent of peripheral airway inflammation is greater in the centrilobular than in the panlobular form. It is possible that in centrilobular emphysema airflow obstruction is primarily a function of peripheral airway inflammation as supported by the correlation between reduced expiratory flow and increased airway inflammation observed in this form of emphysema. By contrast, in panlobular emphysema, airflow obstruction seems to primarily be a function of loss of elastic

recoil and to have little relation to peripheral airway inflammation, as supported by the correlation between reduced expiratory flow and increased compliance observed in this form of emphysema (62,63,68).

Only a few studies attempted to correlate the pathological abnormalities with the degree of airflow obstruction in COPD. The largest study, performed by Nagai et al. (69), showed that subjects with severe COPD had both emphysema and peripheral airway abnormalities. Although the relative contribution of each of these pathologic lesions to the development of airflow obstruction was difficult to establish, the contribution of each may vary according to the stage of the disease. Bronchiolar abnormalities may reveal their contribution to chronic airflow obstruction only when COPD is mild. When the disease becomes severe, loss of elastic recoil assumes overwhelming importance and may mask the effects of bronchiolar disease on causing airflow obstruction (70).

In both centriacinar and panacinar emphysema, the destructive process can be detected microscopically in the alveolar walls of smokers even when there is no evidence of airspaces enlargement on gross examination. Several methods have been designed to microscopically quantify emphysema including the mean linear intercept and the destructive index (71). Both allow an early identification of the disease, before macroscopic emphysema is evident. The functional significance of such early destruction is demonstrated by the positive correlation observed in smokers between destructive index and both airflow obstruction and loss of elastic recoil of the lung.

Since emphysema is defined pathologically, traditionally the presence and extent of emphysema have been determined by macroscopic or by microscopic assessment of lung specimens (72). However, these techniques are time consuming and, more importantly, can hardly be performed in partial lung resections and therefore in living patients. During the last few years, it has become increasingly clear that high-resolution computed tomography (HRCT) is an accurate imaging method for diagnosing emphysema in vivo (73). Indeed, pioneer studies have shown that the extent of emphysema assessed on HRCT scan correlates significantly with the extent of emphysema assessed on macroscopic lung sections (74–78). A good correlation has been reported also between HRCT emphysema assessment and destructive index (76). More recently, Gevenois et al. compared transversal CT slice and transversal anatomical lung slices showing that both macroscopic (79) and microscopic (80) emphysema are well quantified by CT in smokers (81). These studies show that CT scanning represents a significant advance in the ability to identify early emphysema in life and to follow its progression, thus allowing pathogenesis to be better understood and the effects of treatment to be determined (82). Nevertheless, as pointed out by Madani et al., this technique is not yet standardized and further studies are needed (73).

IV. CONCLUSIONS

Lung parenchyma inflammation is associated with all stages of COPD, from the asymptomatic response to irritants such cigarette smoke to the respiratory failure associated with severe COPD. However, the severity of this inflammation appears to increase with the progression of the disease. The main inflammatory cells involved in the pathogenesis of COPD include neutrophils, macrophages, and $CD8^+$ T-lymphocytes. The mechanisms by which each of these cells can damage lung tissue are likely to be distinct, although an interaction between different inflammatory cell types could be hypothesized. The fundamental issue of whether these inflammatory cells are related to emphysema, lung function, and symptoms is much debated and remains an unresolved challenge for the future. The newly emerging application of HRCT scan, by allowing the measurement of parenchymal destruction in living subjects, could help to provide new insights into the pathogenesis and the natural history of COPD.

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3

Small Airways Disease in COPD

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I. INTRODUCTION

The tracheobronchial tree of the human lung provides an unequal branching system of conducting airways that can lead to the gas exchanging surface in as few as eight or as many as 23 branches (Fig. 1A and B) depending on the pathway followed (1,2). The term small airways refers to the bronchi and bronchioles less than 2 mm in maximum internal diameter that are located from the 4th to the 14th generation of airway branching in the adult human lung (Fig. 1C). These purely conducting airways end in many thousands of terminal bronchioles that range from 400 to 600 μm in diameter and supply a gas exchanging unit of lung termed the acinus (2,3). Figure 2A shows a group of several terminal bronchioles surrounded by connective tissue septa that defines a larger unit of lung termed the secondary lobule. Within each acinus (Fig. 2B) the terminal bronchioles branch into respiratory bronchioles that are defined by the appearance of alveolar openings in their walls. The numbers of these openings increase with each successive generation of branching until the bronchiolar epithelium is completely replaced by alveolar openings in the alveolar ducts. The ducts branch several more times and finally end blindly as alveolar sacs (3). On average the distance from the first respiratory bronchiole to alveolar sacs is approximately 9 mm and the airways branch up to 12 times over this distance (3). The parallel branching pattern of the smaller airways rapidly enlarge the total cross-sectional area

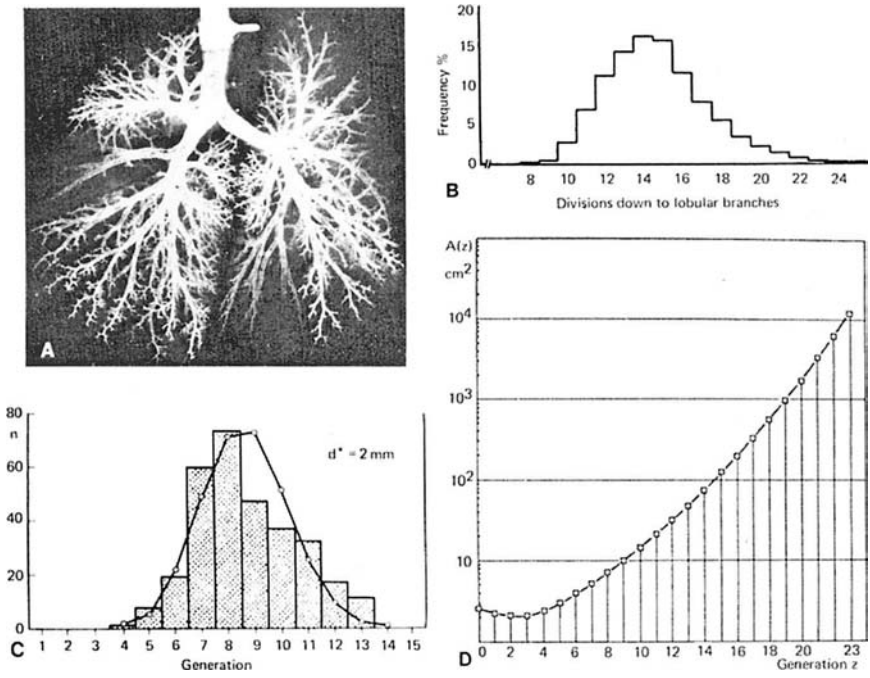

Figure 1 (A) Bronchogram from a normal human lung showing the nature of the branching system of the tracheobronchial tree. (B) Shows that when the trachea is designated 0 the number of branches to the terminal bronchioles varies from as few as eight to as many as 24 depending on the pathway that is followed. (C) Shows that the small conducting airways less than 2 mm in diameter are found from the 4th to the 14th generation of branching in the tracheobronchial tree (D) Shows the remarkable increase in cross-sectional area that occurs beyond the small airways. (Figures B–D are modified from Refs. 1, 2.)

at each successive generation of airway branching (Fig. 1D) which lowers the resistance to the flow in the peripheral lung and shifts the movement of gas from bulk flow to diffusion in the gas exchanging regions.

The total resistance to airflow of a healthy person breathing through the mouth varies with lung volume and is approximately 2 cm H_2O/L/sec during quiet breathing (4). The resistance to flow below the larynx accounts for about 50% of this value (5,6) and the small airways about 10% of total resistance to the bulk flow of gas in the peripheral lung (6,7). For this reason, the small airways have been referred to as the lung's "quiet zone", where considerable disease may develop before there is any effect on total airway resistance (8). The realization that the small airways offer little resistance in health (6,7) and become the major site of airway obstruction in disease (6–8) is consistent with the fact that patients with COPD have had a

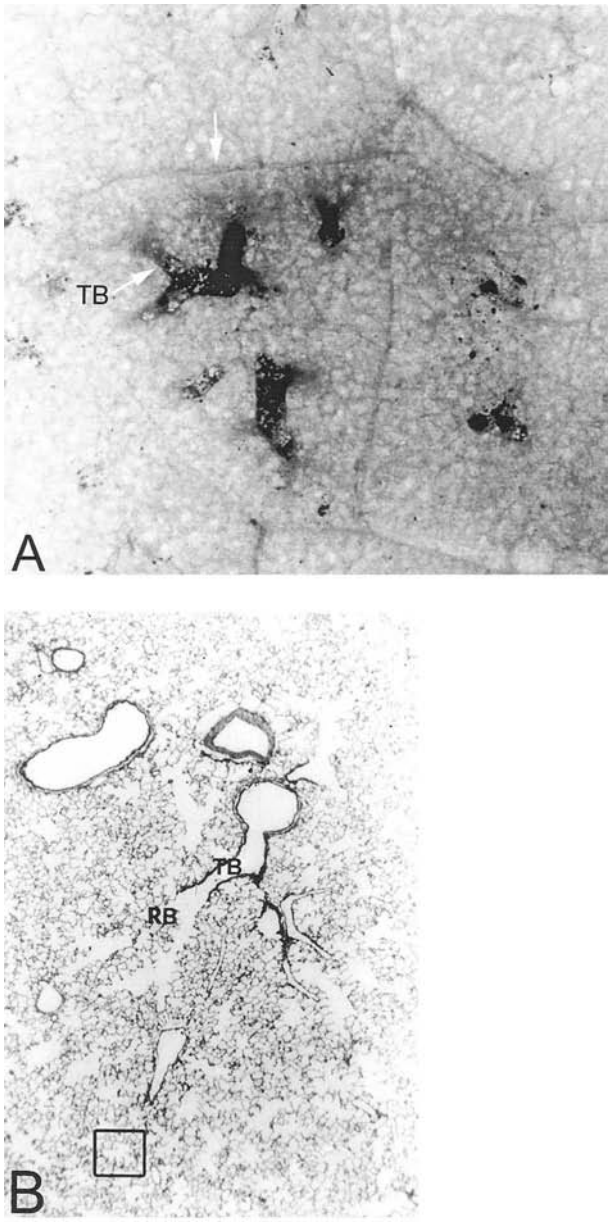

Figure 2 (A) Shows a photograph of the pleural surface of the lung where the boundary of a secondary lobule is outlined by connective tissue septa indicated by the unlabeled arrow. Several terminal bronchioles (TB) can be seen and the fine tuft labeled RB represents the first generation of respiratory bronchioles. (B) Shows a photomicrograph of the histology of a single terminal bronchiole supplying respiratory bronchiole. An alveolar duct is shown in the box.

long subclinical course before they became symptomatic. It also led to the hypothesis that small airways were the lungs quiet zone where disease might be detected and reversed before serious airway obstruction occurred. Although tests were developed that could detect functional abnormalities in the small airways (10) they are no longer used in epidemiological studies because the abnormalities they detect are present in most smokers and do not predict the minority that will go on to develop COPD (11).

Figure 3 summarizes a classic study by Fletcher et al. (11) who made longitudinal measurements of lung function on a group of working men living in London. The lower curved line shows the rapid decline in lung function experienced by a minority of approximately 15% of the cigarette smoking population. The upper curved line represents the minimal decline observed in nonsmokers and the middle two lines represent the decline in FEV_1 observed in the majority of the smoking population that is less susceptible to the cigarette smoking habit. The horizontal lines represent the five stages of severity of COPD recommended by Global initiative on obstructive lung disease (12). Class 0 consists of persons that have normal lung function with respect to these tests but are at risk for COPD either because of their smoking history or the presence of symptoms. Those where FEV_1 remains in the normal range above 80% of the predicted value but the FEV_1/FVC falls below 70% are assigned to the mild disease (Gold Stage 1). In moderate COPD (GOLD-2) the FEV_1 ranges from 80% to 50%, severe disease (GOLD-3) from 50% to 30%, and very severe (GOLD-4) below 30% of the predicted value.

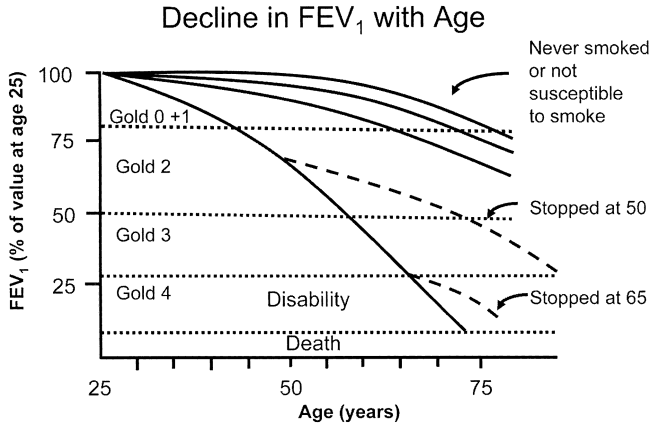

Figure 3 Shows the rate of decline in FEV_1 of men followed longitudinally by Fletcher et al. (see Ref. 15) where a subgroup of heavy smokers lost FEV_1 very rapidly. The horizontal lines indicate the boundaries of the GOLD classification of COPD severity (see text and Ref. 12).

The major risk factor for a rapid decline in FEA_1 that leads to COPD is the chronic inhalation of toxic particles and gases over a person's lifetime (12). The tobacco smoking habit (13) is the dominant factor contributing to this exposure but atmospheric pollution produced by the exhaust from the internal combustion engines, and many other types of exposures that are specific to the home and work place contribute to a lifetime exposure. Although childhood infections of the lower respiratory tract increase the risk for COPD later in life (13) the role of adult infection in the pathogenesis of COPD is a controversial issue. The British hypothesis introduced in the 1950s was based on the concept that there was a progressive change from the chronic cough and sputum production found in smokers to infective bronchitis indicated by the appearance of purulent sputum and finally chronic airways obstruction. However, the study by Fletcher and associates (see Fig. 3) showed that the presence of chronic bronchitis did not predict the rapid decline in lung function that led to severe (GOLD-3) and very severe (GOLD-4) COPD. It is now quite well established that smoking interferes with the clearance of microbes that gain entry into the lower respiratory tract and that the lung becomes colonized by a number of micro-organisms in severe (GOLD-3) and very severe (GOLD-4) COPD. It has also been established that lower respiratory tract infections account for about a third of acute exacerbations of COPD and that these infections are initiated by the appearance of new strains of organisms that chronically colonize the lungs. Although these acute exacerbations are troublesome and expensive, especially if they result in hospitalization they do not appear to influence the rate of decline in COPD except in those that continue to smoke (14).

II. CHRONIC BRONCHITIS AND SMALL AIRWAY OBSTRUCTION

It is a common but incorrect practice to use the terms bronchitis and airways obstruction interchangeably in patients with COPD because the site of obstruction is in the small conducting airways (6–8). The diagnosis of chronic bronchitis is based on the symptom of persistent cough with excessive sputum production that must be present on most days for at least 3 months per year for two consecutive years before the diagnosis can be established (15). Reid (16,17), showed that the presence of these symptoms correlated with increased size of the bronchial mucus glands and she proposed the ratio of the width of the gland to the distance between the reticular basement membrane and cartilage (now referred to the Reid index) as a diagnostic yardstick for the pathologic diagnosis of chronic bronchitis. Mullen et al. (18) and Saetta et al. (19) subsequently showed that the symptoms of chronic bronchitis were associated with an inflammatory process invol-

ving the glands, gland ducts, and epithelial lining of the central airways (Fig. 4) and it is reasonable to postulate that the associated gland hypertrophy is related to growth factors released during this chronic inflammatory process.

The introduction of fiberoptic bronchoscopy provided the opportunity to biopsy the central airways more easily (19–26). These studies have shown that the epithelium of the central airways contains eosinophils as well as areas of both squamous and goblet cell metaplasia in COPD, and that the thickness of the reticular basement membrane remains within the normal range but may overlap the lower range of the values observed in asthma (20,21). The epithelium and subepithelial tissue contain increased numbers of neutrophils (20,21) and smaller numbers of eosinophils that tend to increase during exacerbations of COPD (21), as well as an increase in the numbers of mononuclear cells including mast cells (22) and lymphocytes (23–26). Saetta et al. (23) and Kemeny et al. (24) have shown that the mononuclear cells consist of a mixture T-lymphocytes that express markers of early and late activation (24) and Di Stefano et al. (25) and O'Shaughnessy et al. (26) found that there were greater numbers of CD8 than CD4 cells and

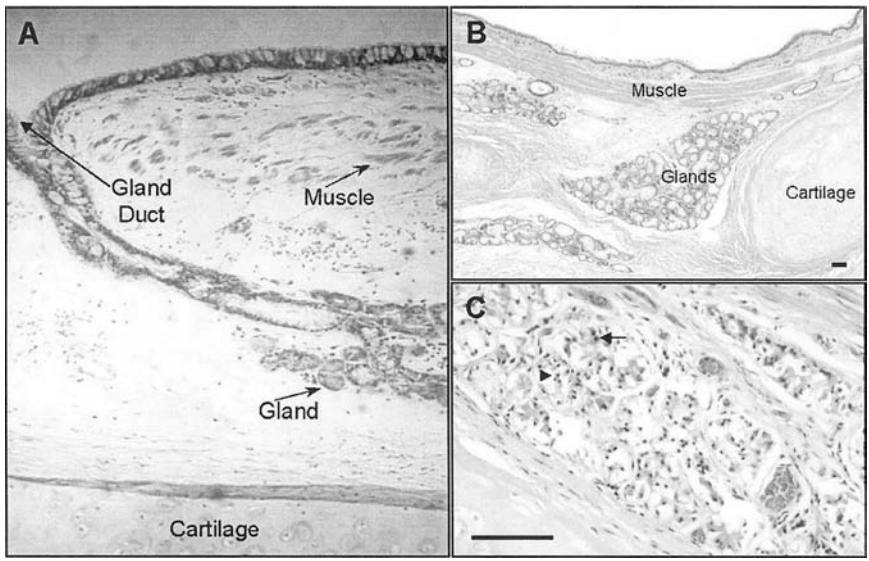

Figure 4 (A) Shows a low power photo micrograph of the bronchial wall that demonstrates a normal mucus producing epithelial gland with its duct connecting to the epithelial surface of the bronchial lumen. B and C show enlarged glands from a patient with chronic bronchitis. The arrow and arrowhead in C point to inflammatory cells in the gland tissue. (Photomicrograph in A was taken by the late professor William M Thurlbeck. From Ref. 12.)

reported an inverse association between CD8 positive T lymphocytes in central airway biopsies and a decline in FEV_1. Unfortunately, there have been very few comparisons of biopsy findings in the larger bronchi with the pathology at site of obstruction in the small conducting airways in the peripheral lung and Mullen et al. (18) data showed that small airways inflammation could be present with or without chronic bronchitis. Therefore, it is safer to conclude that the central airway biopsies need not reflect changes in the peripheral airways.

III. THE PATHOLOGIC FEATURES OF THE SMALL AIRWAYS IN COPD

Direct measurements of peripheral airway resistance in postmortem lungs showed that the major cause of the airway obstruction in COPD is in the small conducting airways (6–8) and one of these studies attributed the obstruction to the structural disease in the airway wall rather than to reduced alveolar support to these airways (6). Niewoehner et al. (27) early study of postmortem lungs from accident victims established that peripheral lung inflammation is present in the lungs of young smokers. Many studies have since confirmed that lung inflammation is present in everyone that smokes (27–30) and others have provided clear evidence that this inflammatory response does not disappear when smoking is stopped (31–36). Dunnill (37) recognized that a predominant inflammatory process was in the outer wall of the small airways of emphysematous lungs and Matsuba and Thurlbeck (38) reported that connective tissue is deposited around these airways in cases of severe emphysema.

Figure 5A shows a group of inflammatory cells migrating through the epithelial cell layer onto the surface of the airways of a guinea pig following acute exposure to cigarette smoke and Fig. 5B shows the microvascular bed of a human small airway where there are on set of microvessels just below the epithelium and a second set of vessels outside the muscle in the adventitial layer. The subepithelial microvasular bed delivers the cells that migrate into the lumen and the microvascular bed outside the muscle is the source of the inflammatory cells that collect in the adventitial compartment of the small airways. The opportunity to study surgically resected lobes and sometimes entire lungs from patients being treated for tumor made it possible to relate the histological findings to measurements of lung function made in the immediate preoperative period. Cosio et al. (39) used this approach to show that there were progressive inflammatory changes in the airways of patients with different levels of COPD severity. And Wright et al. (40) showed a progressive worsening of the disease in both the small conducting airways and respiratory bronchioles as FEN_1 declined. Bosken et al. (41) found that the PMN infiltrate in the subepithelium correlated with the amount smoked

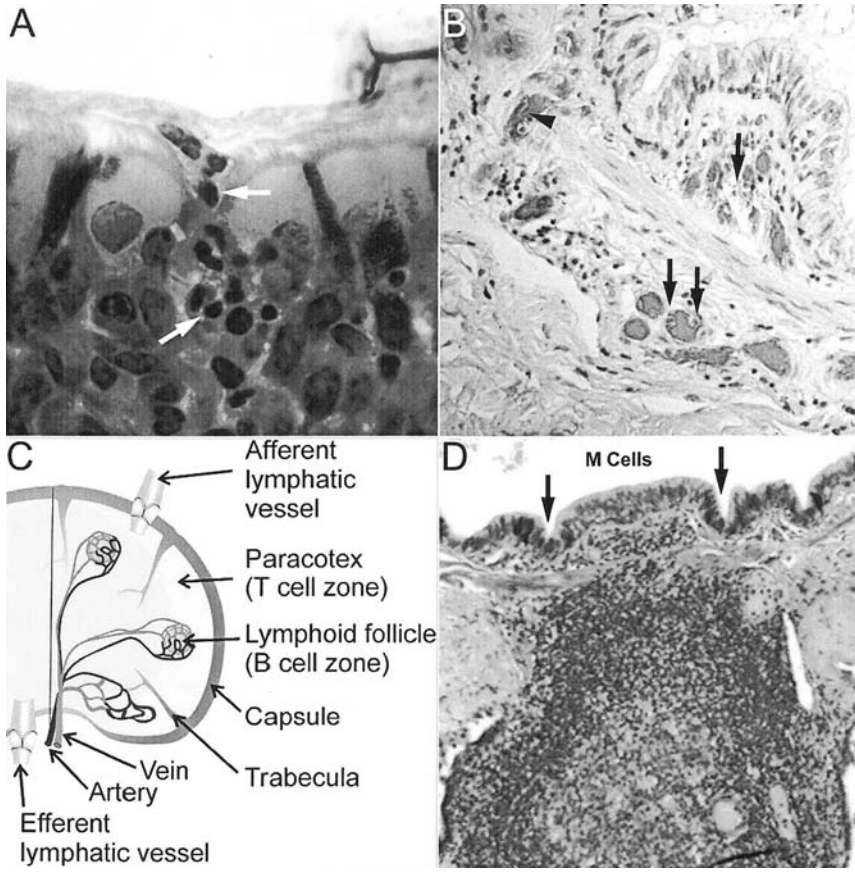

Figure 5 (A) Shows a photomicrograph of the epithelium where a liquid layer can be seen lining the airway surface in a guinea pig lung that has been expose to cigarette smoke. Inflammatory cells can be seen migrating to the surface between the epithelial cells (arrows). (B) Shows a diseased small airway in cross-section where one microvascular bed can be seen beneath the reticular basement membrane (single arrow) and a second in the adventitial space outside the muscle layer (double arrow). These two microvascular beds are connected by vessels that penetrate the muscle layer (arrow head). (C) Shows a diagram of a regional lymph node where the afferent vessel can be seen penetrating the capsule and the efferent vessel leaves at the hilum of the node. The lymphoid follicles are supplied by a cuff of vessels that allow the lymphocytes to migrate out of high endothelial venules into the lymphoid tissue. (See text foe further explanation). (D) Shows a collection of bronchial associated lymphoid tissue (BALT) with a surface covering of M-cells and underlying lymphoid follicle that has an active germinal center.

whereas the collections of B lymphocytes in the adventitial compartment of the peripheral airways that had a tendency to aggregate into lymphoid follicles (Fig. 5C) in mild to moderate COPD. Kuwano et al. (42) showed that the walls of the small airways became thick in COPD and a recent comprehensive study of resected lung tissue from 169 cases over the full range of the GOLD classification confirmed and extended many of these earlier reports (36). These studies established that progression of COPD is associated with a greater number of small airways containing each inflammatory cell type as well as an absolute increase in the accumulated volume of CD8 and B-cell lymphocytes and the number of lymphoid follicles, however, a multiple regression analysis indicates that a decline in FEV_1 was more closely associated with a repair and remodeling process that thickened the walls of the small airways and an accumulation of inflammatory exudates containing mucus in their lumen (36). Collectively, these data suggests that small airway obstruction in COPD is based on an innate inflammatory immune response that is associated with an exudation of fluid and cells into the wall and lumen and an active repair and remodeling process that thickens the walls of these airways (36). The progressive increase in lymphoid follicles with active germinal centers also suggests that an adaptive immune response in the later stages of COPD when the peripheral lung is known to become colonized and infected with a variety of organisms (43).

IV. THE INNATE AND ADAPTIVE HOST DEFENSE SYSTEM

A. Innate Immunity

The first line of the innate defense system of the lung is the mucus producing and clearance system that physically removes foreign material deposited on the airway surface (44). When this system fails plugs of exudates containing mucus accumulate in the airway lumen, interfere with the clearance of microbes from the lower airways and predispose the lung to infection. Although, it is easy to demonstrate this type of airway occlusion in postmortem studies it is not easy to separate the chronic disease from the effects of bronchopneumonia near the end of life. Cosio et al. (39) study of surgically resected lung specimens suggested that the occlusion of the small airways could be reliably estimated but did not correlate with a decline in lung function. But a recent study of a much larger number of cases over the full range of COPD severity suggests that there is an association between occlusion of the airway lumen with inflammatory exudates containing mucus and the development of flow limitation (36).

The second line of the innate defense system is provided by the tight junctions that join the airway epithelial cells beneath the mucociliary layer and provide protective barrier that is breached by chronic cigarette smoke exposure (45). Cytokines released by macrophages and epithelial cells during

the innate response attract polymorphonuclear neutrophils (PMN), alveolar macrophages (AM) and natural killer (NK) cells into the damaged tissue (46–50). Molecules produced by cells at the site of injury (lysozyme, iactoferrin, the surfactant protein A and D, and the defensins) (51–53) as well as others produced in the liver and transported to the inflammatory site in the blood (c-reactive protein, mannose binding protein, and compliment protein C-3) cooperate with these migrating cells to enhance the phagocytosis and the killing properties of the neutrophils and macrophages (46). Although the innate system has primarily been studied in relation to infection it is also stimulated to respond by nonliving particulates suspended in the atmosphere (47,49,50,54). Most of the larger atmospheric particles are efficiently removed from inhaled air by the filter provided by the upper airway but some of these particles trapped in this way are subsequently aspirated into the lower airways during sleep (55). The smaller atmospheric particles as well the fine particulates generated in tobacco smoke escape the upper airway filter and are deposited directly in the lungs. The particles that reach the lower airways from either inhalation or aspiration from the upper airway provide the major stimulus to for the inflammatory process responsible for COPD (12).

The alveolar macrophages produce a variety of cytokines as the phagocytose foreign particles deposited on the lung surface that include TNFα, IL-6, GM-CSF, MIP-1α, and IL-1β (47). The bronchial epithelial cells also take up these particles and produce LIF, GM-CSF, IL-1α, and IL-8 during this process (49) and the macrophages and epithelial cells appear to cooperate to increase local cytokine production (50). Some of these cytokines (TNFα and IL-1β) function in a paracrine fashion to activate the endothelium and epithelium to express the adhesion proteins that control the migration of leukocytes (46). Cytokines that enter the blood have an endocrine function that stimulate the hypothalamus to initiate fever (TNFα and IL-1β), activate the synthesis of acute phase proteins in the liver (TNFα, IL-1β, and IL-6) and increase the production and release of leukocytes and platelets from the bone marrow (TNFα, GM-CSF, and IL-6). The production of IL-12 by macrophages also stimulates the NK cells to produce IFN-γ which promotes MHC class 1 and class 11 expressions on surrounding cells and induces the macrophages to enhance their respiratory burst. This IFN-γ production promotes the proliferation of antigen stimulated T cells that initiate the cellular and humoral components of the adaptive immune response (46).

B. Toll-Like Receptors

New insight into the mechanisms responsible for the initiation of the innate response has been provided by the discovery of the Toll receptors (56). These receptors were first discovered in flies (Drosophila) and have

subsequently been shown to be widely distributed in other insects, animals, and plants. In mammals, they are referred to as Toll-like receptors (TLRs) because of their similarities to the original Toll receptors (56). The extracellular domains of these receptors detect specific components of bacterial, fungal, and viral pathogens that serve as ligands for the TLRs. The interaction between ligand and receptor initiates the production of the cytokines that control the innate inflammatory immune response. The mammalian TLR family continues to grow and currently has 10 recognized members. They are expressed on a wide variety of inflammatory immune cells including monocyte/macrophages, dendritic and mast cells. They are also expressed on the epithelial cells of the airway and are located on the lateral surface below the tight junctions in the gastrointestinal mucosa (56). This requires that microbes breach the epithelial barrier and invade the paracellular space from the apical surface before they interact with a TLR and elicit a response.

The chromosomal location of each of the 10 known genes for human TLRs is established and ligands recognized by TLR1,2,3,4,5,7 and 9 have been identified (56). These ligands include components of fungi (TLR2), gram positive bacteria (TLR2), gram negative bacteria (TLR4), mycobacteria (TLR1), bacterial flagella (TLR5), mycoplasma (TLR6) and viruses (TLR3) (56). Activation of the TLRs by these ligands initiates an intracellular signaling pathway that is similar to the mammalian IL-1 pathway. The close association between the Drosophila Toll and mammalian IL-1 receptor is highly conserved and is referred to as the Toll/IL-1 receptor (TIR) domain. Both TLRs and IL-1R associate with an intracellular adaptor protein MyD 88 which has a TIR domain and this interaction leads to the activation of both the JNK and NF-κB signaling pathways that regulate the expression of a many genes associated with the innate inflammatory immune response (56). The distribution of the toll receptors in the human lung is only beginning to be studied and should provide some novel insights into COPD.

C. Adaptive Immunity

In contrast to the innate immune system which responds quickly to many stimuli and mounts a nonspecific response that lacks specificity and has no memory the adaptive immune response develops more slowly, is very specific to individual antigens and has exquisite memory for previous exposure. The development of an adaptive response requires an interaction between T cells and B lymphocytes that have recognized the same antigen but the chance of this happening in the peripheral blood where only one in every 10^5 or 10^6 lymphocytes demonstrate such specificity is very remote. Local lymphatic collections in the regional lymph nodes and bronchial mucosa greatly increase the opportunity for this type of interaction. Because

a proportion of the lymphocytes delivered to this tissue leave the circulating blood by migrating out of veins that are lined by specialized high endothelial cells. The B-cells that enter the lymphatic tissue in this way accumulate at the edge of the lymphoid follicles and T cells in the tissue that surrounds the follicles (57). Antigens picked up by dendritic cells located in the epithelium and lamina propria, are processed as they are transported to regional lymph nodes in the afferent lymphatics (Fig. 5C) and deposited in the marginal sinus of the node. These dendritic cells percolate through the node where they have the opportunity to present antigen to both B cells located at the edge of the follicles and T cells located in the paracortical tissues between and beneath these follicles before they exit in the efferent lymphatic of the node. The CD4 T lymphocytes that are activated by antigen presented to them in paracortical tissue migrate to the edge of the follicles where their chance of meeting a B cell that has been activated by the same antigen is very much improved over that available in peripheral blood. The B cells that receive help signals from the CD4 T cells proliferate and migrate into the germinal center of the follicle where they produce antibody of varying affinity that binds to antigen presented to them by a separate set of follicular dendritic cells. The B cells that produce low affinity antibody receive a death signal and undergo apoptosis whereas those that present high affinity antibody continue to mature into memory cells and plasma cells capable of secreting antibody.

The bronchial associated lymphoid tissue (BALT) differs from that found in regional lymph nodes in that it does not have a capsule and does not receive afferent lymphatic vessels (compare Fig. 5C and D). The BALT collections (Fig. 5D) receive antigen transported directly across the epithelium by specialized epithelial M-cells. The cuff of lymphocytes that surround the B cell rich germinal centers of these lymphoid follicles extends to the epithelium of both the conducting airways and the alveolar surface suggesting that they may receive antigens transported across both the alveolar and small airway surface (36,41,57,59). The dominant class of antibody produced in a mucosal immune response is IgA and the major stimulus for the switching of antibody isotype from IgM to IgA during this response is TGF-β and IL-5 (57). The B cells that mature in the germinal centers of the mucosal lymphatics home back to the lamina propria below the epithelial basement membrane after they enter the circulation and secrete their IgA as a dimer held together by a J chain. This complex binds to poly Ig receptors at the base of the epithelial cells and is actively transported to the airway luminal surface where it is released into the lumen by proteolytic cleavage. Much remains to be learned about the mucosal immune response of the lung in human disease and its precise relationship to the pathogenesis of airway obstruction.

The humoral component of the adaptive immune response generates mature B cells that produce antibodies that protect the host against

microbes that remain in the extracellular space (57). These antibodies are able to neutralize microbial toxins and initiate a much more efficient opsonization and phagocytic process that can be mounted by the innate system. Antibodies that attach to the surface of microorganisms also bind the C1q component of the large multimeric C1 complement molecule circulating in the plasma and initiating the classical complement pathway. Interleukin 2 is produced by T lymphocytes and stimulates the proliferation of T cells, B cells, and natural killer cells. A Th2 subset of CD4 positive lymphocytes secrete IL-4 to initiate antibody isotype switching to IgE production in B cells and IL-5 that stimulates eosinophil production and activation. IL-5 also co-operates with TGF-β to switch B cells to the production of an IgA antibody isotype. Without these stimuli the B cells produce the more common IgM, IgG, and its subclasses.

The cellular component of the adaptive immune response protects the host from microbes that either survive within phagocytes or infect nonphagocytic cells (58). The adaptive immune response to microbes residing within the phagosomes of phagocytes is mediated by a CD4 Th1 lymphocytes as well as CD8 T cells. Both these cells are able to recognize antigens displayed on the surface of macrophages and secrete cytokines that activate them to kill the organisms they have phagocytosed. The CD8 positive lymphocytes are also able to recognize nonphagocytic cells infected by intracellular pathogens and destroy them in three steps. The first is a recognition step where the cytotoxic lymphocyte uses the T-cell receptor to bind to foreign material displayed on the surface of the target cell with the HLA self molecule. The second involves the molecule perforin which creates holes that connect the cytotoxic T cell to its target and the third involves the introduction of the enzyme granzyme that activates the target cell caspases and introduces apoptosis.

There is a substantial increase in mucosal lymphoid follicles in the small airways of patients with severe (GOLD-3) and very severe (GOLD-4) COPD (36). This probably reflects an adaptive immune response to the colonization of the peripheral lung in the later stages of COPD severity. The bacteria that commonly colonize and infect the lungs of patients at this stage of their disease include *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, Enterobacteriaceae, and *Haemophilus parainfluenzae* (43). Sethi and colleagues have reported an important longitudinal cohort study of patients with COPD that was designed to detect new strains of organisms by pulse field electrophoresis (60). Their results showed that the presence of new strains is common, that some new strains produce exacerbations and that the acquisition of a new strain enhances the risk of an acute episode more than twofold. Abbe Abe (61) and others have also shown that the cellular immune response to these new strains is type specific and that it commonly targets epitopes expressed on the outer membrane proteins (OMPs) of the organism. The antibodies produced against one

strain do not protect against another (62) and a specific decrease in the peripheral blood T lymphocyte proliferation to challenge with a well conserved OMP of *H. influenzae* (OMP P6), appeared to increase the susceptibility to H-Flu induced exacerbations (61). Transgenic mouse experiments indicate *H. influenzae* can induce an innate response by interacting with TLR-4 (63) and it is conceivable that this type of response might induce some of the exacerbations of COPD.

D. Remodeling

The remodeling process that repairs the damage associated with the chronic innate and adaptive immune response in the airways tissue is most closely associated with the decline in lung function in COPD (36). Very little is known about the fundamental mechanisms involved in airway remodeling but it is reasonable to assume that it is similar to the repair process during the healing of wounds. Studies of wound healing indicate that activation of the myofibroblasts to secrete new extracellular matrix (ECM) is a key feature of the tissue repair in wound healing (64). The myofibroblasts are a group of cells of uncertain origin that share features of both fibroblast and smooth muscle cells. They were first described in the lung by Kapanci et al. (65) and are now known to be widely distributed in body tissues where they play a central role in the initiation and control of the inflammatory repair and remodeling processes. Myofibroblasts are capable of synthesizing prostaglandins and expressing both the constitutive cyclooxygenase-1 (COX-1 or PHS-1) gene product and the inducible COX-2 (PHS-2) protein (66–70). They are also avid producers of chemokines and cytokines and express α and β integrins that provide adherence to matrix proteins (71–74). When activated, they express intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM-1), and other adhesion molecules that allow lymphocytes, mast cells, and neutrophils to dock on their surface (64). Walker and his associates have shown that a stellate myofibroblast phenotype populates the interstitial space of the alveolar wall and sends extensions though the endothelial and epithelial basement membranes to make contact with these cells (75–78). This arrangement suggests that the stellate myofibroblast phenotype may be the ''quarterback'' that directs inflammatory cell traffic through the interstitial compartment of normal peripheral lung tissue.

Figure 6 has been modified from the review of Powell et al. (64) to indicate that an activated myofibroblast phenotype capable of synthesizing matrix may arise from stem cells, fibroblasts, and the stellate form of the myofibroblast. The activated phenotype differs from the other forms in that it expresses α-SM actin, has a reduced number of vitamin A lipid droplets and a more expanded rough endoplasmic reticulum than the other forms. It also has fewer extensions and less contact with other cells than the stellate

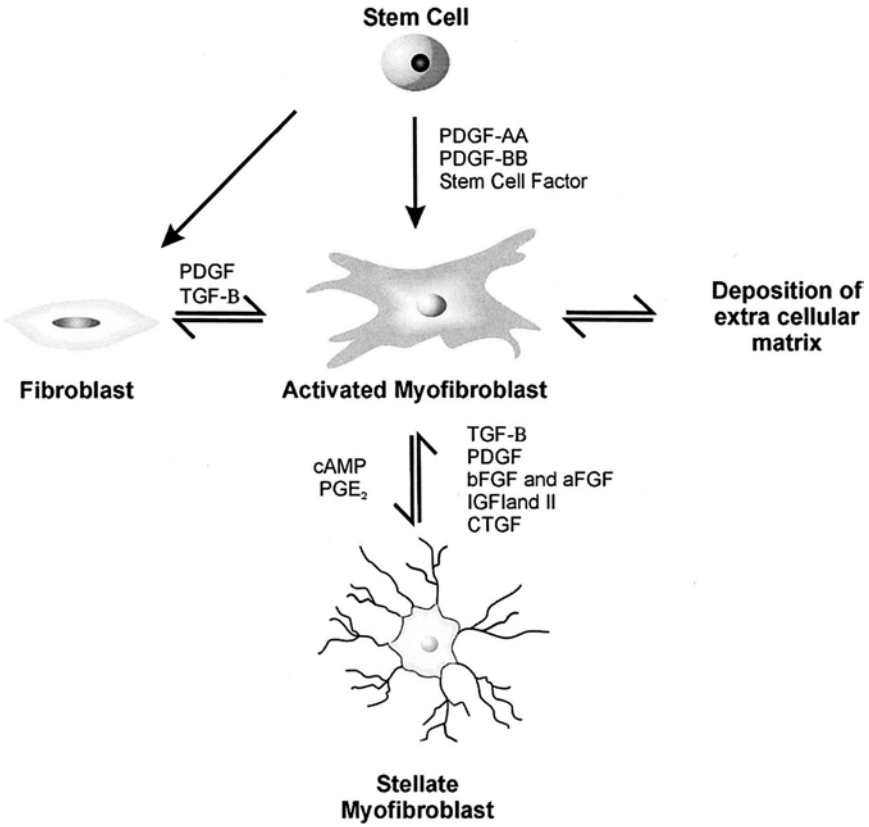

Figure 6 Diagram modified from Ref. 64 showing some of the known features of the interrelationships between stem cells, fibroblasts, myofibroblasts, and activated myofibroblasts. The stellate myofibroblast can be found in the alveolar wall interstitial space and are in a position to provide directional information to migrating leukocytes. A variety of growth factors (indicated in the diagram) act with other mediators that are not shown to stimulate the myofibroblast to secrete matrix proteins. (From Refs. 75, 76.)

myofibroblasts. Although many cytokines and growth factors have been incriminated in the activation process TGF-β1 appears to have the major role in stimulating both the fibroblast and stellate myofibroblast to differentiate to an activated myofibroblast phenotype capable of collagen secretion (64,79–82). Tumor growth factor-β1 also has an indirect role in initiating myofibroblast proliferation by upregulating PDGF receptors and stimulating the production of CTGF (64,83). Other growth factors including TGF-α a member of the epidermal growth factor (EGF) family, as well as EGF itself GM-CSF, both acidic and basic FGF (aFGF and

bFGF), as well as insulin growth factors IGF-I and IGF-II also promote myofibroblast proliferation and ECM synthesis (64,82–84).

Studies performed in transgenic animals by Lee et al have shown that over expression of IL-13 resulted in TGF-β1 production and activation by pathways involving both plasmin and MMP-9 that initiated massive connective tissue deposition in the walls of the peripheral airways and also increased the formation of lymphoid follicles (85). These findings are provocative because the pathology produced in the peripheral airways by this manipulation shares many of the features of human disease where there is growing evidence that susceptibility to COPD is associated with an amplified inflammatory immune response (35,36). But much remains to be done to precisely determine how either the innate or adaptive inflammatory responses are linked to the repair and remodeling process that remodels the airway walls.

In summary the small conducting airways are the major site of airway obstruction in COPD and the pathology responsible for this obstruction can be attributed to the innate and adaptive inflammatory immune response to the chronic inhalation of toxic gases and particles. Although the extent and severity of the infiltration of the airway walls is associated with a decline in lung function in COPD there is a much closer association with the accumulation of inflammatory exudates in the lumen of the airways as a result of failure of the innate response to clear the airways and a thickening of the airway wall by the repair and remodeling process associated with both the innate and adaptive immune system. A clearer understanding of the molecular mechanisms that link the individual components of the overall response in the peripheral lung could lead to new therapeutic targets for this condition.

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4

Asthma vs. COPD: Cellular and Molecular Differences

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I. INTRODUCTION

COPD and bronchial asthma are considered two distinct chronic respiratory disorders sharing two common functional features, airflow limitation and bronchopulmonary inflammation (1,2). For clinical purposes, COPD is defined on a spirometric basis, as a disease state characterized by airflow limitation that is *not fully reversible* postbronchodilator $FEV_1 < 80\%$ predicted with a $FEV_1/FVC < 70\%$ and is progressive in a subject with a current or previous history of tobacco smoking or exposure to noxious agents (2). Similarly, asthma is defined on a spirometric basis, i.e. a disease state characterized by airflow limitation that is *often reversible* either spontaneously or with treatment (1). The difference between *not fully reversible* airflow limitation of COPD and *often reversible* airflow limitation of asthma is minimal and makes it difficult in some cases to distinguish the two disease states. In addition, in asthma, airflow limitation may also progress, even if at lesser extent as compared to COPD, and thus it may shift from reversible to not reversible as in COPD (3). Chronic airway inflammation is present in both COPD and asthma, even though the characteristics of the inflammatory process are markedly different in the two disease states (4,5).

Thus, although the clinical, functional, and inflammatory characteristics are usually markedly different in COPD and asthma, in clinical practice the differential diagnosis between the two respiratory conditions may become complex, particularly in the elderly or when there are overlapping features. Indeed, it is easy to diagnose COPD in a 60-year-old life-long smoker with dyspnea, chronic cough, and sputum, and fixed airflow limitation. Similarly, it is quite easy to diagnose asthma in a young atopic nonsmoking individual, with recurrent episodes of dyspnea, wheezing or chest tightness, and variable and reversible airflow limitation. However, it might become difficult to differentiate between COPD and asthma in adults over 60 with confounding features, e.g., (1) smoking asthmatics, subjects with adult onset asthma, (2) life-long nonsmokers who present with fixed airflow limitation and no history of asthma, (3) smokers with a clear story of COPD but also a significant degree of reversibility of the airflow limitation, (4) nonsmoking asthmatics with fixed airflow limitation, (5) subjects who report both diseases. In these cases, the differential diagnosis between COPD and asthma may be difficult but nonetheless important, as the natural history (Ref. 6) and the response to pharmacological treatment (Ref. 7) are different in the two disease states.

Epidemiologic studies have shown that there is a significant overlap between COPD and asthma. Subjects who report both diseases have lower lung function and more respiratory symptoms than subjects with just one or the other (8). Asthma may be associated with reduced lung function in up to 20% of adults (8), and significant reversibility of airflow limitation to steroids have also been reported to occur in a significant proportion of subjects with COPD and no history of atopy or asthma (9). Data from epidemiological studies show that up to 12% of subjects with COPD are nonsmokers (with a predominance of females) and there is evidence of increasing incidence with increasing age (10–12). Over 20% of asthmatics are current smokers and the 22–43% of adult asthmatics are exsmokers (13,14). Current asthmatic smokers, compared with never smokers, have more severe asthma symptoms, an accelerated decline in lung function, increase in hospitalization rates for asthma, and increased mortality following a near fatal asthma attack (15). Moreover, some data show that active smoking impairs the efficacy of systemic and/or inhaled corticosteroid treatment in chronic asthma (16,17).

II. ASTHMA WITH FIXED AIRFLOW LIMITATION

Although it has been reported that asthma may be associated with the development of fixed airflow limitation (18) and respiratory failure (19), the natural history of asthma is not well defined. In particular, it is unclear whether asthma is associated with progressive decline of lung function over time, and

thus whether (1) asthmatics indeed develop fixed airflow limitation, and if so, (2) whether the pathological basis of fixed airflow limitation occurring in asthmatics is similar to the development of fixed airflow limitation occurring in COPD.

Recent data from a longitudinal epidemiological study conducted in the general population, the Copenhagen City Heart Study, have recently provided an answer to this important question (3). Data were analyzed in terms of changes over time in the forced expiratory volume in one second (FEV_1) in adults with self-reported asthma and adults without asthma (3). The study, conducted between 1976 and 1994, evaluated measurements of lung function from 17,506 subjects, of whom 1095 had asthma, over a 15-year period. The decline in FEV_1 among subjects with asthma was greater than among those without the disease. Among both men and women, and among both smokers and nonsmokers, subjects with asthma had greater declines in FEV_1 over time than those without asthma. This study demonstrated that in a sample of the general population, people who identified themselves as having asthma have substantially greater declines in FEV_1 over time than those who do not (3), suggesting that asthma, similarly to COPD, may indeed be associated with the development of fixed airflow limitation albeit at a lesser extent. Thus, asthma similarly to COPD, but obviously through different mechanisms, may indeed be associated with an increased decline of lung function that can cause fixed airflow limitation (3,20,21).

Patients with fixed airflow limitation are often grouped under the general heading of COPD, and some international guidelines (22) recommend classifying asthma with fixed airflow limitation as COPD. However, although asthmatic patients with fixed airflow limitation are often diagnosed as COPD, the differential diagnosis between asthma and COPD in patients with fixed airflow limitation may be important as the natural history (6) as well as the response to treatment (7) are different, depending on whether fixed airflow limitation is due to asthma or COPD. The course and prognosis in subjects selected from the general population having chronic airflow obstruction (FEVI $< 60\%$ predicted) at the time of their enrollment was analyzed in a longitudinal epidemiological study (6). Mortality and the rate of change in lung function were analyzed in relation to the initial clinical characteristics of the subjects. Subjects with symptoms and signs of asthma had a higher survival rate and a much lower rate of decline in pulmonary function than subjects with clinical characteristics compatible with an emphysematous form of COPD. The 10-year mortality among nonatopic smokers without a history of asthma was close to 60%, whereas it was only 15% in atopic subjects or nonsmokers with known asthma. The mean overall rate of decline in FEV_1 was 70 mL per year in COPD patients, but less than 5 mL per year in subjects with symptoms and signs of asthma. Patients who did not clearly fit into either COPD neither asthmatic subjects had intermediate values for survival and decline in pulmonary function (6).

These data showed that the natural history of asthma is more favorable than COPD once fixed chronic airflow obstruction has occurred.

A pivotal multicenter trial was conducted by Kerstjens et al. (7) who compared the response to bronchodilators (terbutaline or ipratropium bromide) or corticosteroid treatment in 274 patients with airways hyperresponsiveness and airflow limitation who were 18–60 years old. The 56% of patients had allergies and the mean FEV_1 was 64% of the predicted value. The mean FEV_1 increased by 10.3% of the predicted value in the corticosteroid group within 3 months and remained stable thereafter, whereas it did not change in the other two groups. In the corticosteroid group, patients who did not smoke, who had allergies, or who were < 40 years old benefited more from their treatment than did those who smoked, did not have allergies, or were over 40. This study suggested that the addition of an inhaled corticosteroid, but not of an additional bronchodilator (inhaled anticholinergic agent) to maintenance treatment with a beta 2-agonist (terbutaline) substantially reduced airways obstruction, but only in patients with a history of allergy and/or asthma.

III. PATHOPHYSIOLOGY

A. COPD

In COPD, the structural changes occurring in both the large and small airways, and in the lung parenchyma may be related to the characteristic clinical manifestations and lung function changes of the disease, e.g. symptoms (i.e. chronic cough and sputum production), airflow limitation, gas-exchange abnormalities, pulmonary hypertension, and cor pulmonale (2).

Inflammation of the submucosal glands and hyperplasia of goblet cells may contribute to symptoms, such as chronic sputum production, although these pathological abnormalities are not present in all patients with chronic sputum and may be present in subjects without symptoms. The various pathological changes in the central airways responsible for the symptoms of chronic cough and sputum production may continue to be present throughout the course of the disease. Thus, these pathological changes may be present either on their own or in combination with the changes in the peripheral airways and lung parenchyma described below.

Fixed or poorly reversible expiratory airflow limitation is the hallmark functional abnormality of COPD. Several pathological characteristics may contribute to airflow limitation. Airway remodeling and emphysema are most likely responsible for the fixed, poorly reversible component of airflow limitation, whereas airway smooth-muscle contraction, airway inflammation, and intraluminal accumulation of mucus and plasma exudate may be responsible for the small part of airflow limitation that is still reversible either spontaneously or with treatment (1) (Table 1).

Table 1 Causes of Airflow Limitation in COPD (Adapted from Ref. 2.)

Irreversible	Fibrosis and narrowing of airways
	Loss of elastic recoil due to alveolar destruction
	Destruction of alveolar support that maintains patency of small airways
Reversible	Accumulation of inflammatory cells, mucus, and plasma exudate in bronchi
	Smooth-muscle contraction in peripheral and central airways
	Dynamic hyperinflation during exercise

The early decline in lung function in COPD is correlated with inflammatory changes in the peripheral airways, similar to those that occur in the central airways: exudate of fluid and cells in the airway wall and lumen, goblet and squamous cell metaplasia of the epithelium, edema of the airway mucosa due to inflammation, and excess mucus in the airways due to goblet cell metaplasia. The most characteristic change in the peripheral airways of patients with COPD is airway narrowing. Inflammation initiated by cigarette smoking and other risk factors leads to repeated cycles of injury and repair of the walls of the peripheral airways. Injury is caused either directly by inhaled toxic particles and gases such as those found in cigarette smoke, or indirectly by the action of inflammatory mediators; this injury then initiates repair processes. Although airway repair is only partly understood, it seems likely that disordered repair processes can lead to tissue remodeling with altered structure and function. Cigarette smoke may impair lung repair mechanisms, thereby further contributing to altered lung structure (1). Even normal lung repair mechanisms can lead to airway remodeling because tissue repair in the airways, as elsewhere in the body, may involve scar tissue formation. Inflammatory changes such as airway edema and mucus hypersecretion also contribute to airway narrowing in COPD. So does loss of elastic recoil, but fibrosis of the small airways plays the largest role (1).

The relative contribution of airway remodeling and emphysema to airflow limitation is not known. Indeed, there is still no consensus on whether the fixed airflow limitation in COPD is mainly due to inflammation and scarring in the small airways or predominantly to loss of support to the airways resulting from loss of alveolar walls due to emphysema. In general, the studies assessing the lung function in relation to airway and pulmonary structure have shown a relatively poor relationship between macroscopic emphysema and the severity of airways obstruction as measured with spirometry. However, the relative contribution of airway narrowing/fibrosis and emphysema to airflow limitation may depend on the severity of COPD. Bronchiolar abnormalities may contribute more significantly to mild–moderate chronic airflow limitation. When only subjects with less severe

COPD are considered, several indices of bronchiolar inflammation correlate with the degree of airflow obstruction. Indeed, the most consistent relationship between lung function and airway and pulmonary structure found in subjects with severe COPD is between severe emphysema and severe airflow limitation. The most important factor is emphysema and loss of elastic recoil. Most studies in advanced COPD find that the best reflection of the severity of airflow limitation is the extent of pulmonary emphysema. Thus, although both the destruction of alveolar attachments to the outer wall of the peripheral airways and the loss of lung elastic recoil produced by emphysema have been implicated in the pathogenesis of peripheral airways obstruction, direct measurements of peripheral airways resistance show that the structural changes in the airway wall are the most important cause of the increase in peripheral airways resistance in COPD. Thus, when COPD becomes moderate or severe, loss of elastic recoil becomes overwhelmingly important and may mask the effects of bronchiolar disease on chronic airflow limitation (1).

Advanced COPD is also associated with gas-exchange abnormalities, i.e. hypoxemia and, later on, hypercapnia. Abnormal gas exchange may be due to several factors, such as alveolar hypoventilation, altered gas transfer, inequalities in ventilation–perfusion (V/Q) ratio, and right–left blood shunting. Several studies have demonstrated a negative relationship between single breath or steady-state carbon monoxide transfer factor (TLCO) and the degree of emphysema (23,24). In COPD, regardless of the stage of disease and the presence or absence of emphysema, V/Q inequality is generally accepted to be the major mechanism that impairs gas exchange and leads to arterial hypoxemia. Impaired V/Q relationships may be caused by multiple pathological changes in different lung structures, including the airways, parenchyma, and pulmonary vasculature. Bronchiolar lesions are associated with V/Q mismatching, as indicated by a significant correlation between bronchiolar inflammation and the distribution of ventilation. Low V/Q units in the lungs may represent areas with a partially blocked airway. Destruction of the lung surface area by emphysema reduces the diffusing capacity and interferes with gas exchange (23). The severity of pulmonary emphysema appears to be related to the overall inefficiency of the lung as a gas exchanger. This is reflected by the good correlation between the diffusing capacity of carbon monoxide per liter of alveolar volume (TLCO/VA) and the severity of macroscopic emphysema. Reduced ventilation due to loss of elastic recoil in emphysematous lung together with loss of the capillary bed and generalized inhomogeneity of ventilation due to the patchy nature of these changes leads to areas of V/Q mismatching which result in arterial hypoxemia. Of the four classic mechanisms determining hypoxemia and/or hypercapnia—alveolar hypoventilation, alveolar-end capillary diffusion limitation to oxygen, increased intrapulmonary shunt, and ventilation–perfusion mismatching—the last is by far the most common intrapulmonary determinant of hypoxe-

mia in COPD. The role of shunt is almost negligible, even in the most life-threatening conditions, and diffusion limitation is conspicuously absent. Hypercapnia can be induced by ventilation–perfusion imbalance and/or alveolar hypoventilation, the latter being predominant during exacerbations.

Pulmonary hypertension develops late in the natural history of patients with COPD is usually associated with the development of severe hypoxemia ($PaO_2 < 8$ KPa or 60 mmHg), and often of hypercapnia as well. It represents the main cardiovascular complication associated with the development of right ventricular hypertrophy (cor pulmonale). Several factors are known to contribute to the development of pulmonary hypertension in patients with COPD, i.e. (a) thickening of pulmonary vessel walls and reduction of lumen, (b) hypoxia, which causes pulmonary vascular smooth muscle to contract and further reduces the lumen, (c) impaired endothelium-dependent vasodilation (reduction of nitric oxide (NO) synthesis or release in response to hypoxemia), (d) abnormal secretion of vasoconstrictor peptides such as endothelin-1, (e) destruction of the capillary bed, which further increases the pressure required to perfuse the pulmonary circulation.

Cor pulmonale is defined as "hypertrophy of the right ventricle resulting from diseases affecting the function and/or structure of the lungs, except when these pulmonary alterations are the result of diseases that primarily affect the left side of the heart, as in congenital heart disease." This is a pathological definition and the clinical diagnosis and assessment of right ventricular hypertrophy is difficult in life. The prevalence and natural history of cor pulmonale in COPD are not yet clear. Pulmonary hypertension and reduction of the vascular bed due to emphysema can lead to right ventricular hypertrophy and right heart failure. Right heart failure is associated with venous stasis and thrombosis that may result in pulmonary embolism and further compromise the pulmonary circulation.

B. Asthma

Similar to COPD, also in asthma structural changes occur in both large and small airways and may lead to the characteristic clinical manifestations and lung function changes of the disease, i.e. respiratory symptoms (i.e. wheezing, dyspnea, chest tightness), often reversible airflow limitation, and in some cases, gas-exchange abnormalities (1). At variance with COPD, asthma is usually not associated with emphysema, i.e. the lung parenchyma is usually preserved even in most severe asthmatics.

Asthma is a complex inflammatory disease state characterized by a specific pattern of inflammation involving many inflammatory cells, mediators, with multiple inflammatory effects, including bronchoconstriction, plasma exudation, mucus hypersecretion, and sensory nerve activation. Genetic factors are involved in the predisposition to atopy, the most important risk factor for asthma together with allergen exposure. There is

accumulating evidence that in addition to the airway "functional" change, also airway structural changes or "remodeling" may occur in asthma, probably secondary to the airway inflammatory process (19,25,26). Airway remodeling might be linked to the development of physiologic dysfunction, providing a possible mechanism for the development of fixed airflow limitation observed in some patients with asthma (27).

The relationship between airway inflammation and clinical symptoms is not yet clear. Inflammation of the airways may increase airway responsiveness, but inflammation may also directly lead to an increase in asthma symptoms, such as cough and chest tightness, by activation of airway sensory nerve endings. The airway chronic inflammation may lead to the structural changes in the airways, including subepithelial fibrosis, airway smooth-muscle hypertrophy/hyperplasia, angiogenesis, and mucus hyperplasia.

C. Asthma with Fixed Airflow Limitation

The pathophysiology of asthma with fixed airflow limitation has been poorly investigated. In particular, we do not know whether the development of a fixed airflow limitation in asthma is due to structural abnormalities similar to those present in COPD. A recent study has addressed this issue (28), i.e. whether subjects with fixed airflow limitation have distinct pathological and functional characteristics depending on their history of asthma or COPD. The study showed that patients with a history of asthma and fixed airflow limitation maintain distinct airway inflammation as compared with those with a history of smoking-induced fixed airflow limitation. By comparing patients with similar age and similar degree of fixed airflow limitation, the study showed that FEV_1 and FEV_1/FVC ratio, $PC_{20}FEV_1$, and TLC (total lung capacity) were no different between the group of patients with history of asthma and the group of patients with COPD, but patients with COPD had increased residual volume, lower diffusing capacity and oxygen tension in arterial blood, decreased response to inhaled albuterol and steroids, and a higher HRCT scan emphysema score. The lower diffusing capacity and PaO_2, the higher residual volume, and the higher

Table 2 Pathophysiology of COPD and Asthma

	COPD	Asthma	Asthma with fixed airflow limitation
Reversibility to bronchodilators	+/−	+++	+
Reversibility to corticosteroids	+/−	+++	+
HRTC emphysema	+++	+/−	+/−
Diffusing capacity	<	+/−	+/−
Gas-exchange abnormalities	+++	+/−	+/−

HRCT emphysema score in patients with fixed airflow obstruction due to COPD suggest that parenchymal destruction, i.e., emphysema, is present in subjects with fixed airflow limitation due to COPD but not in subjects with fixed airflow limitation due to asthma (Table 2).

IV. CHARACTERISTICS OF INFLAMMATION IN COPD AND ASTHMA

A. COPD

Many studies have been conducted in the last 20 years on the pathology of asthma and COPD, mainly directed to understand the mechanisms of these two complex syndromes. The studies on the pathology have revealed quite remarkable differences between the pathology of asthma and the pathology of COPD, so that there has been an increasing interest in using cellular and/or biochemical markers for the diagnosis, differential diagnosis, and management of these two diseases.

The pathology of COPD and asthma is markedly different (4,5,29,30). In general, in COPD the fixed airflow obstruction is associated with an airway inflammatory profile consisting mainly of an increased number of T-lymphocytes (predominantly $CD8^+$), macrophages, and neutrophils (31,32). In contrast, the variable airflow obstruction in asthma is associated with a characteristic airway inflammation consisting of an increased number of T-lymphocytes (predominantly $CD4^+$) and eosinophils and an increased thickness of the reticular layer of the epithelial basement membrane (19,25) (Table 3).

COPD is associated with inflammation of central and peripheral airways, lung parenchyma, and pulmonary vessels. In the central airways, the characteristics of inflammation are: (1) an increased number of mononuclear cells, particularly macrophages and T-lymphocytes of the $CD8^+$ type, associated in few cases with neutrophils, eosinophils, and mast cells in the airway mucosa; (2) increased number of neutrophils and, in few cases, of eosinophils in lavage fluid; (3) infiltration of submucosal glands by neutrophils; (4) hyperplasia of goblet cells and enlarged mucous glands; (5) metaplasia of airway epithelium that is otherwise well preserved; (6) no change of the structure of the lamina reticularis of the basement membrane. The contribution of these pathological abnormalities to airflow limitation and gas-exchange abnormalities remains unclear. However, as airflow limitation progresses, the number of T-lymphocytes and macrophages increases in the submucosa, and a particular subset of T-lymphocytes, the $CD8^+$type, correlates significantly with the evolution of airflow limitation (33,34). Peripheral airways show pathological abnormalities similar to the ones present in central airways with an increased number of mononuclear cells in the airway mucosa, and, similar to central airways, a particular subset of T-lymphocytes, the $CD8^+$, that correlates significantly with the evolution of airflow limitation (33,34), an increased number of neutrophils in the

Table 3 Characteristics of Inflammation in COPD and Asthma

	COPD	Asthma
Cells	Neutrophils	Eosinophils
	Large increase in macrophages	Small increase in macrophages
	Increase in $CD8^+$ T-lymphocytes	Increase in $CD8^+$ T-lymphocytes
		Increase in $CD4^+$ Th2 lymphocytes
		Activation of mast cells
Mediators	LTB4	LTD4
	IL-8	IL-4, IL-5
	TNF-α	(Plus many others)
Consequences	Squamous metaplasia of epithelium	Fragile epithelium
	Parenchymal destruction	Thickening of basement membrane
	Mucus metaplasma	Mucus metaplasia
	Glandular enlargement	Glandular enlargement
Response to treatment	Glucorticoids have little or no effect	Glucorticoids inhibit inflammation

Source: adapted from Ref. 2.

airway fluid, a metaplasia of airway epithelium with hyperplasia of goblet cells. In addition, peripheral airways show increased intraluminal mucus and exudate, increased mass of smooth muscle, airway wall fibrosis, distortion, and obliteration, loss of alveolar attachments to the bronchiolar walls. In the lung parenchyma, the characteristics pathological abnormalities are the presence of (a) panlobular emphysema (PLE) and centrilobular emphysema (CLE) in various combination; (b) paraseptal emphysema; and (c) loss of vascular bed linked to emphysema.

Up to 20% of clearly defined COPD patients have a significant reversibility of airflow limitation to bronchodilators and/or glucocorticosteroids (9,35,36). These patients have the same pathologic abnormalities of COPD patients, but also some pathological features of asthma, namely a small but significant increase of eosinophils in bronchoalveolar-lavage (BAL) fluid, and a slight but significant increase of the thickness of the reticular layer of the basement membrane (9). Moreover, in COPD with partial reversibility of airflow limitation, the bronchodilator response is associated with increased exhaled NO and sputum eosinophilia (37).

B. Asthma

In asthma, most studies have included mild asthma, and have concentrated on biopsies from central airways. These studies have consistently shown that

asthma symptoms and airway hyperresponsiveness are associated with pathological abnormalities in central airways, whereas lung parenchyma is unchanged, at least in baseline conditions (19,38). In the central airways of asthmatics, the characteristics pathological abnormalities are: (1) an increased number of mononuclear cells, particularly T-lymphocytes of the $CD4^+$ Th2 type, associated in most cases with eosinophils and mast cells in the airway mucosa and in the airway fluid, (2) damaged airway epithelium, and (3) increased thickness of the lamina reticularis of the basement membrane. The contribution of these pathological abnormalities to symptoms of asthma and airway hyperresponsiveness remains unclear.

The peripheral airways of asthmatics have been examined only in one study that showed substantially similar, although more severe, infiltration by T cells and eosinophils (Ref. 39). Peripheral airways show (4) increased intraluminal mucus and exudate, even of different composition with respect to the one observed in COPD (5) increased mass of smooth muscle, (6) airway wall fibrosis, distortion, and obliteration (7) loss of alveolar attachments to the bronchiolar walls. These latter pathological abnormalities, however, have been described in the lungs of subjects who died of asthma, and it remains unknown whether they are present in the peripheral airways of living asthmatics.

The pathology of severe asthma is less clear. Some studies have shown that the airway pathology of severe asthma is similar to the airway pathology of mild asthma, in particular involving eosinophils (40), even if the eosinophilic infiltrate looks more severe and more proximally distributed. By contrast, other studies have shown that severe asthma has a different pathology, with a predominance of neutrophils over eosinophils on BAL, endobronchial biopsies, and transbronchial biopsies (41,42). Interestingly, the thickness of the reticular layer of the basement membrane is no different in subjects of different severity (26). A possible explanation for the differences in the inflammatory cells observed by some authors between severe, steroid-dependent asthma and mild asthma is that the two forms of asthma have different pathologies, with a prominent neutrophilia in steroid-dependent asthma, and a prominent eosinophilia in mild asthma. An alternative explanation is that neutrophilia in steroid-dependent asthma may be the effect of steroid therapy itself, since glucocorticosteroids have been shown to inhibit neutrophil apoptosis (43). Finally, it is possible that different inflammatory cell profiles in the airways may have been caused by different inflammatory stimuli, i.e. infectious stimuli in case of neutrophilia, and allergic stimuli in case of eosinophilia.

C. Smoking Asthmatics

The pathology of smoking asthmatics, late-onset asthmatics, nonsmoking subjects with COPD, or subjects with COPD and large reversibility to treat-

ment is less characterized. Some data suggest that in addition to the typical eosinophilic airway inflammation observed in patients with asthma, smoking asthmatics develop a neutrophilic airway inflammation that has been suggested to be responsible for the development of resistance to steroid treatment in these subjects (44). Only one study has assessed airway inflammation in nonsmoking subjects and COPD, suggesting that two phenotypes may exist, with and without airway eosinophilia (10). COPD patients presenting with a certain degree of reversibility of airflow limitation show some inflammatory abnormalities similar to asthmatics, i.e. higher airway eosinophilia (9,37), and higher levels of exhaled NO (37).

D. Asthmatics with Developed Fixed Airflow Limitation

The inflammatory characteristics of asthmatic patients who have developed fixed airflow limitation, and thus, functionally similar to COPD patients, has been poorly investigated. Previous studies have compared airway inflammation in predefined patients with either asthma or COPD (45–47). The limitation of those studies is that they compared young asthmatics with variable airflow obstruction with older COPD patients with fixed airflow obstruction. The results of those studies showed that, in asthma, the variable airflow obstruction is associated with a characteristic airway inflammation consisting of an increased number of T-lymphocytes (predominantly $CD4^+$) and eosinophils and an increased thickness of the reticular layer of the epithelial basement membrane (25). In contrast, in COPD the fixed airflow obstruction is associated with an airway inflammatory profile consisting mainly of an increased number of T-lymphocytes (predominantly $CD8^+$), macrophages, and neutrophils (31,32).

Based on this evidence, one could predict that in asthmatics developing fixed airflow limitation, airway inflammation would also change with the development of fixed airflow limitation and become similar to the airway inflammation present in COPD. If so, asthma could become COPD not only functionally but also pathologically. The recent investigation (28) of subjects with fixed airflow limitation with history of asthma or COPD, has shown that, compared with the patients with a history of COPD, patients with a history of asthma have more eosinophils in peripheral blood, sputum, BALF, and airway mucosa (Fig. 1) and have fewer neutrophils in sputum and BALF. Patients with a history of asthma have more bronchoalveolar lymphocytes and more $CD4^+$ cells, a higher $CD4^+/CD8^+$ ratio, and a thicker reticular layer of the basement membrane in bronchial biopsy specimens. Finally, they have a higher level of exhaled NO. Moreover, the statistical analysis showed that both the percentage of sputum and BALF eosinophils and the NO level were good predictors of a history of asthma (28).

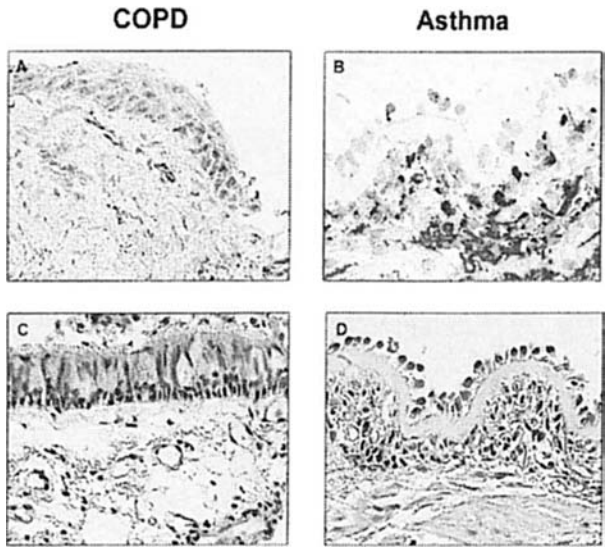

Figure 1 (A) and (B): Photomicrographs showing bronchial biopsy specimens immunostained with anti-EG-2 (eosinophil cationic protein) from a patient with fixed airflow obstruction and a history of COPD (A) and from a patient with fixed airflow obstruction and a history of asthma (B). The two patients had a similar degree of fixed airflow obstruction. In (B), there is prominent eosinophilia beneath the destroyed epithelium that is not present in (A). (C) and (D): Photomicrographs showing bronchial biopsy specimens stained with H&E from a patient with fixed airflow obstruction and a history of COPD (C) and from a patient with fixed airflow obstruction and a history of asthma (D). The two patients had a similar degree of fixed airflow obstruction. In (D), there is a thicker reticular layer of the epithelial basement membrane compared with (C). (From Ref. 28.)

The finding of this study suggests that asthmatic airway inflammation does not change with the development of fixed airflow limitation and does not become similar to the airway inflammation characteristic of COPD (5,28). Thus, even when they develop fixed airflow limitation, patients with a history of asthma have the same airway inflammatory changes that are present in asthmatics with variable airflow limitation, in terms of both cellular infiltrates and increased thickness of the reticular layer of the basement membrane, indicating that, even when fixed airflow limitation is present, asthma should be diagnosed and treated as asthma and not as COPD.

V. INFLAMMATORY MEDIATORS IN COPD AND ASTHMA

A. COPD

Migration and activation of inflammatory cells is regulated by cytokines and chemokines, small proteins secreted by a variety of structural, such as

epithelial, endothelial, smooth muscle, and fibroblasts, as well as by inflammatory cells. Chemokines are a family of more than 40 small (8–11 kDa) cytokines that have been defined primarily by their ability to mediate leukocyte chemotaxis. Chemokines have been divided into four major families on the basis of the spacing of the most amino-terminal of four conserved cysteine residues, including the C, CC, CXC, and CX3C families, where X represents any amino acid (48).

Bronchopulmonary inflammation of COPD is characterized by CD8, Tc1 cells, and neutrophils. Recent reports demonstrate that T cells from the bronchial mucosa of patients with COPD predominantly express the chemokine receptor CXCR3 (49,50) and produce interferon-γ (IFN-γ), suggesting that these cells elaborate type I cytokines (Fig. 2).

The CXCR3 chemokine receptor binds three highly potent, inflammatory-inducible CXC chemokine agonists, e.g. interferon (IFN) -γ-inducible protein 10 (CXCL10/IP-10), monokine induced by IFN-γ, and interferon-inducible T-cell α-chemoattratant (CXCL11/I-TAC) (52). CXCR3 is selectively expressed by activated T cells, B cells, natural killer cells (NK) and a subset of circulating blood T cells consisting mainly of $CD45RO^{+}$ memory cells; a subset of dendritic cells as well as intraepithelial lymphocytes in normal and inflamed tissues. In addition, CXCR3 is constitutively expressed by endothelial vessels of medium and large caliber but not in small vessels.

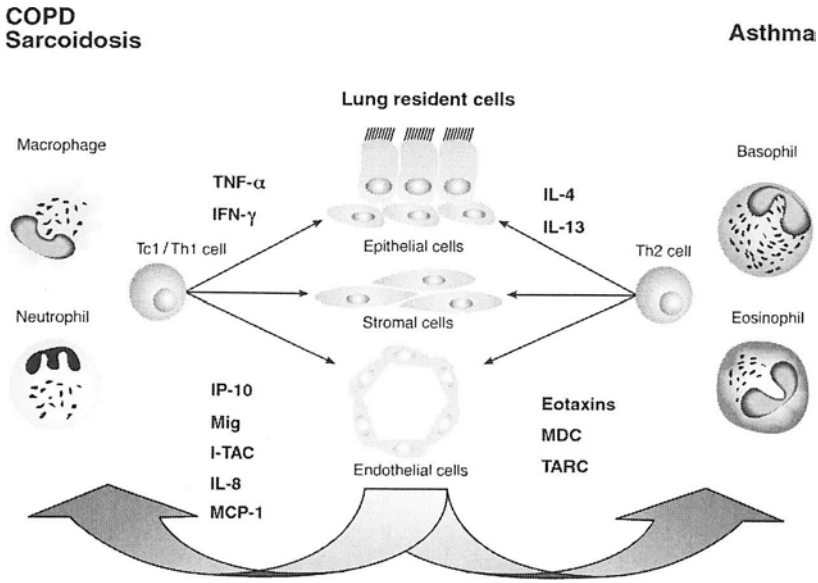

Figure 2 The potential role of cytokine to chemokine regulatory networks in the development of inflammation in asthma and COPD. (From Ref. 51.)

Interestingly, neutrophils produce, among other chemokines, all the three ligands for CXCR3 (53). These data suggest that neutrophils are capable of participating in the selective recruitment of T-lymphocytes and in the process of tissue homing of Th1/Tc1 lymphocytes, in keeping with the frequent association of neutrophils with T-cell-mediated type-1 inflammatory response.

$CD8^+$ cells are potent producers of IFN-γ in COPD. It has been demonstrated that IFN-γ causes emphysema with alveolar enlargement, enhanced lung volumes, enhanced pulmonary compliance, and macrophage and neutrophil-rich inflammation when inducibly targeted, in a transgenic fashion, to the adult murine lung. Prominent protease and antiprotease alterations were also noted in these mice. They included the induction and activation of matrix metalloproteinase (MMP) -12 and cathepsins B, H, D, S, and L, the elaboration of MMP-9, and the selective inhibition of secretory leukocyte proteinase inhibitor (SLPI). Therefore, IFN-γ causes emphysema and alterations in pulmonary protease/antiprotease balance when expressed in pulmonary tissues (54).

Several chemokines are involved in neutrophil chemotaxis and mainly belong to the CXC family, of which interleukin-8 (IL-8, CXCL8) is the most prominent member relevant in COPD. IL-8 levels are markedly elevated in the sputum and BAL of patients with COPD and correlated with the extent of neutrophilic inflammation and disease severity (55,56). The blocking of IL-8 reduces the chemotactic response of neutrophils to sputum from COPD patients in vitro (55). IL-8 activates human neutrophils by binding to two G-protein-coupled receptors, designated CXCR1 and CXCR2. CXCR1 is selectively activated by two chemokine ligands IL-8 and granulocyte chemoattractant protein (CXCL6/GCP2), the binding of which is coupled to activation and degranulation. Whereas, CXCR2 can be activated not only by IL-8, but also by other members of the CXC chemokine family and is important in chemotaxis (57). IL-8 has similar binding potency for CXCR1 and CXCR2 receptors, which are present in equivalent number on neutrophils.

CC chemokines are also thought to be involved in COPD. Increased expression of monocyte chemoattractant protein 1 (CCL2/MCP-1) and its cognate receptor CCR2 have been demonstrated in macrophages and epithelial cells from patients with COPD (58). CCL2/MCP-l may play a role in recruitment of blood monocytes to the lungs of COPD patients (59) as well as elastin fragments generated at the diseased sites are potent chemoattractants for monocytes in the lung in pulmonary emphysema (60).

Leukotriene B4 (LTB_4) has been implicated as the major chemoattractant responsible for neutrophil recruitment in COPD and particularly in α_1-AT deficient patients (61). Studies indicated that the major source was probably the alveolar macrophage and it was proposed that the recruitment of neutrophils could actually amplify the effect. The concentration of LTB_4,

which is chemotactic for neutrophils, is increased in the sputum of patients with COPD (62).

The elastase, poorly inhibited because of the reduced α_1-AT in the deficient subjects, could stimulate the macrophages to release more LTB_4, resulting in further neutrophil recruitment, and thereby perpetuating and amplifying the neutrophil-associated lung damage (63). The concentration of LTB_4, which is chemotactic for neutrophils, is increased in the sputum of patients with COPD (62).

Tumor necrosis factor-α (TNF-α) is also present in high concentrations in the sputum of COPD patients and is detectable in bronchial biopsies specimens, and can induce IL-8 expression in airway cells by activation of the transcription factor, nuclear factor-κB (NF-κB) (31). TNF-α increases the expression of ICAM-1, which is increased in COPD and which may activate macrophages to produce MMP (64).

In COPD, alveolar macrophages and neutrophils play a central role in destruction of lung parenchyma (65,66). Various proteases break down connective-tissue components, particularly elastin, in lung parenchyma to produce emphysema. Most attention has focused on neutrophil elastase and proteinase 3, which are neutrophil-derived serine proteases, and on cathepsins, all of which can produce emphysema in laboratory animals (31). However, there is now increasing evidence that MMPs derived from macrophages and neutrophils have a role in patients with emphysema. Studies of human samples show increases in many proteases, including MMP-1, -2, -8, -9, and -14 in smoking-related emphysema (67). As discussed above, $CD8^+$ cells are potent producers of IFN-γ in COPD. IFN-γ causes emphysema and alterations in pulmonary protease/antiprotease balance when expressed in pulmonary tissues (54). Three MMPs degrade elastin: MMP-2, -9, and -12. Particularly, MMP-9 has been found elevated in homogenates of lung removed from patients underwent to lung volume reduction surgery, which represents samples obtained by advance stage of emphysema (67). Homogenates of lung removed in volume reduction reveal MMP-9-related elastolytic and gelatinolytic activity and significant elevations in MMP-9, with no significant increase in neutrophil elastase by ELISA (68). There is an increase in the activity of MMP-9 and MMP-2 in the lung parenchyma of patients with emphysema. The role of MMP-9 in the pathogenesis of emphysema is likely to be complex; α-antitrypsin can be cleaved by MMP-9. Increased chemotactic activity of BALF is associated with the development of smoking-related emphysema (69), and potentiation of IL-8 by MMP-9 could amplify the alveolar inflammation and destruction in smokers who develop emphysema (70).

Also, oxidative stress may have an important role in COPD. In smokers and in COPD patients, there is an oxidant/antioxidant imbalance in favour of oxidants (71). The presence of oxidative stress has been assessed by measuring markers of the effects of radicals on lung biomolecules such

as lipid proteins or DNA, or by measuring the stress responses to the increased oxidant burden. Hydrogen peroxide in breath condensate is a direct measurement of airspace oxidant burden, and is detected in higher levels in patients with COPD than in normal subjects, and is present even in higher levels during exacerbations of COPD (72).

Oxidative stress may exacerbate COPD through several mechanisms, including the activation of the transcription factor, NF-κB, which switches on the genes for TNF-α, interleukin-8, and other inflammatory proteins, and oxidative damage of antiproteases, such as α_1-antitrypsin and secretory leukoprotease inhibitor, thus enhancing inflammation and proteolytic injury. Smoking produces a fall in alveolar and lung glutathione (GSH) metabolism, which are widely recognized as a central feature of COPD. It has been demonstrated that the severity of airway obstruction, as measured by FEV_1 in smokers with COPD, correlated negatively with the concentration of GSH in BALF: the higher the BALF GSH, the lower the FEV_1 (72). Patients with acute exacerbations of COPD show increased production of superoxide anion from their peripheral blood neutrophils compared with the measurement in stable patients. Products of lipid peroxidation are significantly increased in plasma or BALF in healthy smokers and patients with acute exacerbations of COPD, compared with healthy nonsmokers (72). It has been demonstrated that the severity of airway obstruction, as measured by FEV_1 in smokers with COPD, correlated negatively with the concentration of GSH in BALF: the higher the BALF GSH, the lower the FEV_1 (72). The GSH concentration in BALF from patients with COPD was similar to that in chronic smokers with no airflow obstruction. This emphasizes the effects of smoking on GSH metabolism rather than reflecting the disease severity in the COPD patients. The actual relevance of these studies in the lungs of these patients is not known. It is possible that BALF GSH levels are influenced by the recent smoking of these patients. Finally, the levels of the antioxidant capacity in the plasma negatively correlate with the increased release of oxygen radicals, from circulating neutrophils in patients with exacerbations of COPD, suggesting, at least in part, that the systemic oxidative stress in this condition derives from reactive oxygen species (73). Exposure to cigarette smoke is reported to induce goblet cell metaplasia and mucus production (74), but the mechanism is still unknown. Recently, mucin synthesis in airways has been reported to be regulated by the epidermal growth factor receptor (EGFR) system. In particular, the exposure of airway epithelial cells to cigarette smoke upregulates EGFR expression and activates EGFR tyrosine phosphorylation, causing mucin synthesis in epithelial cells (75). The mechanisms by which cigarette smoke induces EGFR activation are not completely defined (75). However, it has been recently observed that cigarette smoke is able to transactivate EGFR stimulating the transmembrane metalloproteinase TNFα converting enzyme (TACE) or a disintegrin and metalloproteinase (ADAM) -17. This

metalloproteinase is able to cleave transmembrane amphiregulin, a ligand for the EGFR. The binding of amphiregulin to EGFR then triggers receptor's activation (76). Of interest, cigarette smoke can also cause neutrophil migration into the airways (77). Neutrophils are also increased in the bronchial glands (78). In vivo and in vitro studies show that these neutrophils can stimulate goblet cell degranulation (79). Purified neutrophil elastase has been shown to be a potent secretagogue for goblet cells in vitro (80). However, it is not clear whether this molecule is responsible for the effect of neutrophils on degranulation, since the cells themselves release little or no elastase in vitro, even after activation with a variety of molecules (IL-8, LTB4, TNF-α). Rather, the activation of neutrophils causes a translocation of elastase from the azurophilic granules to the cell surface (81). However, in a guinea pig model, the elastase inhibitor ICI 200355 inhibited the neutrophil-dependent goblet cell degranulation seen after the trachea instillation of neutrophil chemoattractants (82). These results confirmed the role of neutrophil elastase in degranulation, but the question of how elastase exerts its effect still remains since neutrophil activators and chemoattractants do not appear to promote its release (83). The potential role of elastase in goblet cell degranulation implies a direct interaction between neutrophils and goblet cells. Some experimental evidence suggests a direct interaction between neutrophils and goblet cells in the airway epithelium, involving ICAM-1, CD11b, or CD 18 cells (83). These leukocyte adhesion receptors play an important role in inflammation via their regulatory effects on leukocyte adhesion, transmigration, and function (84).

B. Asthma

Like in COPD, airway wall inflammation is thought to play a central role also in the development and progression of asthma. The currently available knowledge on associations between inflammation and airway function, including airflow obstruction and bronchial hyperresponsiveness, is based on both acute inflammatory events, such as airway smooth-muscle contraction and/or (sub) mucosal swelling, secondary to activation of inflammatory and resident cells within the airway wall, as well as chronic airway inflammation, associated with subepithelial collagen deposition, smooth-muscle growth, and increased mucosal vascularity (85,19). The pattern of inflammation varies considerably from COPD patients and depends on the stage of the disease. The degree of airway inflammation varies with the severity and chronicity of the disease and may also determine the responsiveness of the patient to treatment (86).

Infiltration of the airway by lymphocytes and eosinophils have been found in patients with mild-to-moderate chronic asthma. However, inflammation in chronic asthma appears to be far more complex than a simple eosinophilic inflammation alone. All cells of the airways, including T cells,

eosinophils, mast cells, macrophages, epithelial cells, fibroblasts, and even bronchial smooth-muscle cells are involved in asthma and become activated (19,87). At the cellular epicenter of this process are $CD4^+$ T-helper memory cells. These produce an array of cytokines that directly or indirectly "program" the leukocytes that are responsible ultimately for acute and chronic inflammation in the airways (87). The principal type 2 helper (Th2) cytokines implicated in this process include interleukin (IL) -4, which is required to drive production of allergen-specific immunoglobulin E (IgE) (88), IL-3, which controls mast cells and basophil development (89), and IL-5 in conjunction with IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF), which regulate eosinophil recruitment and activation. In addition, there is growing interest in the role in Th2-derived IL-9 in regulation of IgE production and mast cell growth, and in I1-13 in relation to airways hyperreactivity. Furthermore, it has been recently observed that T cells infiltrating the airway mucosa of atopic asthmatic express the Th2 chemokine receptors CCR4 and, to a lesser extent, CCR8, and that their number increases after allergen challenge, but not the Th1 chemokine receptor CXCR3 (49). CCR4 is the high-affinity receptor for the CC chemokines thymus- and activation-regulated chemokine (TARC) and monocyte-derived chemokine (MDC). CCR4 is very highly expressed by Th2 cells. It is also found on dendritic cells, monocytes, basophils, and platelets (51). After allergen challenge, it has been demonstrated that virtually all T cells obtained from asthmatic patients express CCR4. Also CCR8 is coexpressed on T cells, but to a lower extent. The expression of CCR4 was paralleled by strong expression of its ligands MDC and TARC in the airway epithelial cells after allergen challenge, suggesting that the CCR4/ligands axis may be involved in the Th2 cell recruitment (Fig. 2).

Eosinophils store and release, on appropriate activation, a wide spectrum of proinflammatory mediators including cationic granule proteins, major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (90). They have also been shown to synthesise up to 28 cytokines, chemokines and growth factors, many of which are stored in their crystalloid granules (91). Major basic protein directly increases smooth-muscle reactivity by causing the dysfunction of vagal muscarinic M2 receptors. It also triggers the degranulation of mast cells and basophils. In addition, eosinophils amplify the inflammatory cascade by producing their own chemoattractants [e.g., RANTES (regulated upon activation normal T-cell expressed and secreted), eotaxin, and platelet-activating factor), which accelerate the recruitment of eosinophils into the inflammatory focus (92).

Further damage is caused by hydrogen peroxide and halide acids, which are generated by eosinophil peroxidase, and by superoxide, which is generated by the respiratory-burst-oxidase pathway in eosinophils. Eosinophils also generate large amounts of the cysteinyl leukotriene, LTC_4, which is metabolized to LTD_4 and LTE_4. These three lipid mediators are

the slow-reacting substances of anaphylaxis that increase vascular permeability and mucus secretion and are potent stimulators of smooth-muscle contraction. Lastly, activated eosinophils produce a wide range of inflammatory cytokines that have the potential to modulate multiple aspects of the immune response (92).

It has also recently become clear that eosinophils are not only key effector cells in mediating asthmatic inflammation, but also have a role in presenting allergen to stimulate $CD4^+$ T-helper (Th) cells (93). Epithelial damage and loss caused by eosinophil-derived mediators, particularly their highly basic granule proteins—MBP, eosinophil peroxidase and ECP—is thought to be a major event in asthma pathogenesis (94). Thus, it can be appreciated that eosinophils have the ability to make a major contribution to the inflammatory processes underlying asthmatic and allergic disease. Furthermore, it is also becoming increasingly apparent that eosinophil–epithelial cell interactions are an important facet of asthmatic disease in relation to both the initiation and resolution of inflammation (95).

Further evidence of an inflammatory response in asthma is the presence of cytokines that mediate inflammation and chemotactic chemokines in BALF or pulmonary secretions. Since these cytokines and chemokines are elaborated by resident and inflammatory cells in airways and have many effects on these cells, a variety of autocrine, paracrine, and endocrine networks could participate in asthma (19). Some cytokines initiate inflammatory responses by activating transcription factors, which are proteins that bind to the promoter region of genes. Transcription factors involved in asthmatic inflammation include NF-κB, activator protein-1, nuclear factor of activated T cells, cyclic AMP response-element binding protein, and various members of the family of signal transduction–activated transcription (STAT) factors (19). These transcription factors act on genes that encode inflammatory cytokines, chemokines, adhesion molecules, and other proteins that induce and perpetuate inflammation. Corticosteroids modulate immuno inflammatory responses in asthma by inhibiting these transcription factors (19).

The ability of cytokines to induce the expression of adhesion molecules such as intercellular adhesion molecule 1, vascular-cell adhesion molecule 1, and endothelial-leukocyte adhesion molecule provides a mechanism for the adhesion of inflammatory cells to the endothelium and the migration of these cells from the circulation into the lamina propria, the epithelium, and in many cases, the airway lumen itself.

VI. CONCLUSIONS

Inflammation is important both in COPD and asthma, and the inflammatory response in COPD is markedly different from that in asthma (Table 3).

Since inflammation is a feature of COPD, it follows that anti-inflammatory therapies may have clinical benefit in controlling symptoms, preventing exacerbations, and slowing the progression of the disease. However, at variance with asthma, the inflammatory response in COPD appears to be poorly responsive to the glucocorticosteroids that are effective anti-inflammatory medications in asthma. Although the clinical, functional, and inflammatory characteristics are usually markedly different in COPD and asthma, in clinical practice the differential diagnosis between the two respiratory conditions may become complex in some subjects with overlapping characteristics. In these cases, the differential diagnosis between COPD asthma may be difficult but nonetheless important, as the natural history and the response to pharmacological treatment are different in the two disease states.

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5

Airway Mucus Secretion

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I. INTRODUCTION

Airway mucus hypersecretion was long dismissed as a benign, albeit aggravating, component of chronic obstructive pulmonary disease (COPD) (1,2). This doctrine is exemplified in omission of the term 'mucus hypersecretion' from recent definitions of COPD (3). However, new epidemiological studies (4) demonstrate that mucus is far from innocent. In fact, airway mucus hypersecretion is now recognized as a potential risk factor for an accelerated loss of lung function in COPD. Thus, current wisdom declares that mucus hypersecretion is a key pathophysiological feature in many patients (Fig.1). Mucus hypersecretion, implicit in the term chronic bronchitis, is one of three pathophysiological entities that comprise COPD, the other two being chronic bronchiolitis (small airways disease), and emphysema (alveolar destruction with airspace enlargement). The relative contribution of each component to pathophysiology varies between patients, with the impact of mucus hypersecretion on clinical symptoms varying accordingly. In many patients, hypersecretion has clinical significance, for example in patients with low lung function or who are prone to chest infections (5) (Fig. 2). Consequently, it is important to understand the pathophysiology of mucus hypersecretion in COPD. This in turn should allow identification of therapeutic targets and rational development of pharmacotherapeutic drugs. The present chapter: 1) assesses the contribution of airway mucus hypersecretion and impaired mucociliary clearance to pathophysiology of the 'bronchitic' component of COPD, 2) considers the epidemiology and

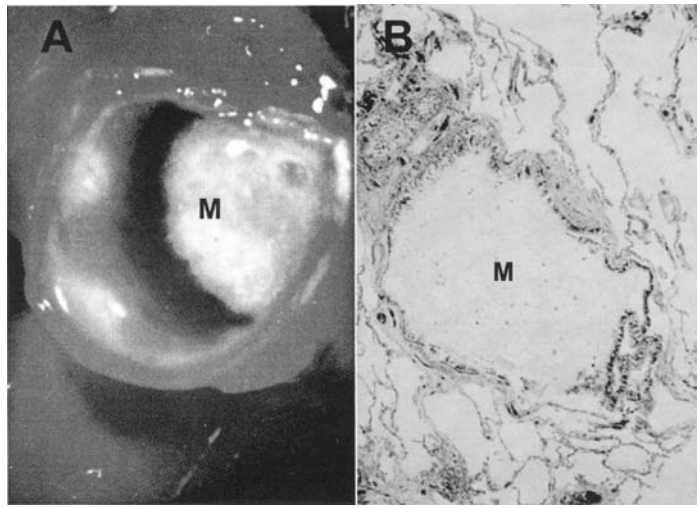

Figure 1 Airway mucus hypersecretion in COPD. Panel A: Gross pathology: mucus (M) partially blocking an extrapulmonary bronchus in a cigarette smoker with chronic sputum production. Panel B: Histopathology: mucus (M) occluding a small airway in a patient with COPD.

changing view of the clinical impact of mucus hypersecretion in COPD, 3) discusses current therapy and outlines potential novel therapy for this condition, and 4) presents some of the options for chest physiotherapy to clear airway mucus in COPD. To set these issues in context for the nonexpert

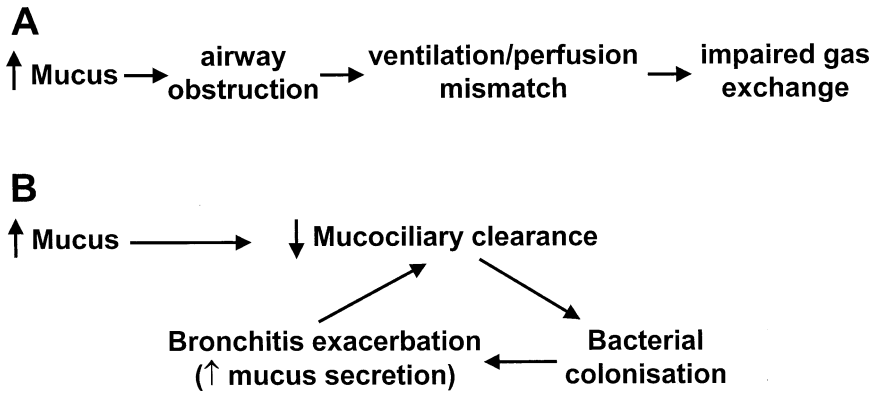

Figure 2 Putative schemas for mucus pathophysiology in COPD. Panel A: Impact of mucus hypersecretion on lung function. Panel B: "Vicious circles" of mucus hypersecretion and bacterial infection.

reader, the following section gives a condensed summary of airway mucus, mucins, and mucin (MUC) genes.

II. AIRWAY MUCUS, MUCINS, AND MUC GENES

In healthy individuals, a thin film of slimy liquid protects the airway surface (6). The liquid is often referred to as 'mucus' and is a complex, nonhomogeneous dilute (1-2)% aqueous solution of salts, enzymes and antienzymes, oxidants and antioxidants, bacterial products, antibacterial agents, cell-derived mediators and proteins, plasma-derived mediators and proteins, and cell debris such as DNA. The mucus forms a bilayer comprising an upper gel layer and a lower sol layer. A thin layer of surfactant appears to separate the gel and sol (7,8). Cilia beat in the sol layer, often termed periciliary fluid. Inhaled particles are trapped in the gel layer and, by transportation on the tips of beating cilia, are removed from the airways, a process termed mucociliary clearance (Fig. 3). Airway mucus requires an optimal combination of viscosity and elasticity for efficient ciliary interaction. Viscoelasticity is conferred primarily by high molecular weight mucins that comprise up to 2% by weight of the mucus (9). Airway mucins are primarily produced in, and secreted by, goblet cells in the surface epithelium (10) and by mucous cells in the submucosal glands (11). Mature mucins are long thread-like molecules composed of monomers joined end-to-end by disulphide bridges (Fig. 4). These threads form a 'tangled network' (12) that contributes to the formation of the mucus gel. The mucin monomers comprise a highly glycosylated linear peptide sequence, termed apomucin, that is encoded by specific mucin (MUC) genes. Nineteen human MUC genes are reported to date, namely MUC1, 2, 3A, 3B, 4, 5AC, 5B, 6–9, 11–13,

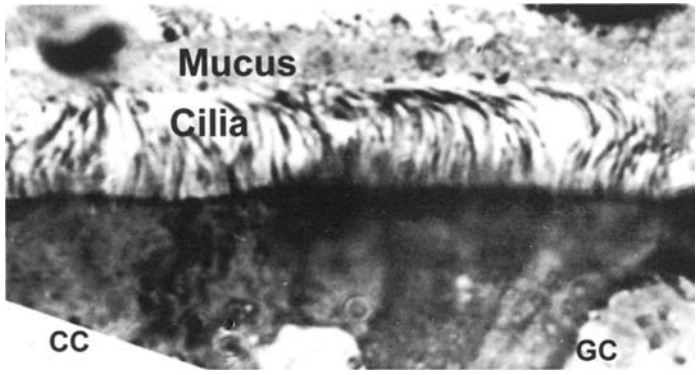

Figure 3 Mucus movement on cilia in bovine trachea in vitro. Note differences in bending of the cilia at different stages of the beat cycle. CC, ciliated cell; GC, goblet cell.

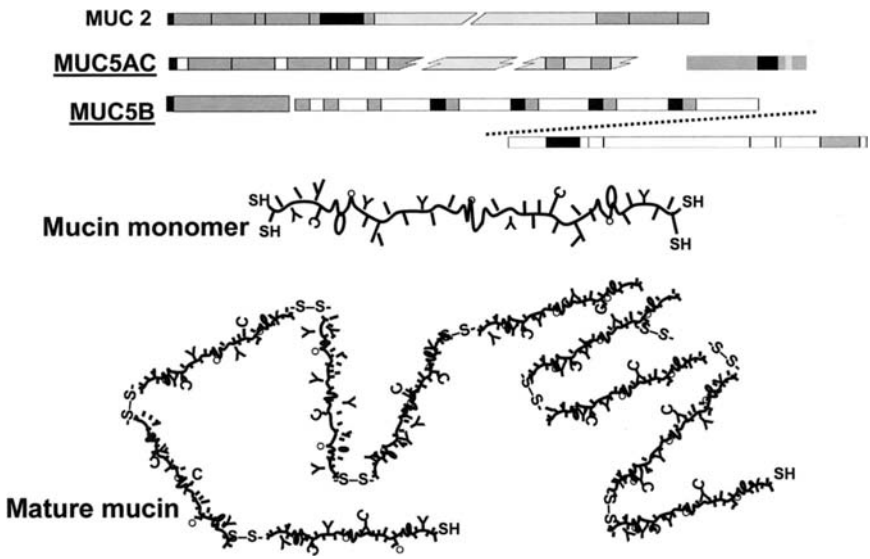

Figure 4 Respiratory tract mucins. Top panel: Schematic representation of gene products of MUC2, MUC5AC, and MUC5B. These gene products comprise the mucins in airway secretions from patients with COPD. Lower panels: Schematic representation of a gel-forming mucin molecule. The mucin subunit (~500 nm in length) comprises an amino acid backbone with highly glycosylated areas and folded regions, stabilized via disulphide bonds, with little or no glycosylation. Glycosylation is via O-linkages and is highly diverse. In the mature secreted molecule, the subunits are joined end-to-end by disulphide bonds (S-S) into long thread-like molecules.

and 16–20 (13–18). Although a number of these genes are expressed in the airways (9), it is the MUC5AC and MUC5B gene products that are the major gel forming mucins in 'normal' respiratory tract secretions (9), although MUC2 may be upregulated in COPD (see below) (Fig. 4).

III. MUCUS HYPERSECRETORY PHENOTYPE IN COPD

Airway mucus hypersecretion in COPD has characteristic pathophysiological features. Many of these features, for example sputum production and goblet cell hyperplasia, are common to other hypersecretory respiratory diseases, for example asthma and cystic fibrosis. Other features appear to be specifically associated with COPD (see below). Differences in mucus pathophysiology between COPD and asthma have been discussed previously (19), and are summarized in Figure 5. Presumably, differences in the pulmonary inflammatory 'profile' of COPD and asthma (the former essentially a macrophage-driven neutrophilia and latter a Th2 lymphocyte-driven

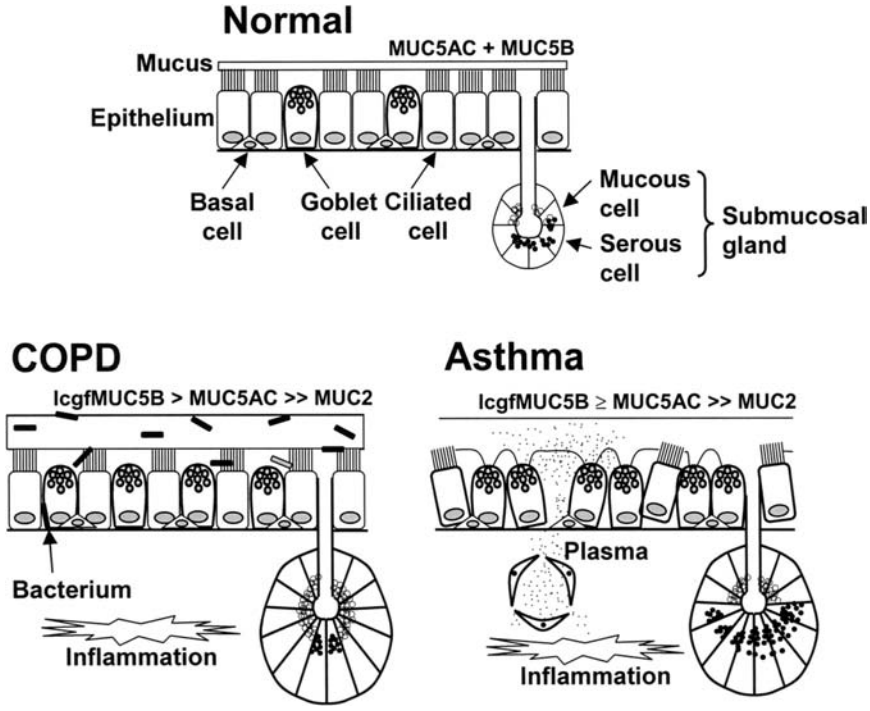

Figure 5 Putative differences in airway mucus pathophysiology between COPD and asthma. Compared with normal, in COPD there is increased luminal mucus, goblet cell hyperplasia, submucosal gland hypertrophy (with an increased proportion of mucous to serous acini), an increased ratio of mucin (MUC) 5B (low charge glycoform, lcgf) to MUC5AC, small amounts of MUC2, and respiratory infection (possibly due to reduced bacterial enzymatic 'shield' from reduced serous cell number). Pulmonary inflammation includes macrophages and neutrophils. In asthma, there is increased luminal mucus, epithelial 'fragility', marked goblet cell hyperplasia, submucosal gland hypertrophy (although without an increased mucous to serous ratio), 'tethering' of mucus to goblet cells, and plasma exudation. Airway inflammation includes T lymphocytes and eosinophils. Many of these differences require more data from greater numbers of subjects.

eosinophilia) (20) underlie the differences in hypersecretory phenotype between these two conditions.

Sputum production, up to 100 mL per day in many patients, is associated with excessive mucus in the airways (Fig. 6) (21–23). The increased mucus is associated with goblet cell hyperplasia (21,24) and submucosal gland hypertrophy (Fig. 7) (21,22,25,26). Of particular note is that the gland mucous cells are markedly increased relative to the serous cells (27). This is in contrast to asthma, where the glands, albeit hypertrophied, are

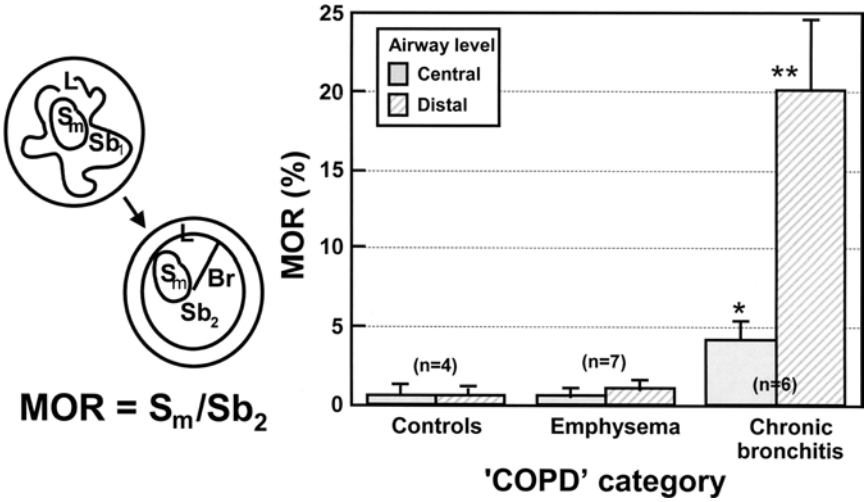

Figure 6 Airway mucus hypersecretion in COPD. Mucus occupying ratio (MOR) is significantly greater in both central and distal airways of patients who die with a diagnosis of chronic bronchitis compared with those with emphysema or controls without respiratory disease. L, luminal perimeter; Sb_1, size of bronchus before computer-based image analysis conversion to Sb_2 (Br, bronchial radius); S_m, size of stained area of mucus. Redrawn after 22.

morphologically normal. Gland size in COPD correlates with the amount of luminal mucus and daily volume of sputum (25). Although not necessarily causal, the latter observation suggests a strong relationship between gland hypertrophy and mucus hypersecretion in COPD.

The above features of hypersecretion are not common to all COPD patients. Not every patient expectorates, and there is overlap in gland size with healthy nonsmokers, and also between sputum producers and nonproducers (24,26,28–30). Goblet cell hyperplasia is not noted in all patients (22,27). Interestingly, although goblet cell hyperplasia is associated with degree of airway inflammation, gland size is not (24). Thus, although considered a general feature of COPD, mucus hypersecretion does not characterize all patients.

The mucin composition of airway mucus may be abnormal in COPD. Mucins in sputum are less acidic than normal (31), which may relate to disease-related alterations in glycosylation. MUC5AC and a low charge glycoform of MUC5B are the major mucin species in patients with COPD (32–35). Intriguingly, the low charge glycoform appears to be relatively increased above normal levels (36), a potentially significant observation that requires confirmation, or otherwise, in a greater number of samples. It is possible that the change in MUC5B glycoforms, coupled with the reduc-

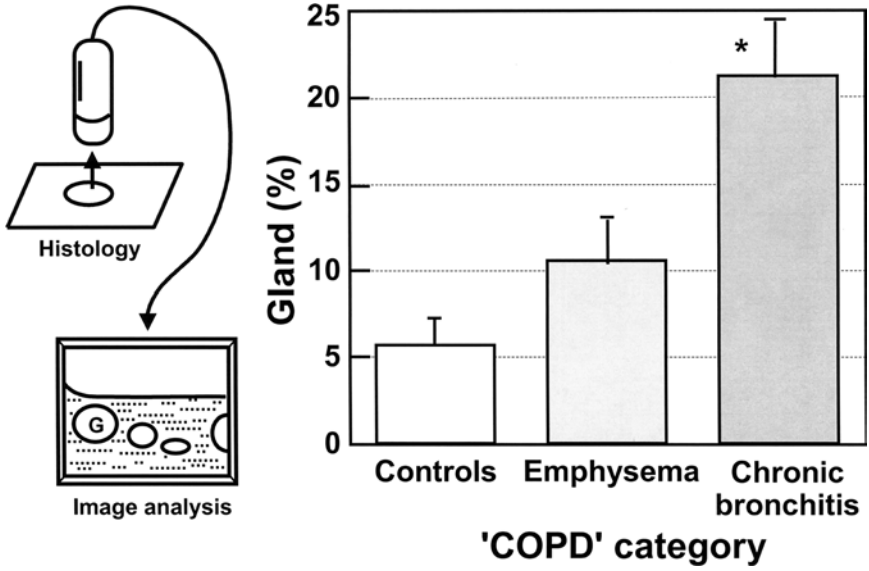

Figure 7 Airway submucosal gland hypertrophy in COPD. The percentage of the airway wall occupied by the glands is significantly greater in the airways of patients who die with a diagnosis of chronic bronchitis compared with those with emphysema or controls without respiratory disease. Redrawn after 22.

tion, mentioned above (27), in gland serous cells, a rich source of antimicrobial enzymes such as lysozyme and lactoferrin (37), contributes to the bacterial colonization of the airways that is a clinical feature of many COPD patients (3).

In contrast to normal airways, goblet cells in COPD contain not only MUC5AC but also MUC5B (34,38) and MUC2 (9). This distribution is different to that in patients with asthma or cystic fibrosis (CF), where MUC5AC and MUC5B show a similar histological distribution to normal controls (39,40). Although not found consistently (23,33), there is a growing impression that MUC2 is increased in 'irritated' airways, including COPD (9,36,41).

IV. ABNORMALITIES IN MUCOCILIARY CLEARANCE IN COPD

Aberrant airway mucus in COPD goes in concert with aberrant ciliated cells and cilia. The number of ciliated cells and ciliary length of cilia is decreased in patients with chronic bronchitis (42). Ciliary abnormalities include compound cilia, cilia enclosed within periciliary sheaths, cilia with abnormal

axonemes and cilia with intracytoplasmic microtubule doublets (43). These abnormalities coupled with mucus hypersecretion are associated with reduced mucus clearance and airway obstruction (44). However, differences in methodology (45) and patient selection, especially, exclusion of patients with asthma (46), can confuse overall interpretation of the results of these studies. Lung clearance is significantly reduced in heavy smokers (47) and in patients with chronic bronchitis (48). However, it should be noted that forced expirations and cough, compensate relatively and effectively for decreased mucus clearance in patients with chronic bronchitis, although they are much less effective in patients with emphysema where lung elastic recoil is impaired (49,50).

V. EPIDEMIOLOGY OF MUCUS HYPERSECRETION IN COPD

The perception of the role of airway mucus hypersecretion in pathophysiology and clinical symptoms in COPD has shifted from being a condition independent of disease progression to now being positively associated with morbidity and mortality (4,19). Epidemiological studies sampling hundreds to thousands of subjects in the late 1970s to early 1990s found scant evidence for the involvement of mucus in either the mortality or accelerated age-related decline in lung function associated with COPD (1,2,51–55). In all studies, sputum production, assessed by standardized questionnaire, was the index of mucus hypersecretion. However, the relationship between sputum production and mucus hypersecretion, particularly in the small airways, the main site of airflow obstruction in COPD (56), is unclear. It is also noteworthy that these studies were primarily in occupational cohorts, and were exclusively in men. Nevertheless, the consensus of these studies was that airflow obstruction and mucus hypersecretion were largely independent disease processes.

In contrast, a number of studies over the last 18 years, again with large sample numbers, and with an emphasis on general population samples rather than occupational cohorts, found positive associations between sputum production and decline in lung function, hospitalization and death (57–65). Some of these reports were re-examinations of the same patients, now older, reported previously (57). Of note is the observation that incidence of death was related to increased risk of patients with phlegm production to die of respiratory infection (5). Additionally, the association between chronic mucus hypersecretion and frequency of lower respiratory illness extends to an association with an accelerated decline in lung function with successive bouts of pulmonary infection (66). In summary, although not associated with disease progression in all cases, mucus hypersecretion contributes to morbidity and mortality in many patients with COPD, particularly those prone to infection, those with low lung function (58) and, possibly, as patients age. This highlights the importance of developing drugs that inhibit mucus hypersecretion in these patients.

VI. PHARMACOTHERAPY OF MUCUS HYPERSECRETION IN COPD

As discussed above, airway mucus contributes to morbidity and mortality in patients in whom the mucus hypersecretory phenotype impacts significantly on pathophysiology. Consequently, drugs affecting the bronchitic component of COPD should be beneficial in these patients. It should be noted that COPD has specific trigger factors, 'profile' of pulmonary inflammation and mucus hypersecretory phenotype (Fig. 5), and specific drugs may be required to fulfil the theoretical requirements for treatment of hypersecretion in COPD (Table 1). The following two sections consider different approaches to inhibition of mucus hypersecretion in COPD, starting with the effect of conventional pharmacotherapy on mucus hypersecretion and followed by a brief section on novel pharmacotherapeutic approaches. Each section includes some aspects of suppression of lung inflammation, possibly, the most beneficial therapy overall. Other approaches include neural inhibition, inhibition of mucin output, inhibition of MUC gene expression, and mucin synthesis and goblet cell hyperplasia.

A. Conventional Pharmacotherapy

The medications used currently in clinical management of COPD, namely, bronchodilators (anticholinergics, β_2-adrenoceptor agonists, and methylxanthines) and anti-inflammatories, primarily glucocorticosteroids (3), are not administered necessarily to target airway hypersecretion, but may nevertheless exert some of their beneficial effects via actions on mucus.

Table 1 Objectives for Pharmacotherapy of Mucus Hypersecretory Pathophysiology in COPD

Overall objective	Component objective
Facilitate mucus clearance (short term relief of symptoms)	Reduce viscosity (?increase elasticity)
	Increase ciliary function
	Induce cough
Reverse hypersecretory phenotype (long term benefit)	Reduce submucosal gland size
	Correct increased gland mucous:serous cell ratio
	Reduce goblet cell number
	Reverse increased lcgf-MUC5B:MUC5AC ratio

lcgf, low charge glycoform; MUC, mucin gene product.

Anticholinergics

The parasympathetic (cholinergic) nervous pathway is the dominant neural drive to mucus output in the airways (67). Cholinergic stimulation has been shown experimentally to affect a variety of responses pertaining to mucus hypersecretion in the airways, including stimulation of mucin output, electrolyte, water, and albumin flux and, when administered repeatedly to induce goblet cell hyperplasia and submucosal gland hypertrophy (Table 2). The muscarinic M3 receptor mediates airway mucin secretion, whilst the Ml receptor type appears to mediate water secretion. Cholinergic stimulation also increases albumin flux across the epithelium (68). The combined effect of stimulation of these receptors would be expected to be secretion of a hydrated, albumin-rich mucus (Fig. 8). Interestingly, in concert with increasing mucus secretion, cholinergic agonists increase mucociliary clearance in a number of experimental preparations (69–71). Anticholinergics block muscarinic receptors on secretory cells and, theoretically, may reduce production of airway mucus (Fig. 8) and have an effect on cough-induced clearance, as well as reduce the influence of vagal tone on bronchial smooth muscle. However, beneficial effects on mucociliary clearance with ipratropium bromide in COPD patients have been difficult to demonstrate. Notably, ipratropium bromide reduced the effectiveness of cough for clearing mucus from the airways compared with placebo (72). This may be a result of changes in airflow dynamics caused by bronchodilation, or altered rheology or depth of airway secretions following treatment. A clinical study with oxitropium bromide in patients with COPD showed a reduction in mucus secretion (73). The onset of the effect was slow and the mechanism by which it was mediated was not elucidated. The newer long-acting anticholinergic agent, tiotropium, has kinetic selectivity for both the Ml and M3

Table 2 Effect of Cholinergic Stimulation on Production of Airway Mucus

Stimulus	Target	Response	Species
Nerve stimulation	Goblet cell	Mucin secretion	Rodent
	Submucosal gland	Mucin secretion	Human
	Epithelium	NR	NR
Cholinoceptor agonists	Goblet cell	Mucin secretion	Rodent
		Hyperplasia[a]	Rat, cat
	Submucosal gland	Mucin secretion	Human
		Hypertrophy	Rat, cat
		Electrolyte/water flux	Pig
	Epithelium	Electrolyte/water flux	Human
		Albumin flux	Ferret

NR, not reported;
[a]After subacute repeated injection.

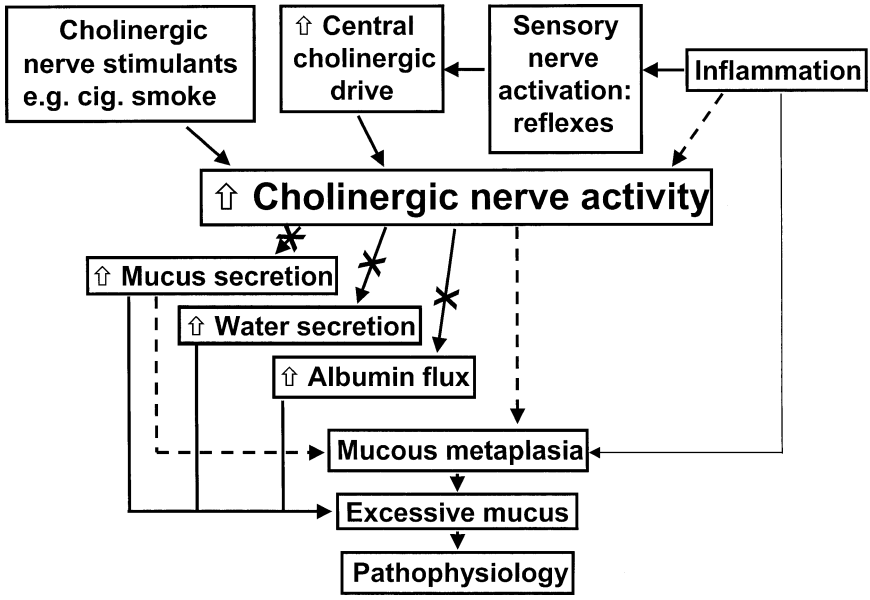

Figure 8 Cholinergic airway mucus pathophysiology in COPD. Cholinergic nerves are activated directly and indirectly to induce mucin, water, and albumin secretion with consequent mucus hypersecretion. X, inhibition by anticholinergic drugs. Dotted lines, pathways uncertain.

receptors over the M2 (auto-inhibitory) receptor. There are no current reports of its effects on cholinergic mucus secretion output. In one clinical study, tiotropium did not significantly improve tracheobronchial clearance compared with placebo, despite improving airway patency (74).

There are novel options for inhibiting the effects of cholinergic nerves (68), none of which are used clinically. These include inhibition of neurotransmitter (acetylcholine) release by activation of prejunctional receptors, for example opioid μ and δ receptors, cannabinoid CB_2 receptors, or vasoactive intestinal peptide VPAC1 receptors, and activation of large conductance calcium-activated potassium (BK_{Ca}) channels (Fig. 9). There is also the possibility that sensory nerve activation may mediate mucus output in COPD (75). These nerves can also be inhibited in a similar fashion to cholinergic nerves (see above), and also by inhibition of the effects of their tachykinin neurotransmitters, including substance P and neurokinin A, by tachykinin antagonists (76).

Short-Acting β_2-Agonists and Methylxanthines

Short-acting β_2-agonists and methylxanthines stimulate mucus clearance in healthy subjects and patients with chronic bronchitis or asthma, as assessed

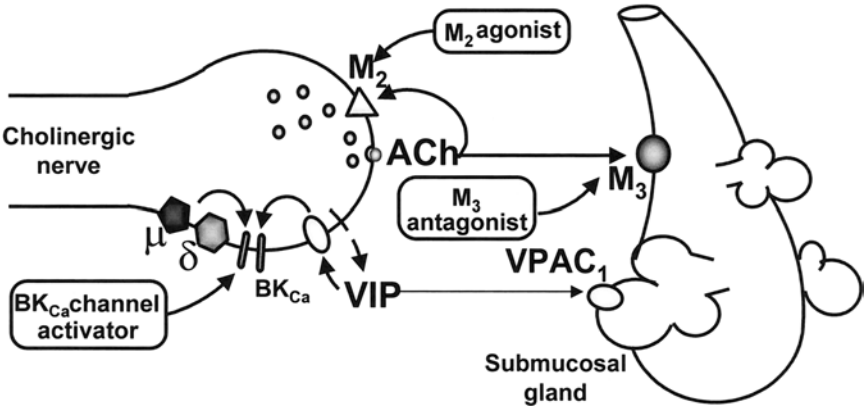

Figure 9 Inhibition of cholinergic airway mucus secretion. ACh, acetylcholine; M, muscarinic receptor; $VPAC_1$, vasoactive intestinal peptide (VIP) receptor; μ and δ opioid receptors; BK_{Ca}, large conductance calcium-activated potassium channel.

by radioaerosol clearance (77–80). However, stimulation is more dramatic in healthy individuals compared with patients with respiratory disease (81). β_2-Agonists may mediate these effects through an increase in intracellular cyclic adenosine monophosphate (cAMP) levels, which increases ciliary beat frequency (82,83). Methylxanthines have been shown to promote mucus secretion, augment airway epithelial ion transport, with consequent increases in luminal water flux, and stimulate ciliary beat frequency, with net enhancement of mucociliary clearance (84). However, interpretation of the mucus clearance effects of β_2-agonists and methylxanthines in clinical studies could be hindered by the bronchodilator action of these agents, which may allow greater penetration of the radioaerosol into the smaller bronchi where mucociliary clearance is slower. However, a placebo controlled study in healthy individuals treated with salbutamol or theophylline found no such effect (85), indicating that, at least in this subject group, beneficial effects on clearance were unrelated to effects on airway calibre.

Long-Acting β_2-Agonists

Salmeterol and formoterol are the two inhaled long-acting β_2-agonists currently available for treatment of COPD. They have a duration of action of at least 12 hr and can be administered twice-daily. Both drugs appear to exhibit anti-inflammatory activity, with consequent reduction in mucus hypersecretion. In terms of specific beneficial effects on the mucus hypersecretory phenotype, salmeterol increases ciliary beat frequency in cultured human bronchial epithelial cells (83) and improves mucociliary transport compared with placebo in asthmatic patients (86). Similarly, formoterol improves mucociliary clearance in 10 patients with bronchitis (87). Larger

studies of the effects of these two long acting β_2-agonists on mucus clearance in COPD are required.

Anti-inflammatory Drugs

Suppression of inflammation should indirectly suppress mucus hypersecretion. Anti-inflammatory drugs also directly affect the mucus hypersecretory phenotype. For example, in experimental systems glucocorticosteroids inhibit mucus secretion, MUC gene expression, mucus synthesis, and goblet cell hyperplasia (88). However, in contrast to asthma where they are clinically effective (89), in part due to an antihypersecretory action, glucocorticoids have limited effectiveness in stable COPD (3). This limited effectiveness appears to be due to a relative lack of effect of corticosteroids on the pulmonary inflammation in COPD (90). A limited effect on pulmonary inflammation will, therefore, hinder suppression of the component of hypersecretion that is inflammation driven (for example, by neutrophil elastase). In addition, it may be that the COPD-specific aspects of mucus hypersecretion (for example, increased proportion of gland mucous cells) are similarly limited in their response to corticosteroids. In contrast to the lack of effect of corticosteroids, inhalation of the nonsteroidal anti-inflammatory drug indomethacin, a cyclooxygenase (COX) inhibitor with no obvious clinical benefit in asthma, markedly reduced mucus output in patients with chronic bronchitis (91). Clinical trials of selective COX-2 inhibitors in COPD, for example rofecoxib, celecoxib, and eterocoxib which have reduced gastrointestinal activity, would be of great interest. The difference in efficacy of conventional anti-inflammatory drugs between COPD and asthma highlights the need to define the differences in inflammatory profile and hypersecretory phenotype between the two conditions.

Combination Therapy

The combination of a long-acting β_2-agonist and glucocorticosteroid has greater efficacy in asthmatic patients than merely increasing the dose of steroid (92). Consequently, combination therapy with long-acting β_2-agonists and glucocorticosteroids is now recommended in clinical management of asthma (89). There is now evidence that the combination of a long-acting β_2-agonist and glucocorticosteroid has greater clinical benefit in COPD than either drug alone, in particular in reducing exacerbations (93), Whether or not any beneficial effect of this drug combination in COPD is due to effects on airway mucus is not reported. Theoretically, the steroid would inhibit mucus synthesis and mucus secretion, whilst the β_2-agonist would have beneficial effects on mucus clearance (see above). Studies measuring these parameters are required to address these issues. Similarly, the effects of other combination therapies, for example long-acting β_2-agonists with either anticholinergics or theophylline (94), on mucus hypersecretory parameters need to be assessed.

Mucolytics

Decreasing the viscosity of, or 'thinning', viscous airway mucus with mucolytic drugs is one way of improving mucus clearance, both by cough and mucociliary transport. However, although numerous mucolytic drugs are available worldwide, their effectiveness in treatment of stable COPD has not been established (95). In addition, there are safety issues with a number of mucolytic preparations, for example iodinated glycerol. Consequently, mucolytics are not generally recommended in current guidelines on clinical management of COPD (3). Nevertheless, two rigorous meta-analyses found that treatment for at least 2 months with certain mucolytic drugs, with a heavy bias towards *N*-acetylcycsteine, reduces number of exacerbations and days of illness (96,97). Cost effective treatment would be in severe patients (98). However, it is not clear whether the beneficial effects of *N*-acetylcysteine (or the other drugs) are due to its mucolytic or antioxidant properties (or both).

Contraindications to Mucolytic Therapy

Severe airflow limitation reduces the effectiveness of cough to clear secretions (49). Consequently, there is a theoretical risk that, in the presence of reduced effectiveness of cough clearance, if secretions are thinned or loosened the mucus could lodge deeper into the lung, thereby causing greater obstruction. However, patients with moderately severe CF lung disease (FVC $<40\%$ predicted) given human recombinant DNAse (Dornase alfa) to thin their secretions did not demonstrate a worsening of pulmonary function (99). Nevertheless, any mucoactive therapy, except those that increase airflow or do not require active expectoration on the part of the patient, should be used with caution in patients with end-stage pulmonary disease or neuromuscular weakness.

Patients with acute mucus retention, for example during exacerbations of COPD, appear less responsive to mucoactive medications than stable patients (97). This may be due to decreased airflow caused both by the increase in infection and to muscular weakness in association with the pulmonary exacerbation, further reducing airflow dependent clearance mechanisms. In some patients with COPD (especially those with hyperreactive airways, sputum production, and small airways disease) lung function, wheezing, and bronchoconstriction may follow the use of chest physical therapy (100). In patients in whom mucus transport is increased by chest physiotherapy and cough, mucociliary clearance may be slowed or absent due to airway compression during the procedure. Patients with gastroesophageal reflux coupled with reduced mucus clearance, for example by impaired cough, are at risk from postural drainage. They may be at additional risk with mucus mobilizing or expectorant therapy, particularly when the medication increases the volume of secretions produced (101).

Macrolide Antibiotics

Antibiotic treatment against acute infections is recommended in clinical management of COPD (3). Erythromycin, clarithromycin, and flurythromycin are macrolide antibiotics that have a variety of beneficial effects on airway mucus, for example inhibition of mucin secretion in a variety of experimental preparations including human airways in vitro (19,102). Anecdotally, erythromycin reduces excessive sputum production in patients with airway mucus hypersecretion (103,104). The mechanism of action of erythromycin is relatively unexplored, but may involve anti-inflammatory effects (105,106) as well as direct inhibitory effects on MUC gene expression, mucin synthesis and mucin secretion (107). Formal clinical studies of its effects on the pathophysiology of mucus hypersecretion, for example sputum production and lung function, in COPD would be of interest.

B. Novel Pharmacotherapy

The clinical symptoms of cough and sputum production, coupled with a perception of the importance of mucus hypersecretion in the pathophysiology of a number of severe lung conditions, including COPD, has prompted renewed interest in research into airway hypersecretion and, in concert, in development of drugs targeting mucus and the hypersecretory phenotype in COPD. These drugs and their purported targets have been discussed in detail recently (10,19,94,108,109), and will only be briefly highlighted herein (Table 3). It should be noted that the activity of many of these compounds is not as selective for the target as may be thought and, in any event, whether or not any beneficial activity of the drug is due to activity at the target is, for the most part, substantially unproven.

Epoxygenase Inducers

Cytochrome P-450 enzymes (epoxygenases) metabolise arachidonic acid and regulate inflammation (110). Benzafibrate, an inducer of epoxygenase, inhibits airway goblet cell hyperplasia in a rat model of chronic bronchitis (111). The mechanisms underlying the inhibition include production of anti-inflammatory mediators and reduction in amount of 'available' arachidonic acid. Epoxygenase inducers, or of selective eopxyeicosanoids, would be potential therapy for both the inflammation and mucus hypersecretion of COPD.

Inhibitors of Reactive Gases

Oxidant stress is considered a pathophysiological feature of COPD f(112) and exhaled nitric oxide (NO) is elevated in COPD (113). Oxidants and NO have marked effects on airway mucus and goblet cells (114,115). Consequently, antioxidants and inhibitors of inducible NO synthase (iNOS) may have therapeutic benefit for mucus hypersecretion in COPD.

Table 3 Novel Targets for Inhibition of the Mucus Hypersecretory Phenotype in COPD

Target	Requirement	Example drug(s)	Reference
Bax	Inducer	–	(131)
Bcl-2	Inhibitor	Antisense oligonucleotides (e.g., ODN64 and ODN83)	(130)
EGFR tyrosine kinase	Inhibitor	AG1478, BIBX1522, and ZD1839 (Iressa)	(115)
Epoxyeicosanoids	Adjunct therapy	–	–
Epoxygenase	Inducer	Benzafibrate	(111)
hCLCAl	Inhibitor	MSI1956 (talniflumate)	(149)
MAP kinase	Inhibitor	SB203580, SB202190	(120)
MARCKS	Inhibitor	Synthetic peptide to N-terminal region, antisense oligonucleotide	(116)
MEK	Inhibitor	PD98059 and U0126	(121)
MUC gene expression	Inhibitor	Antisense oligonucleotide	(129)
Muncl8B	Inhibitor	Antisense oligonucleotide	(117)
Nitric oxide (NO)	iNOS inhibitor	GW273629, L-NIL	(114)
Oxidants	Inhibitors	N-acetylcysteine, superoxide dismutase mimetics, and spin trap compounds	(150)
$P2Y_2$ purinoceptors	Agonist	INS365	(132)
$P2Y_2$ purinoceptors	Antagonist	? reactive blue 2 derivatives	(134)
PtdIns 3-kinase	Inhibitor	LY294002	(121)
RAR-α	Antagonist	RO-41-5253	(127)

hCLCAl, human calcium-activated chloride channel 1; EGFR, epidermal growth factor tyrosine kinase receptor; iNOS, inducible nitric oxide synthase; MAP, mitogen-activated protein (kinase); MARCKS, myristoylated alanine-rich C kinase substrate; MEK, mitogen-activated protein/extracellular signal-regulated kinase (ERK) 1/2 kinase; PtdIns, phosphatidylinositol; RAR-α, retinoic acid receptor-α

Inhibitors of Mucin Exocytosis

Inhibition of mucin exocytosis is a therapeutic option for mucus hypersecretion in COPD. However, it should be noted that inhibition of secretion could lead to excessive accumulation of intracellular mucins, with unknown, and potentially detrimental, effects on secretory cell function. Myristoylated alanine-rich C kinase substrate (MARCKS) protein is a key intracellular molecule involved in intracellular movement and exocytosis of mucin granules (116). Blockade of MARCKS by a synthetic peptide to its N-terminal region inhibited mucin secretion by normal human bronchial epithelial

cells in vitro. In addition, an antisense oligonucleotide to MARCKS down-regulated both mRNA and protein levels and also attenuated mucin secretion. Similar to MARCKS, the Secl/Muncl8 family are critical to exocytosis in airway goblet cells. Experimental induction of Muncl8B induces a marked airway hypersecretory phenotype (117). Inhibition of Muncl8B using antisense technology is under investigation.

Inhibitors of Goblet Cell Hyperplasia

Increased MUC gene expression, mucin synthesis, and goblet cell hyperplasia appear to be linked processes that are regulated by a number of inflammatory mechanisms. For example, airway epidermal growth factor receptor (EGF-R) expression is induced by experimental procedures pertinent to COPD (115). EGF-R upregulation and signaling via EGF-R tyrosine kinase is a signaling event for induction of mucin synthesis and goblet cell hyperplasia. Inhibitors of EGF-R tyrosine kinase block these responses. One of these, Iressa, is in clinical trial for cancer, but not yet for COPD or similar respiratory diseases.

Unsurprisingly, the p38 mitogen activated protein (MAP) kinase pathway, MEK/ERK pathway, and phosphatidylinositol 3-kinase pathway are all involved, to a greater or lesser extent, in intracellular events leading to mucin synthesis and goblet cell hyperplasia (118–121). Inhibitors of these pathways inhibit mucus hypersecretory endpoints in experimental systems.

Calcium-activated chloride (CLCA) channels also appear to be critically involved in development of an airway hypersecretory phenotype. In mice, suppression of mCLCA3 inhibits goblet cell hyperplasia, whilst overexpression increases goblet cell number (122). 'Lomucin' (MSI 1956, or talniflumate) is a small molecule putative inhibitor of hCLCAl that is currently in clinical trial for hypersecretory airway diseases. The results of these trials are awaited with great interest.

Retinoic acid (vitamin A) is perceived to be of clinical benefit in a variety of clinical conditions. Agonists at the retinoic acid RAR-γ are currently being intensely investigated as inhibitors and reverses of alveolar destruction in emphysema (123). In contrast, the RAR-α receptor appears to be involved in mucin expression (124–127) and in the development and maintenance of a hypersecretory phenotype (127). RAR-α antagonists such as RO-41–5253 inhibit a number of these activities (128). Consequently, there is interest in development of selective RAR-α antagonists for mucus hypersecretion in a number of respiratory diseases, including COPD.

Finally, antisense technology is also being explored as a new approach to inhibition of goblet cell hyperplasia. For example, an 18-mer MUC antisense oligomer suppressed mucin gene expression and wood smoke-induced epithelial metaplasia in rabbit airways (129).

Inducers of Goblet Cell Apoptosis

Hyperplastic airway goblet cells in COPD models express the antiapoptotic factor Bcl-2 (130). Conversely, the pro-apoptotic factor Bax is crucial for resolution of hyperplasia (131). Thus, the balance between Bcl-2 and Bax may affect the persistence of goblet cell hyperplasia. Reduction of Bcl-2 expression by antisense oligonucleotides induces a dose-dependent resolution of hyperplasia.

Mucus Inhibition vs. Mucus Hydration

The purine nucleotides, adenosine 5'-triphosphate (ATP) and uridine triphosphate (UTP), increase airway mucin and water secretion via interaction with P_{2Y2} purinoceptors (132,133). Consequently, P_{2Y2} antagonists might be effective in inhibiting airway hypersecretion (134). However, mucus hydration is associated with improvements in mucociliary clearance and stimulation of water secretion may have greater therapeutic potential than inhibition of P_{2Y2}-mediated mucin secretion (6). Consequently, there is considerable interest in development of P_{2Y2} agonists. In phase I clinical trial, a second generation P_{2Y2} agonist, INS365, was safe, well tolerated and significantly enhanced sputum expectoration (132). However, uncontrolled thinning of airway mucus may have adverse clinical effects (see the section on Contraindications to Mucolytic Therapy).

VII. CHEST PHYSIOTHERAPY

Chest physiotherapy aims to facilitate mucus clearance and encompasses forced expiratory manoeuvres, postural drainage, chest percussion, clapping, vibration, high frequency oscillation, breathing exercises, and induction of cough (135–137). In general, these techniques have been poorly studied in COPD and there is little evidence of clinical benefit, particularly in exacerbations. The efficiency of airflow transport and cough are dependent on three factors:

1. Airflow velocity. At high airflow velocities there is a characteristic interaction between gas and liquid called annular flow which increases mucus transport to a level where airflow velocity is directly related to mucus transport (138).
2. Thickness of the mucus layer. The minimal flow velocity at which mucus transport by gas liquid interaction arises is dependent on the thickness of the mucus layer (139). With increasing thickness of the mucus layer, the airflow velocity needed for cough transport decreases.
3. Peak expiratory flow. The peak expiratory flow developed during a forced expiration is important because a decreased PEF minimizes the effectiveness of forced expiration. The rapid application

of a high expiratory (peak) flow, may also decrease the viscosity of mucus. This phenomenon of shear stress reduction of viscosity in non-Newtonian fluids is known as thixotropy. A high peak flow can facilitate detachment of adherent airway secretions from the epithelial surface and can be facilitated by physiotherapy techniques such as chest percussion (140). Once a critical airflow is reached, termed detachment velocity, there is marked augmentation in mucus clearance with improvements in ciliary efficacy.

Cough is best at clearing secretions from central airways (139,141). Chest physiotherapy may trigger cough receptors and, when combined with physiotherapy, vigorous, directed cough is effective in clearing the airways of patients with retained secretions (142). Inhaled radioaerosol studies show that cough alone and cough combined with chest physiotherapy were equivalent in promoting central airway mucus clearance whereas combined techniques were better for accelerating clearance from small airways (143).

High expiratory airflow rates that develop mucus shearing forces depend upon generation of large positive intrapleural pressures (144). Consequently, reasons breathing exercises are often combined with chest physiotherapy. Addition of percussion to conventional physiotherapy does not improve sputum yield or mucociliary clearance in most studies, except for patients with cystic fibrosis. Postural (gravity assisted) drainage, as distinct from chest percussion, adds little to the effectiveness of chest percussion and increases the risk of aspiration from increased gastroesophageal reflux (145,146). High frequency oscillation are also used in an attempt to improve mucus transport, although there is little evidence for clinical efficacy in COPD (147). The effectiveness, if any, of the technique is frequency-dependent, with a frequency of about 10–15 Hz, which is outside the range of the manual techniques, being optimal for enhancing mucus transport. Oscillations are applied either at the mouth using a modified loudspeaker (147), or at the thorax using an inflatable vest (148). Other, newer, techniques aimed at improving mucus clearance include the active cycle of breathing, positive expiratory pressure (PEP) mask, 'flutter' breathing, and autogenic drainage. All of these techniques seek to avoid the problem of dynamic airway compression which may inhibit sputum mobilization. Most have not been adequately examined in terms of their effectiveness in patients with COPD.

VIII. SUMMARY AND CONCLUSIONS

Airway mucus hypersecretion and the pathophysiological changes that accompany it, for example goblet cell hyperplasia, are features of many patients with COPD. The impact of airway hypersecretion on morbidity and mortality is now more fully understood, albeit that it may be limited

to certain groups of patients, particularly those that are prone to respiratory tract infection. Nevertheless, it is important to develop drugs that inhibit mucus hypersecretion in these susceptible patients. However, before addressing these issues in a rational manner, considerably more information is required on basic mucus physiology and, in particular, mucus pathophysiology. For example, more detail is required concerning the biochemical and biophysical nature of airway mucins in normal healthy subjects. Answers to the questions of whether or not there is an intrinsic abnormality of mucus in COPD, and whether any abnormality is specific for COPD are urgently required. In addition, the factors that regulate MUC gene expression in health and disease, and the relationship between this regulation and development of a hypersecretory phenotype that appears to be specific to the bronchitic component of COPD, need to be determined. The above information could then be used in delineation of therapeutic targets which, in turn, should lead to rational design of anti-hypersecretory drugs for specific treatment of airway mucus hypersecretion in COPD.

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6

Macrophages

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I. INTRODUCTION

Alveolar macrophages play a critical role in innate and acquired immunity, in the defense of the respiratory tract against pathogens, and in the clearance of inhaled particles. They also play an important role in the inflammatory response and there is increasing evidence that they play a pivotal role in the pathophysiology of COPD. Because of the extraordinary diversity of macrophage responses they can account for most of the known features of COPD (1–5) (Fig. 1).

II. INCREASED MACROPHAGE NUMBERS IN COPD

There is a marked increase in the numbers of macrophages in airways, lung parenchyma, BAL fluid and sputum of smokers and patients with COPD. A careful morphometric analysis of macrophage numbers in the parenchyma of patients with emphysema showed a 25-fold increase in the numbers of macrophages in the tissue and alveolar space compared with normal smokers approximately matched for cigarette exposure (6). Furthermore, macrophages are localized to sites of alveolar wall destruction in patients with emphysema (7,8). Indeed, there is a correlation between macrophage numbers in the airways and the severity of COPD (9).

Clusters of macrophages are also found around small airways and associated with peribronchiolar fibrosis in respiratory bronchiolitis (RB) (10). This is exclusively seen in smokers and ex-smokers and may persist

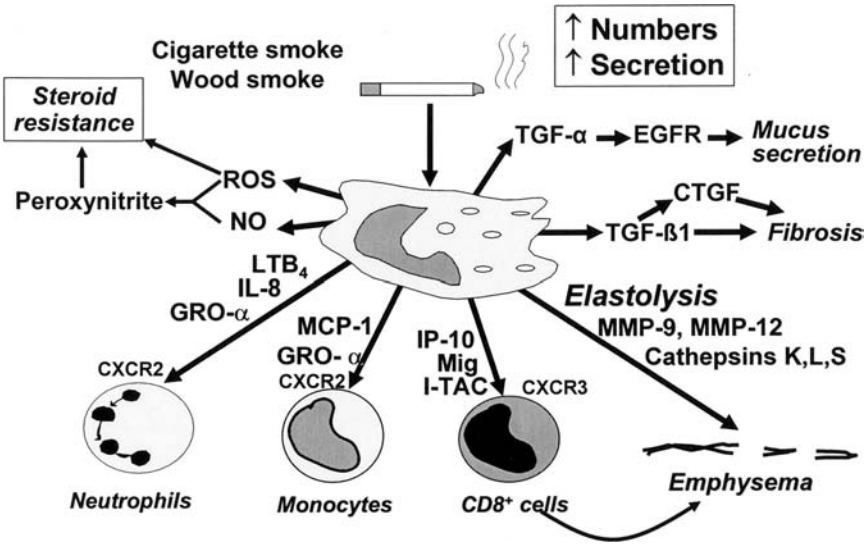

Figure 1 Macrophages may play a pivotal role in COPD as they are activated by cigarette smoke extract and secrete many inflammatory proteins that may orchestrate the inflammatory process in COPD. Neutrophils may be attracted by interleukin (lL)-8, growth related oncogene-α (GRO-α) and leukotriene B_4 (LTB_4), monocytes by macrophage chemotactic protein-1 (MCP-1), and $CD8^+$ lymphocytes by interferon-γ inducible protein (IP)-10, monokine-induced by interferon-γ (Mig) and interferon-inducible T-cell α-chemoattractant (I-TAC). Release of elastolytic enzymes including matrix metalloproteinases (MMP) and cathepsins cause elastolysis, and release of transforming growth factor (TGF)-β1 and connective tissue growth factor (CTGF). Release of TGF-α activates epithelial growth factor receptors (EGFR) which stimulate mucus hypersecretion. Macrophages also generate reactive oxygen species (ROS) and nitric oxide (NO) which together form peroxynitrite and may contribute to steroid resistance.

long after smoking cessation. Respiratory bronchiolitis is represented by ill-defined parenchymal micronodules detected on high resolution computer ized tomography in some cigarette smokers. Long-term follow-up suggests that these nodules may evolve into centrilobular emphysema and may therefore represent the evolution of emphysema (11).

The increased numbers of macrophages in COPD may be explained by increased recruitment of blood monocytes into the lung, prolonged survival within the lung, or impaired clearance of macrophages from the respiratory tract.

A. Increased Recruitment

The increased numbers of macrophages in smokers and COPD patients may be explained by increased recruitment of monocytes from the circulation in

response to monocyte-selective chemokines produced in the lungs. The monocyte-selective chemokine monocyte chemoattractant protein-1 (MCP-1) is increased in sputum and BAL of patients with COPD (12,13), with increased expression in alveolar and small airway macrophages (14).

CXC chemokines also act as monocyte chemoattractants and act via the low affinity chemokine receptor CXCR2. The concentration of the CXC chemokine growth-related oncogene-α (GRO-α, CXCL1), which selectively activates CXCR2, is markedly increased in sputum and BAL of patients with COPD (12). Monocytes from patients with COPD show a greater chemotactic response to GRO-α than cells from normal smokers and nonsmokers, but this is not explained by an increase in CXCR2 density (15). Interestingly, while all monocytes express CCR2, the receptor for MCP-1, only $\sim$30% of monocytes express CXCR2. It is possible that these CXCR2-expressing monocytes transform into macrophages that behave differently – e.g., release more inflammatory proteins.

B. Macrophage Proliferation

The increased numbers of macrophages in COPD may be due to increased recruitment of monocytes from the circulation, but may also be due to increased proliferation and prolonged survival of macrophages in the lungs. Macrophages have a very low proliferation rate in the lungs, but there is some increase in cell proliferation measured by proliferative cell nuclear antigen (PCNA) in macrophages from smokers compared to normal and asthmatic macrophages, with approximately 5% of cells staining positive in smokers compared with only 2% of cells in nonsmokers (16). These small but significant differences could be important over time as macrophages have a long survival in lung tissue. Alveolar macrophages also show signs of increased proliferation in other chronic inflammatory lung diseases (17).

C. Macrophage Survival

Macrophages have a survival time of several months so this is difficult to measure directly. There is a report of cigarette particulates in alveolar macrophages over 2 years after cessation of smoking (18). In alveolar macrophages from smokers, there is markedly increased expression of the antiapoptotic protein Bcl-X_L and increased expression of $p21^{CIP/WAFI}$ in the cytoplasm, which is associated with prolonged survival (16). This may be secondary to oxidative stress which mimics these effects in vitro. This suggests that macrophages may have a prolonged survival in the lungs of smokers and patients with COPD. On the other hand, acute exposure of rodent and human alveolar macrophages to cigarette smoke extract induces apoptosis, an effect that is also mediated via oxidative stress (19). This suggests that acute and chronic effects of cigarette smoking on macrophage survival may differ.

D. Macrophage Clearance

Alveolar macrophages are presumed to be cleared from the airways predominantly by mucociliary clearance, which is significantly impaired in patients with COPD. Macrophages may also be cleared via lymphatic drainage, and this might also be impaired in COPD patients as a result of emphysema and fibrosis of small airways interrupting lymphatic channels.

III. MACROPHAGE HETEROGENEITY

There is increasing evidence for distinct populations of macrophages that may play different roles, although the tools that can differentiate these subpopulations are poorly developed.

A. Alveolar and Interstitial Macrophages

At least two subpopulations of macrophages are found in the lungs (20,21). Alveolar macrophages are localized to the alveolar spaces and are strategically placed to interact with inhaled pathogens and particles. Interstitial macrophages make up about half of the total numbers of macrophages in the lung and are located within the alveolar walls and in airway walls. This location may give them an important role in the regulation of matrix proteins in the lung. Interstitial macrophages may represent an intermediary stage in the maturation of alveolar macrophages from monocytes recruited from the pulmonary circulation. Alveolar macrophages are large mature cells with a high cytoplasm/nucleus ratio and resembling other tissue macrophages, whereas interstitial macrophages are smaller, more uniform in size, contain few intracytoplasmic lamellar inclusions or lysosomes, and more closely resemble peripheral blood monocytes. There is increasing evidence that alveolar and interstitial macrophages are distinct cell populations with differing functions and that each population may contribute to inflammatory and immune responses in the lungs. Alveolar macrophages release more inflammatory mediators and show increased chemotaxis, phagocytosis, cytotoxicity, and release of reactive oxygen and nitrogen intermediates than interstitial macrophages. Interstitial macrophages express greater quantities of complement C3 receptor and intercellular adhesion molecule-1 (ICAM-1), are more active in secreting interleukin (IL)-1 and IL-6 and exhibit greater Ia antigen expression, suggesting that they are more specialized in immune responses and immunoregulation. They may also have a greater capacity to proliferate compared to alveolar macrophages and this may maintain the lung macrophage pool. There is a need to further investigate the differences between alveolar and interstitial macrophages, particularly in COPD patients; alveolar macrophages may be obtained by bronchoalveolar lavage, whereas interstitial macrophages may be isolated by digestion of lung parenchyma removed at surgical resection. There

may also be differences between alveolar macrophages, possibly through different recruitment mechanisms or local factors that may alter their function, but there are currently no surface markers that allow separation of subpopulations. Higher density alveolar macrophages are smaller and appear less mature and may represent the most recently arrived cells. They have greater chemotactic and phagocytic activity and release greater amounts of reactive oxygen species and inflammatory mediators than lower density cells (22). Alveolar macrophages may also be differentiated by different antibodies which appear to discriminate a more dendritic phenotype of cell (23).

B. Dendritic Cells

Dendritic cells are a specialized form of macrophage that play a central role in the initiation of the innate and adaptive immune response (24). The airways and lungs contain a rich network of dendritic cells that are localized near the surface, so that they are ideally located to signal the entry of foreign substances that are inhaled (25). Dendritic cells can activate a variety of other inflammatory and immune cells, including macrophages, neutrophils, T- and B-lymphocytes (26). It therefore likely that dendritic cells may play an important role in the pulmonary response to cigarette smoke and other inhaled noxious agents and may therefore be a key cellular element in COPD. The mechanisms by which tobacco smoke actives the immune system is not yet understood, but a glycoprotein isolated from tobacco has powerful immunostimulatory actions (27). There is an increase in the number of dendritic cells in rat lungs exposed to cigarette smoke (28) and in the airways and alveolar walls of smokers (29,30). Pulmonary histiocytosis is a disease caused by dendritic cell granulomata in the lung. It is characterized by destruction of the lung parenchyma that resembles emphysema and the adult form of the disease occurs almost exclusively in smokers (31,32). In mice exposed to chronic cigarette smoke, there is an increase in dendritic cells in the airways and lung parenchyma (33). The role of dendritic cells in recruiting other effector cells in COPD deserves further study. MIP-3α (CCL20) acts on the chemokine receptor CCR6 that is expressed by immature dendritic cells and is a potent chemoattractant of dendritic cells. It is expressed by airway epithelial cells in response to interferon-γ (IFN-γ) and therefore may be involved in COPD (34).

IV. SECRETORY ROLE

Alveolar macrophages have the capacity to secrete a very large number of inflammatory mediators, including lipid mediators, chemokines, cytokines, growth factors, and reactive oxygen and nitrogen species. They may therefore play many roles in the respiratory tract and may be proinflammatory

or anti-inflammatory. They may be activated by several stimuli, including cigarette smoke, proinflammatory cytokines, endotoxin, and immune stimuli. Overall, alveolar macrophages from COPD patients show greater release of inflammatory mediators than cells from normal smokers, which in turn secrete more than cells from nonsmokers.

A. Lipid Mediators

Alveolar macrophages have the capacity to generate leukotrienes (LT) and prostaglandins (PG). In patients with COPD, there is an increase in LTB_4 concentration in sputum and exhaled breath (35–37), but this is likely to be derived predominantly from neutrophils rather than alveolar macrophages. Human alveolar macrophages express cytosolic phospholipase A_2 and release LTB_4 and platelet-activating factor on activation (38). Alveolar macrophages also secrete PGE_2 and may contribute to the increased PGE_2 in exhaled breath of COPD patients (37). This is likely to be derived from cyclo-oxygenase-2 (COX-2) which is expressed in macrophages (39). There is increased COX-2 expression in alveolar macrophages from patients with COPD compared to normal control subjects (40).

B. Chemokines

Alveolar macrophages have the capacity to release multiple chemokines leading to the recruitment of several cell types from the circulation, including monocytes, neutrophils, and T-lymphocytes.

The best studied chemokine is IL-8 which is released by alveolar macrophages in response to several stimuli, including cigarette smoke extract, endotoxin, and IL-1β. Hypoxia also releases IL-8 from alveolar macrophages and this mechanism might be relevant in severe COPD and exacerbations (41). It is likely that macrophages contribute to the high concentrations found in the airways of patients with COPD (42), although neutrophils recruited by macrophage-derived neutrophil chemokines may be the major source. Alveolar macrophages from COPD patients release significantly more IL-8 at baseline and after stimulation with IL-1β and cigarette smoke than macrophages from normal smokers and nonsmokers and these differences are maintained in cell culture, suggesting that external factors are not responsible for this increased activation (43).

Macrophages also have the capacity to release the chemokines IP-10 (CXCL10), I-TAC (CXCL11) and Mig (CXCL9), which are chemotactic for $CD8^+$ Tcl cells via interaction with the CXCR3 receptor expressed on these cells (44). Macrophages may therefore play a key role in the recruitment of T-lymphocytes, and particularly $CD8^+$ cells, to the lungs. The release of IFN-γ by these cells may further release these chemokines, resulting in a chronic inflammatory cycle.

C. Inflammatory Cytokines

Alveolar macrophages release TNF-α in response to inflammatory stimuli, including cigarette smoke and may contribute to the elevated concentrations of TNF-α in the sputum of COPD patients (42). Granulocyte-macrophage colony stimulating factor (GM-CSF) is also released from macrophages, but unlike chemokines and TNF-α, its release does not appear to be increased in COPD cells.

D. Interleukin-10

Interleukin-10 is a potent anti-inflammatory cytokine that is released from alveolar macrophages in response to inflammatory stimuli. Its secretion is markedly reduced in alveolar macrophages from patients with asthma (45) and its concentrations are reduced in sputum of patients with asthma and COPD, suggesting that a similar abnormality may apply on COPD (46). Its production appears to be increased in macrophages from normal smokers (47), but it is not certain whether macrophages from COPD patients show a relatively reduced production, as in asthma.

E. Growth Factors

Human alveolar macrophages express transforming growth factor-β1 (TGF-β1) and TGF-β3 (48). Alveolar macrophages from chronic bronchitis patients release more TGF-β than cells from normal and asthmatic subjects (49). Interestingly, human alveolar macrophages produce less TGF-β than monocytes (50). This might suggest that interstitial and airway macrophages are likely to be a more important source of TGF-β than alveolar macrophages. Airway macrophages from COPD patients show increased expression of TGF-β1 and this may contribute to the fibrosis found in small airways (51). The increased TGF-β1 by bronchiolar epithelial cells of COPD patients may contribute to the chemoattraction of macrophages to the airways and is correlated with macrophage numbers.

Alveolar macrophages produce TGF-α in much greater amounts than TGF-β (50) and this may be a major endogenous activator of epidermal growth factor (EGF) receptors that play a key role in regulating mucus secretion in response to many stimuli, including cigarette smoke (52).

F. Reactive Oxygen and Nitrogen Species

Macrophages generate reactive oxygen species and therefore contribute to the increased oxidative stress in the airways of patients with COPD (53). Activating stimuli, such as cigarette smoke, cause activation and assembly of a multicomponent NADPH oxidase in the macrophage cell membrane which generates superoxide anions (O_2^-) which are converted to hydrogen peroxide by superoxide dismutase (54). Production of reactive oxygen

species is important for the microbial killing function of macrophages; it is also important in activating mitogen-activated protein (MAP) kinase signal transduction pathways and the transcription factors nuclear factor-κB (NF-κB) and AP-1, which in turn switch on multiple inflammatory genes.

Although NO is readily produced by murine alveolar macrophages, it has proved much more difficult to demonstrate NO production and expression of inducible NO synthase (iNOS) in human macrophages in vitro. However, NO production and iNOS expression is observed in alveolar macrophages in vivo during pulmonary infections and alveolar NO may play an important role in innate immunity of the respiratory tract (55). There is increased expression of iNOS in alveolar macrophages from induced sputum and lung parenchyma in patients with COPD (56–58). As NO is produced under conditions of oxidative stress in COPD, this may lead to increased production of peroxynitrite, accounting for the increase in 3-nitrotryosine immunoreactivity in alveolar macrophages from COPD patients (56).

V. REGULATORY MECHANISMS

A. Transcription Factors

The secretion of multiple inflammatory proteins by alveolar macrophages is largely a result of increased expression of inflammatory genes orchestrated by proinflammatory transcription factors, such as NF-κB and AP-1. There is increased activation of NF-κB in alveolar macrophages from COPD patients, as evidenced by increased nuclear localization of the p65 subunit (59) and this is further increased during acute exacerbations of the disease (60). NF-κB may play a critical role in the increased expression of TNF-α, chemokines such as IL-8, GRO-α and MCP-1, GM-CSF, iNOS, proteases such as MMP-9, and adhesion molecules such as ICAM-1 (Fig. 2). LPS activates NF-κB in alveolar macrophages resulting in secretion of GM-CSF and this is blocked by an inhibitor of NF-κB kinase-2 (IKK2) (61).

B. Signal Transduction Pathways

The signal transduction pathways involved in macrophages activation and secretion have not been well characterized and it is uncertain how they are affected in COPD. The amplification of inflammatory gene expression in COPD may be linked to abnormal activation of signal transduction pathways that regulate transcription factors, such as NF-κB. Endotoxin activates both the extracellular signal related (ERK) and p38 MAP kinase pathways to stimulate expression of cytokine genes (62). PD 098059, an inhibitor of the ERK pathway, blocks GM-CSF release induced by LPS, but this is not affected by SB 203580, an inhibitor of the p38 pathway

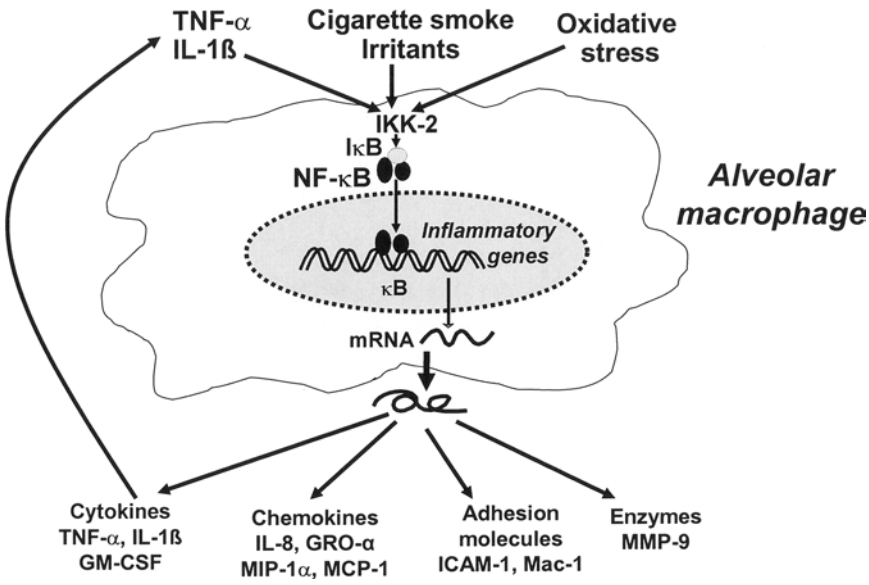

Figure 2 Pivotal role of nuclear factor-κB (NF-κB) in COPD macrophages. Various stimuli activate inhibitor of NF-κB kinase-2 (IKK-2) which causes translocation of NF-κB to the nucleus where it binds to the promoter region of various inflammatory genes, leading the increased transcription and release of cytokines, chemokines and proteases, and expression of adhesion molecules.

(61). More studies in human alveolar macrophages are required as there appear to be important differences in signal transduction pathways between species. There are also important differences in signalling, depending on the activating stimulus and the inflammatory gene that is regulated.

C. Histone Acetylation

Inflammatory genes are regulated by acetylation of core histones around which DNA is wound. Activation of proinflammatory transcription factors, such as NF-κB and AP-1, results in interaction with coactivator molecules that have intrinsic histone acetyltransferase activity (63). The acetylation of histone H4 is involved in the activation of inflammatory genes (64) and this mechanism has been demonstrated in human alveolar macrophages (65). Histone acetylation is reversed by histone deacetylases (HDAC) which show reduced activation in alveolar macrophages from COPD patients (65) and this may account for the increased activation of inflammatory genes and increased release of inflammatory mediators seen in macrophages from COPD patients.

VI. PROTEASES

Alveolar macrophages secrete several elastolytic enzymes, including MMP-2, MMP-9, MMP-12, cathepsins K, L, and S, and neutrophil elastase taken up from neutrophils (66,67). Alveolar macrophages from patients with COPD have a greater elastolytic activity at baseline than those from normal smokers and this is further increased by exposure to cigarette smoke (47,67,68). Macrophages demonstrate this difference even when maintained in culture for 3 days and therefore appear to be intrinsically different from the macrophages of normal smokers and nonsmoking normal control subjects (67). The predominant elastolytic enzyme secreted by alveolar macrophages in COPD patients appears to be MMP-9 (68,69). MMP-9 is also strongly expressed in emphysematous lung at sites of macrophage accumulation (70). MMP-12 (macrophage metalloelastase) appears to play an important role in cigarette smoke-induced emphysema in mice and appears to be necessary for release of activated TNF-α from alveolar macrophages (71). This then leads to increased expression of E-selectin and neutrophil accumulation in the lungs. The role of MMP-12 in human macrophages is less well defined as there is less evidence for its expression in human alveolar macrophages (69,72).

Macrophages also release cysteine proteases, but their role in COPD has not been evaluated. A cysteine protease inhibitor E-64 significantly reduces the elastolytic activity of alveolar macrophages from COPD patients, although this component is less marked and less sustained than MMP-9 secretion (67). Cathepsins K, L, and S have been identified in association with human macrophages (66,73–75).

VII. RESPONSES TO DRUGS

There have been relatively few studies reporting the effects of drugs on human alveolar macrophages, particularly in cells derived from COPD patients. Yet, this is important information if these cells play such a critical role in orchestrating the chronic inflammatory process in COPD.

A. Corticosteroids

Corticosteroids are ineffective in suppressing inflammation, including cytokines, chemokines, and proteases, in patients with COPD (76–78). In vitro, the release of IL-8, TNF-α, and MMP-9 from macrophages taken from normal subjects and normal smokers is inhibited by corticosteroids, whereas corticosteroids are ineffective in macrophages from patients with COPD (43). Curiously, this does not apply to GM-CSF which does not appear to have increased secretion in COPD and is suppressed by corticosteroids,

albeit to a lesser extent than in macrophages from normal smokers. The reasons for resistance to corticosteroids in COPD and to a lesser extent macrophages from smokers may be the marked reduction in activity of histone deacetylase-2 (HDAC2) (65), which is recruited to activated inflammatory genes by activated glucocorticoid receptors to switch off inflammatory genes (64) (Fig. 3). The reduction in HDAC2 activity in macrophages is correlated with increased secretion of cytokines like TNF-α and IL-8 and reduced response to corticosteroids. The reduction of HDAC2 activity in COPD patients may be mediated through oxidative stress and peroxynitrite formation (79).

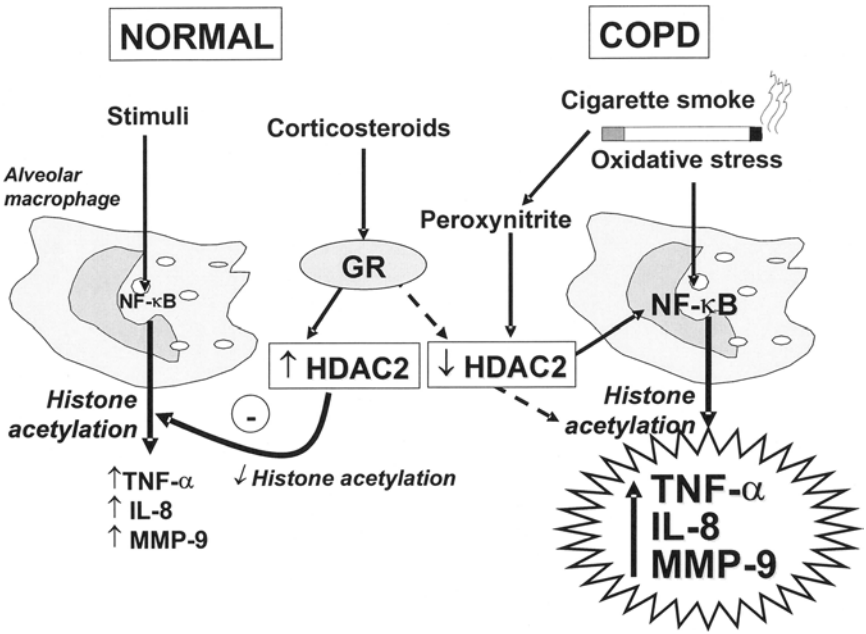

Figure 3 Stimulation of normal alveolar macrophages activates nuclear factor-κB (NF-κB) and other transcription factors to switch on histone acetyltransferase leading to histone acetylation and subsequently to transcription of genes encoding inflammatory proteins, such as tumour necrosis factor-α (TNF-α), interleukin-8 (IL-8), and matrix metalloproteinase-9 (MMP-9). Corticosteroids reverse this by binding to glucocorticoid receptors (GR) and recruiting histone deacetylase-2 (HDAC2). This reverses the histone acetylation induced by NF-κB and switches off the activated inflammatory genes. In COPD patients, cigarette smoke activates macrophages, as in normal subjects, but oxidative stress (perhaps acting through the formation of peroxynitrite) impairs the activity of HDAC2. This amplifies the inflammatory response to NF-κB activation, but also reduces the anti-inflammatory effect of corticosteroids as HDAC2 is now unable to reverse histone acetylation.

B. Theophylline

Theophylline in low concentrations increases HDAC activity in alveolar macrophages and in vitro reverses the steroid resistance induced by oxidative stress (80). This may account for the anti-inflammatory effect of low-dose theophylline seen in COPD, in marked contrast to the resistance to corticosteroids (81). This suggests that theophylline has the potential to even unlock the resistance to corticosteroids in the treatment of COPD (82). In higher concentrations, theophylline has an inhibitory effect on alveolar macrophages through phosphodiesterase (PDE) inhibition (83).

C. Phosphodiesterase Inhibitors

Several PDEs are expressed in alveolar macrophages, suggesting that PDE inhibitors may have inhibitory effects on macrophage function. Alveolar macrophages express PDE 3, 4, and 7A (84). Studies with inhibitors demonstrate that both PDE3 and PDE4 inhibitors suppress macrophage activity, but PDE4 inhibitors are less effective than in monocytes (83). A clinical trial of a PDE4 inhibitor cilomilast has shown a reduction in the numbers of macrophages (CD68+ cells) in the airways of COPD patients, presumably through inhibition of monocyte recruitment into the lungs (85).

D. Resveratrol

Resveratrol, a flavenoid found in red wine, is an effective inhibitor of cytokine expression from macrophages from COPD patients, but its molecular mechanisms of action have not yet been determined (86). Resveratrol appears to inhibit NF-κB and AP-1, suggesting that it inhibits the activation of these transcription factors and thus the expression of multiple inflammatory genes, possibly through the activation of an unknown nuclear receptor.

VIII. PHAGOCYTIC FUNCTION

Macrophages are phagocytic for particles and bacteria and play an important role in host defense. The receptors on macrophages that mediate phagocytosis are largely unknown (87). The macrophage scavenger receptor MARCO appears to play an important role in phagocytosis of bacteria, but its role in COPD has not yet been explored. The phagocytic potential of macrophages from COPD patients has not been explored, but it is possible that impaired phagocytosis may result in the increased bacterial load in the respiratory tract of patients with COPD.

Macrophages recognize apoptotic cells via expression of phosphatidylserine (PS) which interacts with specific receptors on the macrophage surface (88). Ingestion of apoptotic granulocytes by macrophages induces the secretion of TGF-β1 (89). Neutrophil elastase cleaves the PS receptor

and may thus impair the ability of macrophages to take up apoptotic neutrophils, resulting in increased numbers of apoptotic neutrophils in the airways (90). Alveolar macrophages from COPD patients show defective phagocytosis of apoptotic airway epithelial cells (91).

IX. CONCLUSIONS

Macrophages are multipotential inflammatory cells that play a key role in the defense of the respiratory tract. Alveolar macrophages are activated by irritants such as cigarette smoke, leading to a low-grade inflammatory response through the release of neutrophil, monocyte, and T-lymphocyte chemotactic factors. In COPD, this inflammatory response appears to be greatly amplified, resulting in excessive inflammation leads to fibrosis of small airways and release of excessive proteases resulting in destruction of the lung parenchyma. The molecular basis for the amplification of the inflammatory response of macrophages remains to be determined, but may involve impairment of HDAC2 activity as a result of oxidative and nitrative stress. It is likely that genetic factors are important in determining this amplification, although the specific genes involved have not yet been identified. The alveolar macrophages are resistant to inhibition by corticosteroids, reflecting the poor response to this treatment in COPD patients. However, other treatments, such as theophylline, PDE inhibitors and in the future resveratrol and IKK-2 inhibitors may be more effective. Targeting alveolar macrophages is a logical approach, since these cells appear to play such a critical role in orchestrating the chronic inflammatory process in COPD (92,93).

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7

Neutrophils

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I. INTRODUCTION

There is a wealth of data, supporting the neutrophil as the primary effector cell in COPD and that neutrophil proteinases, especially neutrophil elastase (NE), are responsible for the main pathological features. Studies have shown that smokers have increased number of neutrophils in lung tissue (1), sputum (2,3), and bronchoalveolar lavage fluid (BALF) (4). Gas transfer (the most direct physiological measure of emphysema) has been shown to be inversely proportional to the levels of neutrophil-associated markers, such as myeloperoxidase (MPO) and human neutrophil lipocalin in patients with COPD (5). Neutrophils from patients with COPD have increased chemotactic response and proteolytic activity (6) and express more adhesion molecules (7) compared with controls. Both clinical and subclinical emphysema (noted on HRCT) is associated with an increase in NE and other neutrophil proteins in BALF (8,9) and in established emphysema; severity is proportional to NE immunoreactivity in tissue (10) and enzyme activity in BALF (11). Furthermore, neutrophil enzymes have been shown to cause all components of COPD, including emphysema mucous gland hyperplasia and mucus hyper secretion (Table 1), and NE levels decline with smoking cessation (12), the only intervention to date proven to slow progression of the disease.

The proteinase/antiproteinase theory has dominated research in COPD. This states that in health, proteinase activity is limited by

Table 1 The Important Effects of NE in COPD

Cellular interactions	
Neutrophils and macrophages	NE/α_1-AT complexes are chemotactic for neutrophils (193)
	NE/α_1-AT complexes increase α_1-AT secretion by monocytes and alveolar macrophages (194)
	NE increases LTB4 secretion by macrophages (159)
	Increases α_1-AT expression by monocytes and alveolar macrophages (194)
Epithelial cells	Disruption and detachment (167)
	Reduces ciliary beating (163)
	Enhances oxidative stress (165)
	Induces apoptosis (166)
	Enhances production of CXCL8 (195)
	Increase SLPI expression but reduces secretion of SLPI (196)
	Increase elafin expression (197)
	Increase mucin MUC5AC protein content (198)
	Increases bacterial adherence and colonization (199)
Endothelial cells	Induces detachment and apoptosis (168)
Bacteria	NE mediates bacterial killing (207)
Degradation	
	Degrades elastin, fibronectin, and other matrix compounds (17)
	Degrades T lymphocyte surface antigen (200)
	Degrades Cystatin C (201)
	Degrades TIMPs (188)
Activation	
	Activates secreted MMP-2, MMP-2, MMP-3, and MMP-9 (189,202)
	Activates Cathepsin B (201)

antiproteinases such as α_1-antitrypsin (α_1-AT) and secretory leucocyte proteinase inhibitor (SLPI), thereby limiting tissue damage (see Table 2 for antiproteinases important in COPD). In COPD, an imbalance exists, either from the increased activity of proteinases or from a deficiency in antiproteinases, which leads to tissue destruction. The theory originated from the observation that patients with α_1-AT deficiency (α_1-ATD) were predisposed towards the early onset of emphysema (13) and that enzymes normally inhibited by α_1-AT can cause experimental emphysema (see the later section).

This chapter will summarize neutrophil maturation and structure, along with the pulmonary chemoattractants. Neutrophil migration into lung will be outlined, with particular reference to COPD, and the evidence for the actions of neutrophil proteinases in the pathogenesis of COPD will be reviewed, highlighting in vitro and in vivo work.

Table 2 The Inhibitors of Neutrophil Proteinases

α_1-AT	Produced by liver and found in serum. Irreversibly inhibits free NE, CG, and PR3 (203)
SLPI	Produced by mucosal cells. Reversibly inhibits NE and CG (204)
α_2-Macroglobulin	Serum. Irreversibly inhibits all classes of proteinases (205)
Elafin	Produced from mucosal cells. Reversibly inhibits NE and PR3 (204)
Monocyte/NE inhibitor	Produced from neutrophils and monocytes irreversibly inhibits NE, CG, and PR3 (206)

II. NEUTROPHIL STRUCTURE AND DEVELOPMENT

The neutrophil originates from bone marrow where it develops from a bipotential progenitor cell, the granulocyte-macrophage colony-forming unit. Neutrophils have a characteristic multilobed nucleus and abundant storage granules in their cytoplasm. The mature neutrophil has three chemically distinct granule types, which appear at different stages of maturation. The azurophilic (or primary) granules are formed early during the promyelocyte stage and contain MPO, antibacterial proteins (such as defensins, lysozyme, and azurocidin), and serine proteinases such as NE, cathepsin G (CG) and proteinase 3 (PR3) (14). The proteinases are produced as preproenzymes, with gene expression stopping at the metamyelocyte stage (15), and are activated by a lysosomal cysteine proteinase, dipeptidyl peptidase (16). The specific (or secondary) granules are formed later, during the myelocyte stage and contain lysozyme, lactoferrin, and various membrane receptors. The gelatinase-containing granules are formed last, at the metamyelocyte stage, and contain metalloproteinases.

These proteins provide the mechanisms of cell migration, opsonophagocytosis, and a formidable arsenal against pathogens to enable clearance of cellular debris. The neutrophil proteinases (especially NE) also have the potential to be intensely destructive and are able to degrade structural lung proteins (acting as elastases, collagenases, or gelatinases) and are involved in posttranslational processing of other enzymes, cytokines, and receptors (17).

Neutrophils leave the bone marrow fully matured and once activated migrate into the connective tissue. Once in the tissue, apoptosis occurs on average 8 hr after leaving the bone marrow (18).

III. PROMIGRATORY STIMULI

Neutrophils migrate into the lung in response to soluble mediators. Promigratory stimuli can be classified as nonchemotactic cytokines, chemotactic cytokines, or chemoattractants.

A. Cytokines

Cytokines are mediators of short-range signals between cells, and they control growth, differentiation, effector function, and survival of cells. Cytokines comprise of families of molecules including interleukins, lymphokines, monokines, growth factors, interferons, and chemokines. Two of the most important proadhesive cytokines in COPD are tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β).

1. Tumor Necrosis Factor α

Tumor necrosis factor α is mainly produced by activated macrophages [although it can be produced by activated T cells (19), mitogen stimulated B cells (20), natural killer cells (21), and airway epithelial cells (22)]. It is normally synthesized as a 26-kDa precursor (pro-TNF-α), which is stored in a membrane-bound form. With the appropriate stimuli [e.g., bacterial lipopolysaccharide (LPS)], the precursor is converted to TNF-α, a 17 kDa biologically active form. Pro-TNF-α is converted to active TNF-α by a membrane-bound metalloproteinase called TNF-α converting enzyme (TACE) (23) although other matrix metalloproteinases (MMPs) also have a greater or lesser degree of TNF-α converting potential (23). In vitro metalloproteinase 12 (MMP-12) has been shown to release active TNF-α from a synthetic proform, and animal models suggest that (at least in mice) active TNF-α release from macrophages, after acute smoke exposure, is dependent upon both TACE and MMP-12 (24). Tumor necrosis factor α upregulates the expression of adhesion molecules on neutrophils and endothelial cells, but is not chemotactic for neutrophils.

Animal models utilizing acute smoke exposure have suggested that macrophages (as well as their metalloproteinases) are needed for the production of TNF-α which in turn is required for neutrophil recruitment into the lungs, activation of endothelial cells, adherence to endothelium, and migration of neutrophils.

Recently, there has been much interest in polymorphisms of the TNF-α gene in the pathogenesis of COPD, especially TNF-308, the biallelic polymorphism located in the first intron of the lymphotoxin-α gene and exons 1 and 6 of the TNF receptor 1 and 2 genes. These polymorphisms have been associated with enhanced TNF transcription and therefore production of greater concentrations of TNF-α than controls (25) following activation. An Irish study has suggested that homozygosity of the 308 polymorphism is associated with more severe airflow obstruction and a worse prognosis in COPD (26), and the same polymorphism correlates with extent of emphysema on high resolution CT scan in a Japanese cohort (27). However, an Italian study of 63 Caucasian patients with COPD found no increase in prevalence of TNF-308 and TNF receptor gene 1 or 2 compared with healthy controls (28). This discrepancy may reflect ethnic variations in

the pathogenesis of COPD or may highlight that a combination of predisposing factors is required to develop the disease. This observation is clearly worthy of further studies.

2. Interleukin-1β

The macrophage/monocyte is also the primary cellular source of IL-1β, which, like TNF-α, binds to neutrophils and increases expression of adhesion molecules without being directly chemotactic. IL-1β also stimulates expression of metalloproteinases (29). Again, like TNF, increased levels of IL-1β have been found in sputum of patients with stable COPD, which increase further during exacerbations (30). Furthermore, IL-1β production is enhanced by cells cultured from smokers with COPD following cigarette-smoke exposure compared with controls (31). Enhanced production of IL-1β in patients with COPD would lead to greater expression of neutrophil adhesion molecules, thereby recruiting more neutrophils into the interstitium.

Recently, the role of IL-1β in the development of emphysema has been studied comparing an IL-1β type 1 receptor knock-out mouse with double TNF-α receptor deficient, combined IL-β and TNF-α receptor deficient, and wild-type mice after intratracheal instillation of porcine pancreatic elastase. In all cases emphysema was noted after 24 hr of instillation, but continued to progress for more than 10 days after clearance of the elastase.

After 21 days, the emphysema seemed less in all knock-out mice compared with the wild strain. However, only the TNF-α receptor deficient and combined IL-1β and TNF-α receptor deficient mice showed a significant reduction in emphysema compared with the wild strain and also the single knock-out animals. The authors suggest that 27% of total emphysema seen could be related to the IL-1β type 1 receptor, 36% to TNF-α type 1 and 2 receptors, and 81% due to combination of the two receptor groups (18% more than one would expect if the results are simply additive). Using their mathematical model, only 20% of emphysema was due to the initial elastase insult, whereas 80% was due to the subsequent inflammatory process (32). This animal model suggests that both IL-1β and TNF-α are important in the pathogenesis of COPD, but the exact relationship between these important proteins is still unknown.

B. Chemoattractants

Neutrophils possess unique receptors for at least five chemotactic stimuli including leukotriene B4 (LTB4), complement protein C5a, platelet-activating factor (PAF), bacterial peptides, including formyl-methionyl-leucyl-phenylalanine (fMLP), and a variety of other chemokines.

1. Leukotriene B4

Leukotriene B4 is mainly produced by monocytes, alveolar macrophages, and activated neutrophils and is derived from cell membranes. Cytosolic

phospholipase A2 catalyses the hydrolysis and release of arachidonic acid and then undergoes further catalysis, utilizing 5-lipoxygenase and 5-lipoxygenase activating protein, leading to the formation of the unstable oxidation product Leukotriene A4 (LTA4). Leukotriene A4 is then converted to LTB4 by LTA4 hydrolase (33). The formation and release of LTB4 is upregulated by a number of inflammatory mediators including C5a, IL-1β, TNF-α, granulocyte-macrophage colony stimulating factor, PAF, NE, and LTB4 itself (34,35).

Leukotriene B4 is a potent neutrophil activator that acts by enhancing neutrophil aggregation and chemotaxis (36) via by two neutrophil surface receptors. A low-affinity receptor induces degranulation and increases oxidative metabolism, whereas a high-affinity receptor induces aggregation, chemokinesis, and adhesion via the integrin Mac-1 (37). Leukotriene B4 may also activate endothelial cell monolayers in vitro enhancing neutrophil emigration (38), but the mechanism for this is poorly understood. Recent in vitro studies have suggested that LTB4 is responsible for up to 45% of the chemotactic activity of sputum from patients with COPD (39) and may inhibit neutrophil apoptosis, once the cells reach the lung (40). Leukotriene B4 is downregulated by a negative feedback loop whereby LTB4 enhances production of degradative enzymes (35) and is inactivated by oxidation in myeloid cells and hepatocytes, rendering it biologically inactive (41).

Levels of LTB4 have been found to be elevated in sputum (42) and exhaled breath condensate (43) from patients with COPD. Concentrations correlate with the degree of airway neutrophilia (44) and increase further during exacerbations (45) with a corresponding drop once bacteria have been successfully eradicated (46). These results suggest strongly that this chemoattractant plays an important role in the pathogenesis of COPD.

2. C5a

C5a is derived from cleavage of complement protein C5 when the classical complement cascade is activated. Once formed, it can bind immediately to neutrophils in the circulation (47) and acts as a potent chemoattractant. C5 is also produced by tissue macrophages and type II pneumocytes in the lung (48) as a component of the alternative complement cascade. The two pathways promote an inflammatory gradient enhancing subsequent migration. C5a has been shown to enhance adhesion molecule expression [especially intercellular adhesion molecule 1 (ICAM-1)] in airway epithelial cells, and interestingly, this effect was exaggerated in the presence of cigarette smoke (49). There have been few in vivo studies monitoring C5a levels in COPD, and to date, levels have not appeared elevated in COPD patients (50).

3. Platelet-Activating Factor

Platelet-activating factor is produced by endothelial cells, macrophages, neutrophils, and platelets during inflammatory events. It enhances neutrophil

adhesion and acts as a chemoattractant (51), but there have been no studies specifically examining this chemoattractant in COPD.

4. Formyl-methionyl-leucyl-phenylalanine

The tripeptide fMLP is present at the amino termini of proteins from most types of bacteria, but is not found in proteins of human origin. It is released during bacterial degradation and binds to seven transmembrane-spanning G-protein coupled receptors on neutrophils and monocytes. The fMLP-receptor interactions activate multiple transduction pathways enhancing neutrophil adhesion and chemotaxis [via cytoskeletal changes (52)], exocytosis of secretory granules and superoxide anion production (for a review, see Ref. 53). Formyl-methionyl-leucyl-phenylalanine is a powerful chemoattractant, resulting in cell migration and degranulation and therefore potentially increased tissue disruption. The fMLP receptors are expressed on quiescent neutrophils, but expression is increased once they are activated, following the mobilization of secretory vesicles (54). A recent study compared fMLP receptor numbers on peripheral neutrophils from subjects with COPD, healthy smokers, and healthy nonsmokers. Levels of receptors were elevated in both healthy smokers and subjects with COPD who smoked, but not nonsmoking patients with COPD (55), which suggest that fMLP may play a role in the development of disease and its progression during smoking, especially during bacterial exacerbations.

5. Chemokines

Chemokines are a family of approximately 40 structurally related soluble cytokines that exhibit chemotactic activity for a limited spectrum of leukocytes. Chemokines are low molecular weight proteins with cysteine at well-conserved positions that exhibit a basic charge and have an affinity with heparin (56), and they bind to cell-surface G-protein coupled receptors with seven transmembrane domains (56,57). Chemokines also bind to proteoglycans on vascular endothelium and to extracellular matrix proteins in the tissues forming an immobilized gradient of chemokine concentration that begins at the venular endothelium, rises through the tissues, and peaks at the site of injury.

Chemokines are classified by the sequence of NH_2-proximal cysteines. There are four subgroups: C, CC, CXC, and CX_3C chemokines, where "X" is any amino acid placed between the cysteine groups. CXC chemokines are further divided depending on the presence (ELR^+) or the absence (ELR^-) of the tripeptide motif Glu-Leu-Arg at the NH^2 terminus before the first cysteine (57,58). ELR^+ CXC chemokines bind both CXC receptors 1 and 2 (CXCR1, CXCR2) with high affinity and are potently chemotactic for neutrophils and exhibit angiogenic activity. Interleukin-8 (now called CXCL8) is the most important $(ELR)^+$ CXC chemokine in the development of COPD.

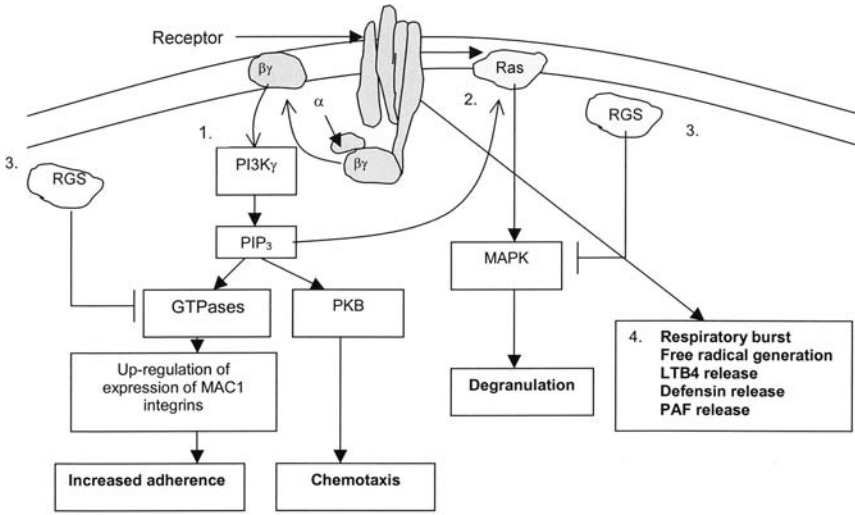
CXCL8 is produced by both leukocytes (monocytes, T cells, neutrophils, and natural killer cells) and nonleukocytes [endothelial cells, fibroblasts (56), and epithelial cells]. Production is not constitutive but is induced by proinflammatory cytokines such as IL-1β and TNF-α (59), bacteria such as *Pseudomonas aeruginosa* (60), bacterial products such as LPS (22), viruses such as adenovirus and rhinovirus (61,62), and oxidants from cigarette smoke (63). CXCL8 is produced as a precursor protein and is processed into its active form by proteinases released from CXCL8-secreting cells, predominantly the neutrophil (64).

Once secreted, CXCL8 binds to CXC receptors (1 and 2) on leukocytes. The G-protein receptor is then converted to the GTP-bound form and dissociates into Gα and G$\beta\gamma$ subunits. The G$\beta\gamma$ subunit initiates a phosphate pathway resulting in activation of protein kinase B and GTPases, which leads to enhanced neutrophil adherence to endothelial cells (by increasing expression of β_2 integrins) and directed cell migration (Fig. 1). CXCL8 also activates Ras and eventually mitogen-activated protein kinases and extracellular signal-related kinases in neutrophils, causing degranulation. These effects can be downregulated by intracellular regulator of G-protein signaling (RGS) proteins that decrease the half life of the active GTP-bound state of CXCR, leading to reduced CXCL8-induced neutrophil migration and adherence (65).

In COPD, sputum CXCL8 correlates with levels of neutrophil activation markers such as MPO and NE (66) and is proportional to airflow obstruction (67). It is believed that oxidative stress caused by cigarette smoke and bacterial and viral infections induce CXCL8 production in both airway epithelial and endothelial cells, leading to neutrophil adhesion, chemotaxis, and degranulation.

In addition to CXCL8, at least five other CXC chemokines mediate neutrophil responses in humans and are believed to be important in COPD; three forms of growth-related oncogenes (GRO) (α, β, and γ), epithelial cell derived neutrophil-activating peptide (ENA)-78, and neutrophil-activated peptide-2. GROα is detectable in bronchial secretions (68), and both GROα and ENA-78 have been measured in BAL fluid (69). Neutrophil-activated peptide-2 is produced following stimulation of pulmonary microvascular endothelial cells (70) although it has not yet been identified in human alveolar or bronchial fluid. A number of other inflammatory mediators including fragments of fibrin, elastin, and collagen are also neutrophil chemoattractants in the lung.

As stated, studies have demonstrated high levels of CXCL8, LTB4, and GROα in bronchial secretions from patients with COPD (66,68,71). However, their presence does not necessarily mean they play a central role in neutrophil migration in this disease. The contribution of CXCL8 and LTB4 has been demonstrated in vitro studies utilizing IL-8 monoclonal antibodies and LTB4 receptor antagonists. Here, neutrophil chemotaxis



1. After CXCL8 binds to the CXC receptor, receptor coupled G proteins are activated and dissociate into Gα and Gβγ subunits. Gβγ remains near the receptor complex and activates phosphatidylinositol 3-kinase γ (PI3K-γ) which generates phosphatidylinositol 3,4,5-triphosphate (PIP_3). PIP_3 activates protein kinase B (PKB) which enhances migration (perhaps by actin polymerisation) and GTPases which cause up regulation of adhesion molecules, enhancing migration.
2. CXCL8 activates Ras which activates mitogen-activated protein kinases (MAPK) leading to degranulation.
3. Regulator of G protein signalling proteins (RGS) down-regulate G-protein associated signals by decreasing the half life of the active GTP-bound state. This reduces CXCL8 driven adherence and degranulation.
4. CXCL8 also enhances the respiratory burst, increases LTB4 release via 5-lipoxygenase, induces PAF synthesis and defensins release in neutrophils

Figure 1 The presumed signaling mechanisms of CXCL8.

towards sputum was reduced by 30% and 50%, although these effects were not completely additive (71,72). Studies have not yet assessed the contribution of other potential chemoattractants such as GROα or C5a.

IV. EPITHELIAL/ENDOTHELIAL CELLS AND NEUTROPHIL CHEMOTAXIS

A. Neutrophil Migration into the Lung

Most of what we understand of neutrophil migration has been elucidated from studies in vitro, using systemic vessels in the mesentery and dermis. Recent work in lung suggests that although migration in postcapillary venules occurs in a similar fashion to migration in other tissues, migration in the pulmonary capillaries may depend on other mechanisms. In larger

vessels, neutrophils migrate from vessel to tissue via a step-like process, dictated by the sequential activation of adhesive proteins and their ligands on neutrophils and endothelial cells.

1. Initiation of Neutrophil Capture

Initiation of migration begins with the "capture" of the neutrophil from flowing blood by the endothelium. The initial tethering and rolling of the neutrophil along the vessel wall is due to reversible binding of transmembrane glycoprotein adhesive molecules called "selectins," which are found on both neutrophils and endothelial cells (52). Selectins have a short intracellular component that is linked to signal transduction proteins, and a longer extra-cellular component that consists of a calcium-dependent lectin domain attached to an epidermal growth factor-like domain and then to a number of short consensus sequences (reviewed in Ref. 73).

2. Leukocyte Selectin

Leukocyte selectin (L-selectin) is constitutively expressed on neutrophils. Greatest expression is seen on young cells released from bone marrow with continued circulation resulting in a gradual reduction (74). L-selectin initiates neutrophil capture and its importance has been demonstrated in vitro where capture was inhibited on noninflamed tissue by the use of L-selectin blocking antibodies (75). It binds an as yet uncharacterized endothelial ligand which is thought to be a sialomucin oligosaccharide and is constitutively expressed, with increased expression induced by inflammatory cytokines and LPS. Neutrophil capture can be transient in noninflamed tissue ("stick and release"), and during both nonpathogenic and pathogenic rolling interactions, L-selectin is shed from neutrophils (76). Both L-selectin binding and shedding are enhanced in the presence of inflammatory products such as TNF-α, CXCL8, fMLP, and LPS (77). Once bound and cleaved, L-selectin molecules cannot be replaced and low expression has been associated with neutrophilic apoptosis that may form a signal for the removal of old neutrophils from the circulation (74).

B. Neutrophil Capture in Inflammation

1. Selectins

Inflammation causes the expression of at least two endothelial bound selectins: platelet selectin (P-selectin) and endothelial selectin (E-selectin). P-selectin is stored intracellularly in Weibal-Palade bodies in endothelial cells and in α-granules in platelets (78). The P-selectin expression is induced by various inflammatory mediators including oxygen free radicals, components of the complement cascade and many cytokines (52), and binds to P-selectin glycoprotein ligand-1 (PSGL1). P-selectin glycoprotein ligand-1 is a homodimer and binds two P-selectin ligands simultaneously (79). It is

uniformly expressed on neutrophils, and binding occurs after and is more long lived than L-selectin–ligand interactions. In the presence of other adhesion molecules, P-selectin/PSGL1 binding slows neutrophil rolling velocities and eventually causes cell tethering to the endothelium, but in the absence of other adhesive events, binding is also transient (80).

E-selectin is not stored, but requires gene transcription for expression with peak expression occuring 4–6 hr after initiation of inflammation (81). In animal studies, E-selectin binds to E-selectin ligand 1, although the human ligand remains uncharacterized (52). It is thought to maintain neutrophil tethering after P-selectin has been down regulated. E-selectin expression is increased in both serum and BAL in patients with COPD and correlates significantly with lung function (82).

2. Firm Adhesion

Firm adhesion is thought to be possible only in the presence of inflammatory stimuli. Once L-selectin and PSGL1 are bound and activated, signal transduction pathways lead to the sequential activation of integrins, through which firm adhesion occurs (73,83).

The integrins are heterodimeric transmembrane glycoproteins that comprise an α and β subunit, which together form an extracellular binding site. Integrins are found on many hematopoietic cells, with differing α and β subunits. The two most important integrins in neutrophil migration through endothelial cells share a β_2 subunit (CD18), these are macrophage antigen 1 (MAC1; CD11b/CD18) and lymphocyte-associated function antigen 1 (LFA-1; CD11a/CD18), with MAC1 being of the greatest importance. A third CD18 integrin, p150,95, can also promote neutrophil trafficking, but has been less well studied.

3. MAC1

Preformed MAC1 is stored in PMNs in secretory vesicles and specific and gelatinase granules (54,84) and is rapidly mobilized to the cell surface after exposure to inflammatory stimuli (including fMLP, TNF-α, and LPS). Inflammatory stimuli also promote transcription and translation of the MAC1 gene via a G-protein Rho (85), increasing expression during inflammation (52). Some MAC1 are expressed constitutively on the neutrophil, but these proteins are incapable of binding ligands unless activated by intra- or extracellular signals, where conformational changes occur exposing a requisite binding epitope (86). This is an important consideration in experimental work, as measured MAC1 does not necessarily equate to functionally active MAC1. However, neutrophils from patients with COPD have increased baseline surface expression of MAC1, which increase further following activation (7), suggesting that this adhesion molecule may play a role in the pathogenesis of the disease.

The main ligand for MAC1 is ICAM1, an immunoglobulin-like protein that is usually expressed in low numbers on the endothelial cell surface, but is rapidly induced in the presence of inflammation (87). Vascular cell adhesion molecule-1 (VCAM-1) is also an immunoglobulin-like molecule expressed by endothelial cells. It binds selectively to β_1 integrins and, in particular, to $\alpha_4\beta_1$ integrin [called very late antigen (VLA)-4] on neutrophils (88). MAC1/ICAM1 interactions cause increased expression of both ICAM1 and VCAM1 on endothelial cells, suggesting that both may be important in inflammatory driven neutrophil migration (89), and levels of ICAM1 are raised significantly in COPD in contrast to asthma or healthy controls and correlate with overall pulmonary neutrophil infiltration (90).

V. MIGRATION

Once the selectins and integrins have caused rolling and firm adhesion, migration begins. This occurs preferentially at tricellular junctions (91) and depends upon activation of platelet endothelial cell adhesion molecule (PECAM1) (92) which is distributed both evenly around the neutrophil and especially at intercellular junctions of endothelial cells. PECAM1 is thought to act as a homing beacon that directs migration towards cellular junctions, and blocking PECAM1 on either neutrophils or endothelial cells using antibodies does not prevent adhesion but does prevent migration through the basement membrane both in vitro and in vivo including a PECAM1 knock-out mouse model (93–95).

Once through the endothelial cell layer, differing mechanisms may allow diapedesis through the basement membrane and extracellular matrix. Leukocytes bind to matrix components such as collagen and laminin via β_1 integrins, and a series of β_1 integrins have been found to be important in allowing neutrophils to move through venule basement membrane and lung tissue, with VLA-6 and -9 being perhaps the most important (96–98). It is believed that endothelial/neutrophil PECAM1 interactions lead to increased neutrophil surface expression of VLA-6 ($\alpha_6\beta_1$) (which is stored intracellularly when cells are quiescent) and VLA-6 facilitates passage through the basement membrane and beyond. To support this, neutrophils from PECAM1 knock-out mice (which display poor migration following stimulation with IL-1β) also do not have the associated rise in VLA-6, which is seen in the wild type (95). Very late antigen-6 binds laminin (which forms a large component of the perivascular basement membrane) and VLA-6/laminin interactions may play a role in neutrophilic migration through this tough interwoven layer (96). Subendothelial transmigration is accompanied by release of neutrophil proteinases especially NE (99), which may facilitate passage by matrix degradation, exposing laminin for VLA-6 binding. Selectin/integrin interactions are summarized in Fig. 2.

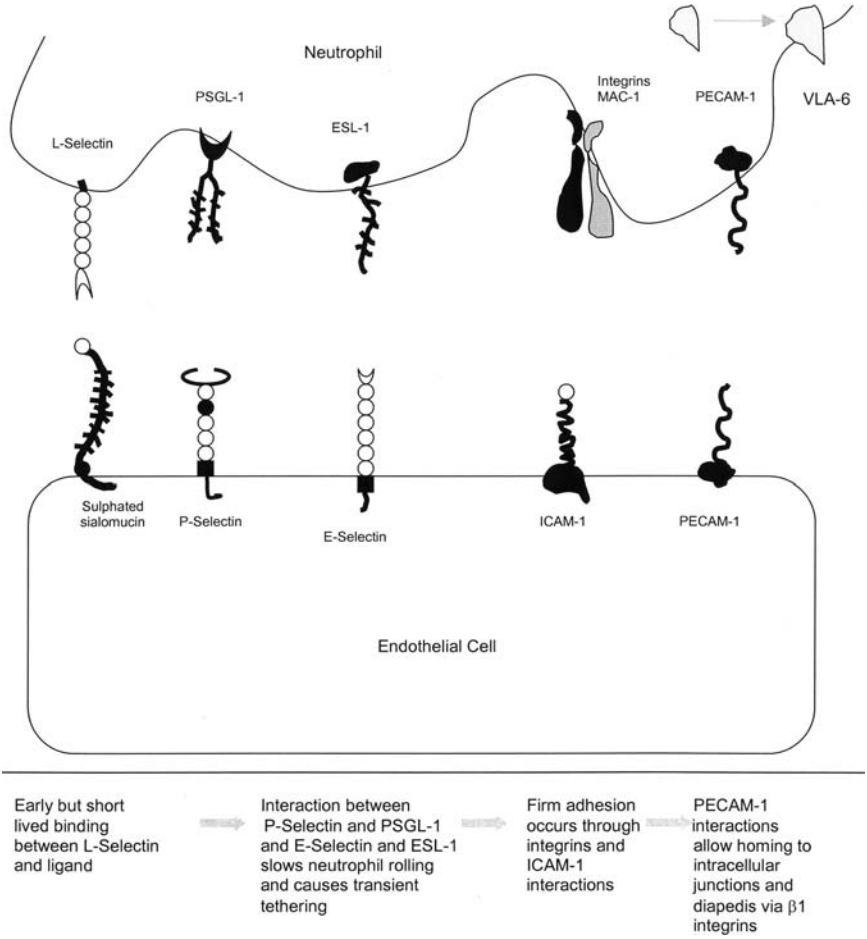


Figure 2 Neutrophil and endothelial cell adhesion molecules.

A. Direction of Migration

Neutrophils migrating within the lung encounter multiple chemoattractant signals in complex spatial and temporal patterns as endothelial, epithelial cells, and immune cells respond to infection or injury.

Most of our understanding about migration is from in vitro models and it has become clear that neutrophils can migrate both up and down chemical gradients, responding to one chemoattractant, migrating to its concentration peak and then migrating up a novel, more distant chemoattractant gradient, from endothelium to tissue. Once a concentration gradient has been traversed, neutrophils can ignore a high-concentration source (due to receptor saturation, desensitization and/or receptor sequestration)

and indeed, if chemoattractants are present in high enough concentrations, leukocytes lose their ability to orientate, but still remain able to respond to novel chemoattractants (100). Migrational models under agarose have suggested that neutrophils are able to respond as if to the vector sum of two or more different signals, integrating directional signals from chemoattractant sources (101). If two sources of the same agonist are used, migration towards the second attractant is poor (most probably due to receptor saturation, desensitization and/or receptor sequestration) (100); therefore, two distinct agonists are required for targeting specificity. These findings may account for the observation that activated cells characteristically secrete multiple induced chemoattractants concurrently. Cells can regain their prior sensitivity, but this process takes time, requiring recycling of receptors. For example, neutrophils, preincubated with low chemotactic levels of LTB4, showed a significant reduction in chemotaxis to LTB4, which improved after 10 min (100) (Fig. 3).

B. The Migrational "Stop Signal"

How cells remain at a specific target is less well understood. Studies have demonstrated that exposing the leukocytes to some chemoattractants diminishes their ability to respond to others (102,103), and a hierarchy of chemoattractants and their receptors has been suggested. Responses to CXCL8 and LTB4 can be suppressed in the presence of even low concentrations of C5a and fMLP (102). Once stimulated, fMLP and C5a receptors induce phosphorylation of the CXCL8 receptor causing functional uncoupling from its signaling apparatus, rendering it inactive (104,105). This prevents neutrophils from migrating away from the source of C5a or fMLP towards other chemoattractants (100), which is logical, as these molecules define some physiologic end targets for neutrophils, because bacteria produce formyl peptides and immune complexes that fix complement generate C5a.

C. Transmigration

Neutrophil proteinases are released during migration through extracellular matrix (99), but it has been difficult to ascertain whether proteinases are necessary for neutrophil migration. However, chemotaxis through artificial substrates in response to fMLP can be inhibited by 50% by α_1-AT (106) and in similar assays, CG antibodies, synthetic inhibitors of CG, α_1-AT, and α_1-antichymotrypsin (ACT), also reduce neutrophil migration (107). In addition, fMLP-stimulated migration across an artificial basement membrane is also reduced by inhibitors of both NE and MMP-9 (108).

On the other hand, it has been shown consistently that proteinase inhibitors are ineffective at stopping neutrophil migration through intact endothelial cell monolayers and basement membrane matrices in vitro (109,110) although they reduce degradation of basement membrane

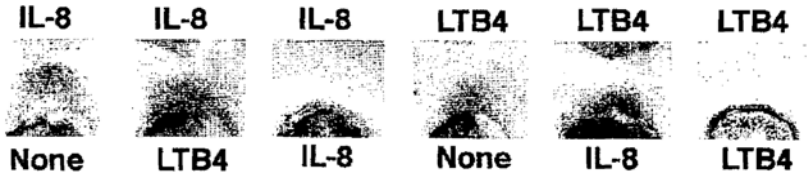


Figure 3 Integrating chemotactic signals. Neutrophils migrate away from one chemoattractant source towards another. Photographs of the stained neutrophils after 2-hr migration towards a distant source of LTB4 or interleukin 8 referred to here as IL-8 (1 pmol) in the presence or absence of an inverse gradient generated by LTB4 or IL-8 (10 pmol). Cells placed with one agonist migrate towards the other agonist almost as well as control cells, but do not migrate well towards a distant source of the same agonist. (Reproduced from Ref. 100 with kind permission.)

components (111). Furthermore, neutrophils from mice whose genes for NE and CG had been "knocked out" showed normal migration both in vitro and in vivo when exposed to LPS, although pathogen clearance was impaired (112) and mice deficient in gelatinase B have normal neutrophilic migration into the lungs (113). However, animal studies of cigarette smoke inhalation suggest that neutrophil influx into the lung is reduced in the presence of proteinase inhibitors. Guinea pigs exposed to cigarette smoke demonstrate an acute response composed of an airway neutrophilia and an increase in connective tissue breakdown products found in BALF, and both of these responses are reduced by pretreatment with synthetic NE inhibitors (114). Intratracheal instillation of a synthetic NE inhibitor reduced neutrophil influx triggered by elastase, but only intravenous instillation of the inhibitor prevented the increased airway neutrophilia seen in response to sputum from patients with cystic fibrosis (115).

In humans, two syndromes provide clues as to the importance of proteinases in neutrophilic migration. In Papillon–Lefevre syndrome, patients have a deficiency of dipeptidyl peptidase 1 that causes a functional deficiency of NE and CG via impaired processing of enzyme precursors. Here, neutrophil migration is impaired, but the main phenotype is periodontitis alone, with no systemic features of immunodeficiency (116). Chediak–Higashi syndrome is associated with deficiencies in NE, CG, and PR3, and here neutrophils do not migrate towards chemoattractants (117) again suggesting that proteinases play a role in cell migration.

This conflicting evidence suggests that migrational dependence on proteinases may vary according to stimuli and the scientific model used. In addition, if neutrophil migration is partially proteinase dependent, migration may not require degradation of extracellular matrix substrates by proteinases to allow cell passage, but instead the generation of inflammatory chemokines and cytokines or modulation of adhesion molecules [enhancing

expression, increasing their activation or exposing binding sites (96)], by proteinase actions and this may be decreased in the presence of inhibitors, producing the results seen earlier.

D. Migration from the Pulmonary Capillary Network

A total of 97% of neutrophils in the lungs are found in the pulmonary capillary network and in the animal models studied to date, most migration into lung tissue occurs at this site (118,119). The average capillary diameter is 6 μm and many capillaries are as small as 2 μm while the average neutrophil is 7–8 μm (120,121), and so neutrophil rolling is not thought to occur due to size constraints. Neutrophils entering the pulmonary capillary network have to undergo a shape change to allow passage through these narrow vessels, slowing their transit time (122,123). In endothelial monolayer models, the presence of LPS and C5a decrease neutrophil deformability by causing actin polymerization thereby increasing neutrophil stiffness (124) and this may favor neutrophil slowing through capillaries and migration into tissue.

Although most neutrophil migration in the systemic circulation is ICAM1/CD11CD18 dependent, both CD18 dependent and independent adhesion pathways are utilized in the pulmonary circulation, depending on the stimulus (125). IL-1, phorbol myristate acetate, and gram-negative bacteria stimuli, including LPS elicit migration via pathways, predominantly mediated by CD18 (126–129). Gram-positive bacteria, hydrochloric acid, CXCL8, and C5a induce CD18 independent pulmonary neutrophil migration. This CD11/18 independent process has been confirmed by the ability of cells from patients with leukocyte adhesion deficiency 1 (who lack these ligands) to migrate across endothelial layers to certain stimuli in vitro (130). The adhesion molecules needed for CD18 independent migration have yet to be identified. Utilization of CD18 may not only be stimulus driven, but also be inflammatory mediator driven. Rabbits produce both CXCL8 and TNF-α during a bacterial pneumonia, but CXCL8 and TNF-α production during a gram-negative pneumonia are 2- and 10-fold greater, respectively, than those seen during a gram-positive pneumonia (131). It may be the varying concentrations of these proinflammatory stimuli which elicit a CD18 dependent response (perhaps by inducing nuclear factor-$\kappa\beta$) (126) rather than the bacterial insult itself.

Selectins may still play a role in enabling neutrophil migration in the pulmonary capillaries, but their involvement will be independent of neutrophil rolling and may be situation specific, although studies have yet to provide a definitive answer. For example, rabbits treated with fucoidan (a polysaccharide that inhibits binding of all selectins) demonstrate a 25% reduction in neutrophil arrest in capillaries and a 50% reduction of the duration of the episodes (132). L-selectin knock-out mice had significantly less neutrophil recruitment into the lungs in response to LPS (a CD18 dependent

stimulus), whereas the recruitment in response to *Streptococcus pneumoniae* (a CD18 independent stimulus) was equal in both knock-out and wild-type mice (133). Similar findings have been reported in P-selectin and E-selectin knock-out mice, suggesting that these selectins are required in CD18 dependent migration (as described earlier), but that they do not play a role in CD18 independent migration. This finding has been supported further by similar work where selectins were blocked with siayl-oligosaccharides (which have a similar structure to selectin ligands). IgG immune complex injury (found to be CD18 dependent in the lung) can be prevented by treating with sialyl compounds (134).

The complexities of these processes and their site and stimulus specificity have received little attention in the study of COPD. Thus, to date, most of the information is speculative and largely derived indirectly.

E. Migration in COPD

Patients with COPD have been shown to have increased neutrophil migration towards common chemoattractants in vitro. Burnett et al. (6) and Stockley et al. (135) demonstrated an enhanced chemotactic response of neutrophils from patients with COPD compared with neutrophils from healthy controls toward fMLP, using the membrane filter chamber method, and this was thought to reflect increased receptor expression. A further potential mechanism for enhanced migration may be upregulation of adhesion molecules, but in vitro studies have shown conflicting results. Noguera et al. (7) measured MAC-1, LFA-1, and L-selectin expression on neutrophils from controls and patients with COPD, prior- and poststimulation with TNF-α. Neutrophils from patients with COPD had enhanced expression of these adhesion molecules compared with controls, and differences were even more pronounced following stimulation with chemoattractants. More recently, Woolhouse et al. (136) demonstrated upregulation of CD11b (a component of MAC1) on neutrophils from smokers with COPD compared with controls. However, Gonzalez et al. (137) found no differences between levels of adhesion molecules in smokers with and without airflow obstruction. Thus, the issue remains unresolved at present.

The increased neutrophilic migration and degranulation seen in COPD has led to a belief that neutrophils in COPD may be "primed," perhaps by inflammatory cytokines, so that they are more responsive, with an increased ability to degranulate compared with those found in healthy individuals. This priming may occur during transmigration, and studies have confirmed clear differences between neutrophils prior to and following migration in healthy controls with increased expression of proteinases on the cell surface, increased adhesion molecule expression, and enhancement of the respiratory burst (96,138), although this has not been studied in respiratory disease. Wherever it takes place, this priming cannot be caused

solely by environmental factors (such as prolonged cigarette smoke exposure), as only a proportion of smokers develop significant airflow obstruction, and so genetic factors must play a role (17). Neutrophil priming may occur only in susceptible individuals, leading to increased neutrophil pooling in the lung, increased degranulation, and therefore increased tissue damage, although it remains unclear whether this is a predisposing factor for COPD or a consequence of the disease.

VI. ACTIONS OF NEUTROPHIL PROTEASES IN VITRO AND IN VIVO

A. The Actions of NE

Neutrophil elastase was the first serine proteinases to be shown to produce emphysema in animal models and has been more extensively studied than CG or PR3. Intratracheal instillation of purified NE induced emphysema in dogs and hamsters (139–141), and intratracheal instillation of other elastases have given similar results in various animal models—Papain (plant elastase) in rats (142), neutrophil lysates in dogs (143), and porcine pancreatic elastase in rats and hamsters (144,145). In fact, the development of emphysema is specific to elastase activity with emphysema severity relating proportionally to the elastolytic potency of the elastase used (144,145), and in these models, emphysema can be prevented by specific elastase inhibitors (146,147).

Free NE activity has been detected in secretions of patients with COPD (66) and this is felt to be fundamental in the development of the condition in vivo. Once at the site of tissue damage, neutrophils degranulate, releasing enzymes into the liquid mileu or directly onto the tissue causing damage by close contact with cells and matrix. During activation, azurophil granule proteinases are expressed on the neutrophil membrane (148) and in vitro, over 95% remain associated with the cell by a charge dependent mechanism while $< 5\%$ is released (149,150). Free NE may accumulate from degranulating neutrophils or, in contrast with apoptotic cells, may be freely released during cell necrosis (151). In addition, the process of phagocytosis may cause the release of significant quantities of proteinases into the media (“sloppy eating”), especially during “frustrated phagocytosis,” when cells attempt ingestion of large particles (152). Free NE can also be released from activated macrophages, which scavenge the proteinase from apoptotic neutrophils via endocytosis and release it during the first 24 hr of their own inflammatory response (153). This is important, as while cell-associated proteinases have partial resistance to native inhibitors such as α_1-AT (148), free NE is more readily inactivated by both serum and tissue-based inhibitors (154) if sufficient quantities of inhibitors are present.

The concentration of free NE released from the granule falls exponentially from 5 mM (155). In healthy subjects, the serum concentration of

α_1-AT is 30 μm, which is at least two orders of magnitude lower than the NE concentrations in the neutrophil granule. α_1-Antitrypsin inhibits NE on a one-to-one molar basis, therefore following degranulation NE cannot be completely inhibited until it has diffused far enough to reduce its concentration to 30 μM, a phenomenon called quantum proteolysis (155). This was elegantly demonstrated in a series of experiments using serum from patients with normal or deficient α_1-AT where an area of obligate enzyme activity existed even with serum from healthy subjects but was far greater using serum from patients with α_1-ATD (156) (Fig. 4). Membrane bound NE is less susceptible to antiproteinases (148), and the combination of increased free and membrane bound NE present in COPD may be sufficient to overcome local inhibitors, causing the tissue destruction which is characteristic of the disease.

Neutrophil elastase interacts with matrix proteins and cells, and this affects not only its own activity, but also the efficacy of its inhibitors. For example, once NE is bound to elastin, it is poorly inhibited by α_1-AT, while

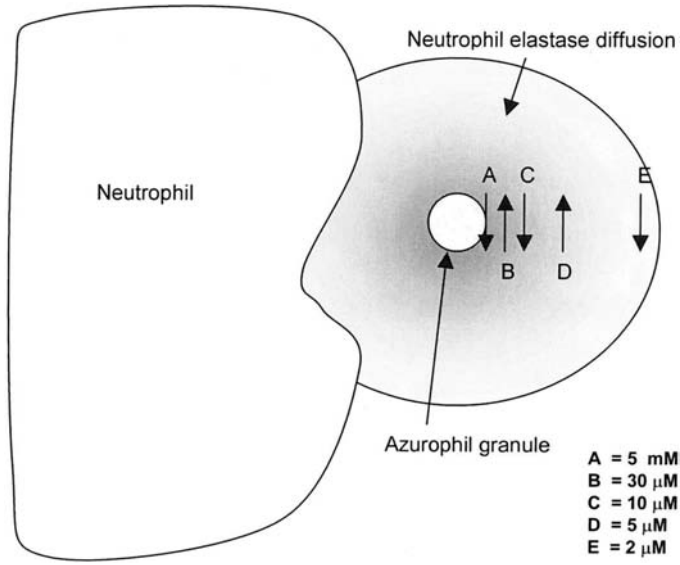


Figure 4 Neutrophil elastase diffusion following degranulation: quantum proteolysis. Once the azurophil has undergone exocytosis from a neutrophil, elastase diffuses into the extracellular mileu. The elastase concentration inside the granule is 5 mM, but this declines in an exponential manner the further away from the cell. The arrows represent the concentrations at given distances from the granule. In patients with normal levels of α_1-AT, enzyme activity would be completely blocked once the concentration was < 30 μM (B), allowing tissue damage proximal to this. In α_1-ATD, the enzyme would not be inhibited until it had diffused far away enough for the concentration to be < 5 μM (D) leading to more extensive tissue damage.

the inhibitory effect of SLPI is unaffected (157). It has also been suggested that adhesion to goblet cells may alter the neutrophil membrane, enhancing the release of membrane bound NE into the intercellular space (158), and this process may result in mucus secretion.

Neutrophil elastase may play a role in recruiting more neutrophils in response to inflammation, further enhancing the inflammatory burden. The proteinase stimulates the release of LTB4 by macrophages (159) and may cause release of CXCL8 from bronchial epithelial cells that would amplify the chemoattractant gradient and hence cellular response. This amplification is greater in patients deficient in α_1-AT as reflected by their higher levels of LTB4 and elastase and thus provides a greater potential for tissue destruction than patients with normal antiproteinase function thereby explaining their more severe and rapidly progressive disease (42).

As well as causing damage leading to emphysema, NE has been shown to cause mucus hypersecretion which is a feature of COPD. Animal models have demonstrated that NE can induce secretory cell metaplasia, which is prevented by NE specific inhibitors (158,160,161). The mechanism for these changes has not been totally clarified, but may involve the mucous gland epidermal growth factor receptor interacting with NE as part of a signaling cascade (162). In human studies, however, there is a clear relationship between the amount of mucus production and the concentration of active NE in the secretions (Fig. 5).

Neutrophil elastase can damage the respiratory epithelium in vitro (163), reduce ciliary beating (164), and trigger a state of oxidative stress in

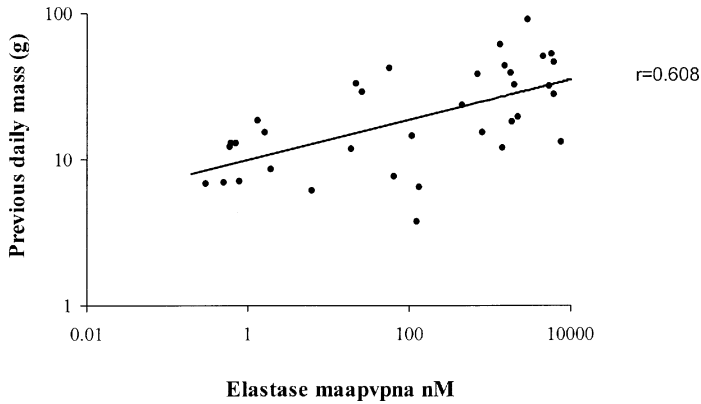


Figure 5 The relationship between sputum volume and NE activity. The active concentration of NE using a synthetic substrate, maapvpna, is shown on the horizontal axis for individual sputum samples from patients with bronchiectasis. The results are plotted against the sputum volume produced (vertical axis). The correlation coefficient of the relationship is shown ($p < 0.002$). (From Ref. 17.)

cells (165), and all of these effects are abrogated by NE inhibitors. Neutrophil elastase can also induce apoptosis of epithelial cells (166) and detachment of bronchial epithelial cells from the extracellular matrix (167), and both PR3 and NE induce detachment and apoptosis of endothelial cells (168), which have been implicated in the pathogenesis of COPD (169). The effects of NE on epithelial and endothelial cells and the extracellular matrix are, therefore, likely to be important in the pathogenesis of COPD.

Recent work has shown that NE activity relates to sputum purulence (which is due to MPO and can be graded visually) (45). During bacterial exacerbations of COPD, sputum purulence and hence neutrophil influx and NE activity increase (45), and therefore it is likely that proteinase-induced damage also increases. Indirectly, this is supported by the finding that tissue degradation product release is increased during the episodes and that the frequency of exacerbations relates to the decline in lung function (170,171).

Animal studies have also studied the effects of smoke exposure and proteinases on the lung. Neutrophil elastase knock-out mice are only partially protected (45%) against the development of emphysema (172). However, these models are limited, as CG and PR3 persist, and CG has been shown to cause secretory cell metaplasia (141) and PR3 causes both emphysema and secretory cell metaplasia (173).

If NE is important in the development of emphysema, it is reasonable to hypothesize that NE inhibitors may limit or prevent the development of the disease. Animal models have suggested that both synthetic (146) and natural NE inhibitors (147,174,175) can limit emphysema when delivered simultaneously with the elastase insult. Certainly, most animal models have supported the concept that neutrophil influx is greatest during the early stages of COPD with macrophage influx, and their metalloproteinases accumulating at a later stage (176,177). However, even when given once disease is established, NE inhibitors can still limit inflammation and connective tissue breakdown (114). Such studies clearly have implications for the design of future preventative strategies for COPD in man.

B. Matrix Metalloproteinases

Neutrophils and macrophages also produce large amount of metalloproteinases (MMPs) and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). The MMPs are proteolytic enzymes that are secreted as proenzymes (activated by other MMPs) and remain bound to cell membranes. The MMPs degrade not only matrix proteins, but also antiproteinases such as α_1-AT and α_1-ACT, activate enzymes involved in the clotting cascade, and interact with cytokines and adhesion molecules, thus leading to widespread interest in their role in the pathogenesis of COPD.

Over 24 mammalian MMPs have been described, and although they are primarily grouped according to the proteins they degrade, most can degrade all extracellular substrates. Matrix metalloproteinases are inhibited by α_2-macroglobulin, as well as the four TIMPs described to date (178). The main MMPs secreted by neutrophils are MMP-9 (gelatinase B), which degrades collagen, elastin, and gelatine, and MMP-8 (neutrophil collagenase), which degrades collagen types I–III. Only MMP-9 has been studied in COPD.

Although there are increased levels of MMP-9 in lung tissue, BALF, and plasma taken from patients with COPD (179,180), levels are negatively correlated with airflow obstruction and relate to the number of sputum neutrophils (181,182). MMP-9 not only acts as a proteinase, but may also modify cellular functions by regulating cytokines and matrix-bound growth factors and therefore may have a role in lung remodeling after inflammatory insult (183,184). MMP-9 knock-out mice did not show reduced neutrophil recruitment or degranulation after exposure to LPS (113). In fact, in one study, MMP-9 knock-out mice displayed greater neutrophil influx, perhaps because MMP-9 can degrade neutrophil chemoattractants (160). Smoke-exposed guinea pigs displayed a reduction in the severity of emphysema and MMP-9 activity in BALF after the introduction of a broad spectrum MMP inhibitor, but this may have been because of inhibition of other metalloproteinases (185). Genetic polymorphisms of MMP-9 have been identified, which cause enhanced protein expression, and in a Japanese study, polymorphism-1562C/T has been associated with an increased risk of smoking induced emphysema (186), but this has not been replicated in other communities (187).

It is likely that the serine proteinases and MMPs act synergistically in lung disease. Neutrophil elastase degrades TIMPs (188), facilitating MMP activity, and can activate several MMPs including MMP-9 (189), whereas MMP-12 inactivates α_1-AT, thereby enhancing NE activity (190). However, the interactions are complex and in mouse models, MMP-9 also degrades some neutrophil chemoattractants, which would reduce PMN recruitment and hence NE release (160). The majority of animal "knock-out" models of emphysema support a multifaceted pathogenic process in the disease, as inhibition of serine, cysteine, or MMPs all show partial protection from the development of emphysema (114,161).

The noticeable exception is MMP-12. The MMP-12 knock-out mice exposed to cigarette smoke do not develop emphysema, although macrophage recruitment to the lungs is normal in response to monocyte chemoattractant protein-1 (191). In a similar study, neutrophil numbers increased in the first month following smoke exposure, but breakdown of connective tissue and the development of emphysema occurred later and related to a subsequent increase in macrophages (176).

Furthermore, neutrophil depletion (with a significant reduction in NE activity) did not protect against smoke induced emphysema while macrophage depletion (with normal neutrophil activity) did. These results questioned the importance of the neutrophil in the pathogenesis of COPD and suggested that the macrophage was the only effector cell (at least in mice). However, this view ignored the weight of evidence amassed over the past three decades favoring the neutrophil and was overly simplistic. It seems likely that in vivo, a combination of cells and chemoattractants is needed.

Bronchoalveolar lavage fluid from cigarette-exposed mice shows an acute influx of neutrophils with evidence of both elastin and collagen degradation in a dose dependent manner at 6 and 24 hr. This resolves by 48 hr and can be reduced by neutrophil depletion or the administration of the serine proteinase inhibitor α_1-AT (177). Other animal models have also found an early rise in neutrophil number and activity, and a corresponding rise in matrix breakdown products after exposure to cigarette smoke (114). In these studies, NE inhibitors reduce TNF-α and emphysema by ~30%, but only if given at the start of exposure. When NE inhibitors are given after 4 months of exposure, these effects are not seen. The TNF-α receptor knock-out mice are also protected from the acute neutrophil infiltrate and connective tissue breakdown seen following cigarette smoke exposure in wild-type mice (192). Therefore, both neutrophils and TNF-α appear important.

Cigarette smoke exposed MMP-12 knock-out mice did not display the early neutrophilic infiltrate or the release of desmosine and hydroxyproline (matrix breakdown products) that are characteristic of wild-type mice, although macrophage infiltration did occur (albeit at lower levels). When MMP-12 knock-out mice underwent intratracheal instillation of normal macrophages, a neutrophil influx was seen. Use of an MMP inhibitor also prevented a neutrophilic infiltrate and subsequent matrix breakdown (192). Later studies demonstrated that cigarette smoke induced production of TNF-α from alveolar macrophages in wild-type mice, but not in MMP-12 knock-out mice. Interestingly, levels of TNF-α mRNA were the same in both groups and it was surmised that MMP-12 processes TNF-α after secretion. Thus, it is likely that MMP-12 is needed to activate TNF-α which in turn initiates neutrophil recruitment, leading to degranulation and tissue damage (Fig. 6).

When interpreting results from in vitro work and animal models, one must consider that there may be important variations in the pathogenesis of COPD between cell types and differing species, and indeed, conflicting results are common. However, it seems likely that neutrophil infiltration remains at least an early event and appears to be a precursor to the pathological changes seen. Macrophages seem central to neutrophil recruitment, probably via activation of TNF-α, and may be needed to sustain the inflammatory process and hence emphysema. The studies have not provided a means of studying the effects of other complex processes, such as the recurrent exacerbations in COPD, or the effect of smoking cessation and

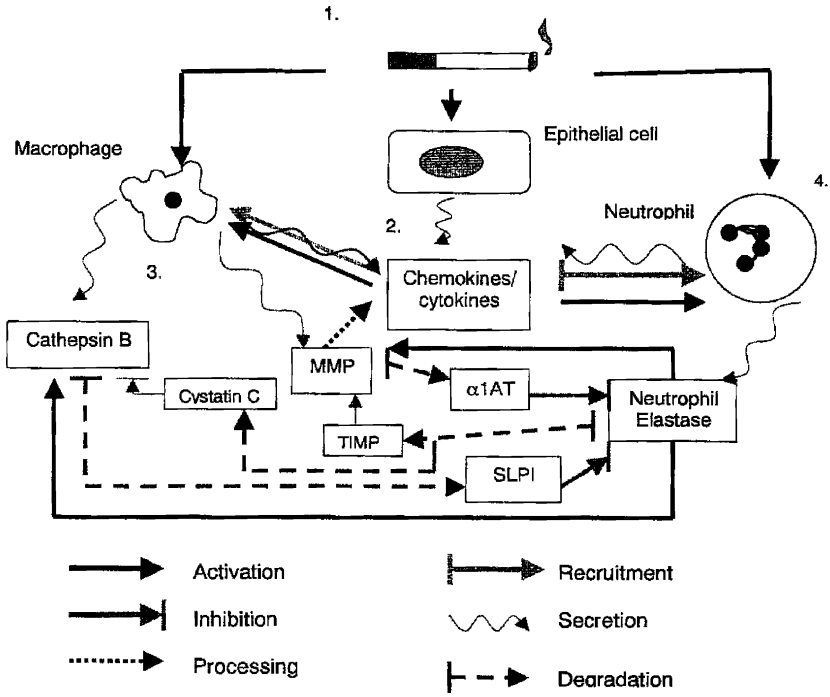


Figure 6 Interactions between neutrophils and macrophages in COPD. 1. Cigarette smoke activates epithelial cells, and macrophages and neutrophils once recruited into the airways; 2. Epithelial cells, macrophages and neutrophils secrete cytokines (such as TNFα and IL-1β) and chemokines (such as CXCL8), which recruit further leukocyte entry into the lung; 3. Once recruited, macrophages secrete MMPs and cathepsin B, which process neutrophil chemoattractants (such as MMP-12 acting on TNF-α) enhancing neutrophil recruitment, and degrade matrix proteins. MMP and cathepsin B also degrades neutrophil elastase (NE) inhibitors (α1AT and SLPI) enhancing NE activity. 4. Neutrophils secrete NE, which leads to protein degradation. NE activates macrophage Cathepsin B and MMPs, and degrades their primary inhibitors, Cystatin C and TIMP (leading to further protein degradation, cytokine modulation and neutrophil recruitment).

do not take us further in understanding the overlapping actions of all proteinases in unison, and in depth studies of cells and chemoattractants in man are awaited.

VII. CONCLUSIONS

There is strong evidence supporting the hypothesis that the neutrophil is fundamental in the pathogenesis of COPD and only the neutrophil has been shown to be able to cause all of the pathological changes of the disease including emphysema, mucus hyper secretion, and reduced ciliary beating.

The diversity of in vitro models, animal models, and human studies have led to conflicting results regarding the importance of various components of the inflammatory response. However, there is a cogent proof that NE is an early step in a proteinase cascade, which leads to a local proteinase–antiproteinase imbalance, causing tissue damage.

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8

Epithelial Cells

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I. INTRODUCTION

The airway epithelium provides much more than a barrier between the host and environment. Epithelial cells are important sources of mediators and play important roles in the pathogenesis of a variety of lung diseases including, asthma and chronic obstructive pulmonary disease (COPD). In this context, epithelial cells are capable of participating in inflammatory cytokine cascades and matrix remodeling. Recruitment, proliferation, and differentiation of epithelial cells are key in epithelial repair following injury. Conversely, persistent damage or metaplasia of the epithelium can contribute to impaired airway function. While less well studied, damage of the alveolar epithelium may play a role in emphysema.

This chapter will review the basic structure of the airway epithelium and potential roles in the pathogenesis of COPD. Roles of epithelial cells in immune processes, wound repair, and airway remodeling will be described. The possible role of the alveolar epithelium in emphysema will be discussed briefly.

II. STRUCTURE OF THE AIRWAY EPITHELIUM

A. Cell Types

The proximal airway epithelium is a pseudostratified epithelium composed of basal cells, nonciliated columnar (secretory) cells, and ciliated columnar cells (Fig. 1) (1). However, as many as eight different epithelial cells have been described and the cellular composition of the epithelium varies with location in the airway. The cellular composition of airway epithelium was extensively reviewed by Harkema et al. (2).

Ciliated cells are thought to be nondividing, differentiated cells of the epithelium (3). They have a columnar shape and attach to the basement membrane. Ciliated cells have motile cilia on the apical surface, which contains dynein, the motor molecule (4). Ciliated cells are damaged in chronic bronchitis and COPD. The cilia themselves are shortened (5) and there are fewer ciliated cells (6).

Mucous secreting goblet cells have secretory granules that produce mucins, which are highly modified proteins resulting from the enzymatic modification of several core peptides (7). Mucous secreting goblet cells are columnar in shape and are present in large airways but less so in the terminal bronchioles where the predominant secretory cells are the Clara cells.

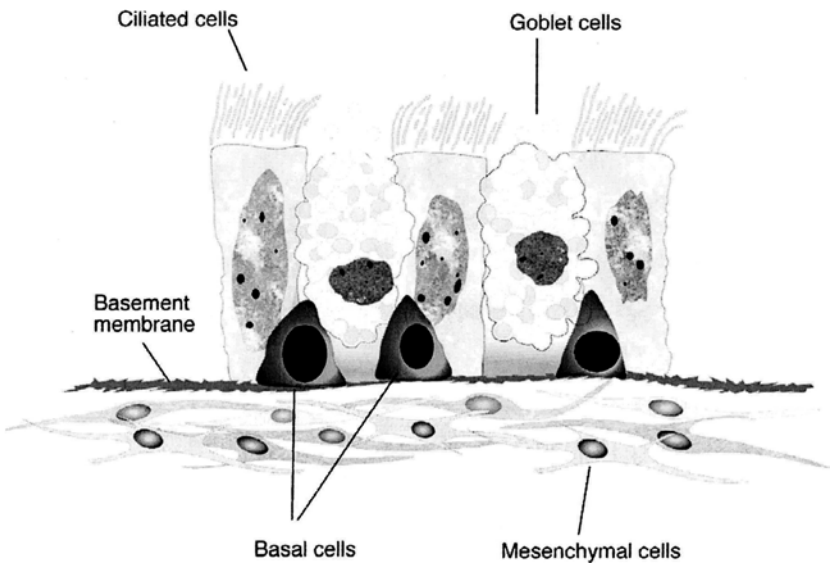

Figure 1 Normal human pseudostratified bronchial epithelium. Schematic drawing of human airway epithelium. Typical epithelium contains a majority of ciliated columnar cells and basal cells (which are thought to enhance the mechanical integrity of the epithelium). There are varying amounts of mucous-secreting goblet cells (see text.) Underlying mesenchymal cells include fibroblasts and smooth muscle cells.

The number of mucous secreting goblet cells in the airways is variable and increases in chronic bronchitis and after inflammatory stimuli (2,8). The small mucous granule cell that has been described in the midepithelial layer may be a precursor of the goblet cell (9).

Basal cells are closely attached to the basement membrane. They do not have a columnar shape and do not normally reach to the airway lumen. It has been postulated that the primary function of basal cells is to enhance the attachment of columnar cells to the basement membrane (10). Basal cells have numerous attachment sites to the basement membrane through hemidesmosomes and an abundance of desmosomes, which mediate attachment to columnar cells. While columnar cells attach to the basement membrane directly, the attachments to basal cells may be more important in maintaining mechanical integrity. There is recent evidence that basal cells are heterogeneous in the expression of some genes (11). The population of basal cells likely contains progenitor cells that can be precursors of the other cell types during injury and repair (11,12).

Clara cells and serous cells are other types of secretory cells. In humans, Clara cells are found primarily in the distal, terminal, and respiratory bronchioles (13). The defining characteristics of Clara cells are electron-dense granules. The main function of Clara cells is likely to provide intralumenal secretions for the distal, nonciliated bronchioles. Clara cells are thought to secrete surfactant proteins, an antileukoprotease, and Clara cell specific peptides such as Clara cell secretory protein (CCSP) (14–18). The CCSP is thought to modulate inflammatory and secretory processes. Clara cells are thought to be progenitor cells during development and it has been suggested that a stem cell for the peripheral airway has Clara cell characteristics (19,20). It has also been shown that CCSP-expressing stem cells that localize to the neuroepithelial body (NEB) can contribute to renewal of the proximal bronchiolar epithelium (20). It has been shown that Clara cells are replaced by mucous-producing cells in the bronchioles of smokers (21,22). Excess mucous in nonciliated airways and a reduction in CCSP may impair clearance of secretions from these small airways. Serous cells are found primarily in submucosal glands but can be found in the surface epithelium of fetal human airways (23).

Pulmonary neuroendocrine cells are rare cells in the small airways of humans. They are hard to identify without immunohistochemical techniques. The subject of pulmonary neuroendocrine cells was comprehensively reviewed in 1991 (24). The role of these cells in normal pulmonary function is difficult to assess, in part, because of their rarity. Pulmonary neuroendocrine cells do proliferate in the setting of airway damage. The mediators produced by these cells include serotonin, bombesin, and somatostatin that can have profound effects on cell growth and development. Increased release of mediators derived from these cells has been suggested to play a role in the response to cigarette smoke and in the development of lung cancer (25–27).

There has been an evolution in our thinking about progenitor cells in the airway. While basal cells were a likely choice as a stem cell, there was evidence that it was not that simple. Breeze and Wheeldon (28) described intermediate cells that have characteristics of basal cells, goblet cells, and ciliated cells. These cells might be precursors of other cell types. Studies in hamsters suggested that secretory cells are the major proliferating cell type after injury (1). The observation in hamsters that basal cells are the last cells to appear in the trachea during fetal development would suggest that they are not the usual precursor cells. Studies with denuded rat tracheas showed that basal cells could repopulate the denuded trachea but only basal and ciliated cells were seen. A secretory cell inoculum gave rise to a more complete epithelium that included secretory, basal, and ciliated cells (29). It is possible that there is more than one type of progenitor cell as has been suggested for the submucosal glands (30). More recent studies of airway injury have emphasized a role for basal cells in repopulating the epithelium. Most of the cells that accumulate after injury express cytokeratin 14, a basal cell marker and not cytokeratin 18, a columnar cell marker (31). Careful studies have shown that the population of basal cells contains progenitor cells that can be precursors of the other cell types (11,12).

Recent studies suggest that there is likely no single progenitor cell for the airways but that several locations in the lung harbor special sites or "niches" where stem/progenitor cells reside (32). In the larger airways, these include the ducts of airway glands (33) and neuroendocrine bodies (34). Niches have also been described in the peripheral airways (20), where Clara cells have also been suggested to play a progenitor role. Finally, recent evidence from animal studies and from human transplant patients suggests that circulating stem/progenitor cells can contribute to the formation of alveolar epithelium (35–38), and possible airway epithelium as well (39).

B. Epithelial Cell Junctions

Epithelia in all organs have specialized cell–cell junctional complexes that allow them to achieve their special barrier functions. Recent advances in understanding the molecular basis for these junctions provide insight not only into how normal epithelial function is achieved, but also promises improved understanding of pathologic processes. The three major structures involved are the tight junctions (or zonula occludens), the adherens junction (or zonula adherens), and the desmosome (or macula adherens) (Fig. 2). These have been recently reviewed (40). These are organized into a complex that is usually located close to the apex of the epithelial cell. Gap junctions are present on the lateral surfaces of secretory cells, ciliated cells, and Clara cells. Their function is to communicate between adjacent cells rather than to form a barrier.

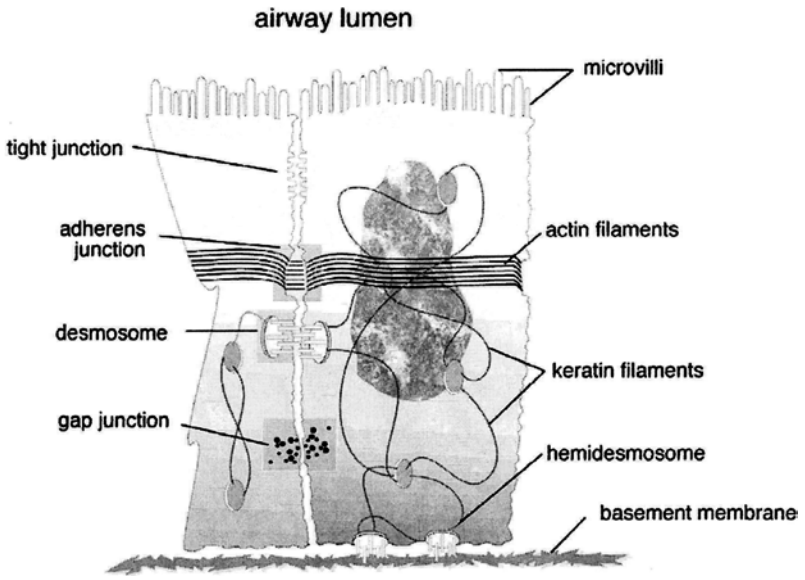

Figure 2 Schematic drawing of the specialized junctions of columnar epithelial cells. The junctional complex includes the tight junction, the adherens junction, and the desmosome. The desmosomes occur more as plaques, rather than as a band. Gap junctions can be located elsewhere. Hemidesmosomes strengthen attachment to the basement membrane. (Adapted from Ref. 245.)

The tight junction has several key roles in the biology of the airway. One of its roles is to form a barrier to water and solute. One of the measures of tight junction performance is the transepithelial electrical resistance measurement (40). The tight junction is composed of a membrane protein, occludin, and cytoplasmic proteins, ZO-1 and ZO-2, claudins, JAM (junction-associated membrane protein), cingulin, and others (41,42). Another role for tight junctions may be to demarcate the boundary between the apex of the cell and basolateral portions of polarized cells. Tight junctions thus separate the external lipid layer of the cell membrane of the apex of the cell from the basolateral cell membrane. This is crucial in maintaining polarized function of epithelia. The function of tight junctions is modified by $Ca2^{+}$, cAMP, and protein kinase C (PKC) (43). Alterations in claudins can affect tight junction barrier function (44) and this has been suggested as a pathway for fatty acid-induced edema formation in an in vivo experimental model (45).

The adherens junction is a special structure that is somewhat analogous to the focal adhesion described later in this chapter. The adherens junction serves to anchor the cell–cell junction to the actin cytoskeleton and contains a collection of molecules including the alpha-, beta-, and

gamma-catenins as well as signaling molecules that facilitate the connection to the cytoskeleton (46,47). When the adherens junction is disrupted, the tight junction is also affected. It is thought that junctional deterioration leads to a progressive decrease in epithelial integrity and induces alterations in epithelial morphology, with consequent enhanced paracellular transit of antigens and pathogens (48). The main cell–cell adhesion molecule is E-cadherin. The cadherins are thought to be critical to the development of all solid tissues. The roles of cadherins during development have been recently reviewed (49–51). The best-described cadherins are E-cadherin (epithelial), N-cadherin (neural), P-cadherin (placental), and L-CAM (liver cell adhesion molecule). E-cadherin is expressed very early in embryonic development and is primarily expressed on epithelial cells in adult tissues. In the human bronchus and alveolus, E-cadherin is expressed at intercellular contacts of epithelial cells and particularly at the lateral aspects of columnar cells (52). This location of E-cadherin is consistent with the location of intermediate/adherens junctions that interact with actin filaments of the cytoskeleton. P-cadherin has been identified in bronchial epithelium but not in alveolar epithelium (53,54).

The expression and function of cadherins are modulated in inflammation and disease states. Kasper and Muller have described cadherin expression in several animal models of lung injury. Using a pan-cadherin antibody, they found that radiation injury of the lung caused increased cadherin expression in bronchial epithelial cells, interstitial cells, and type II pneumocytes (55). Using an antibody to E-cadherin, they found human lung specimens with fibrosis demonstrated spotty, cytoplasmic staining of alveolar epithelial cells rather than the normal basolateral membrane staining (56). McGuire et al. have shown that E-cadherin is a substrate for matrilysin and suggest that shedding of E-cadherin ectodomain is required for epithelial repair (57). The results imply that modulation of cadherin expression and function is important during repair and remodeling of the epithelium. Proinflammatory cytokines alter the function of cadherins and thus cause disrupted epithelial integrity (58,59). Histamine is capable of altering cadherin function and may thus be involved in increased epithelial permeability in allergic inflammation (60).

Desmosomal cadherins are another group of transmembrane adhesion molecules that are involved in cell–cell adhesion and are found in desmosomes. Green and Jones have reviewed the structure and function of desmosomes (61). Similar to the other cadherins described above, desmosomal cadherins, including desmocollins and desmogleins, are calcium-dependent transmembrane adhesion molecules that participate in homotypic cell–cell adhesion. The exact role of desmosomal cadherins in adhesion is not yet clear, however. Desmosomal cadherins apparently have a functional complexity that the classical cadherins, such as E-cadherin, do not have. Three desmocollin and three desmoglein genes have been identified

(62,63). Each of the desmocollin genes also produces splice variants (64). It is not clear that the individual desmogleins and desmocollins intereact through homophilic adhesion in the same way as classical cadherins.

Similar to focal adhesions, desmosomes include a complex of cytoplasmic proteins that enable connections to the cytoskeleton. Plakoglobin is related to beta-catenin, which is present in adherens junctions with E-cadherin (61,65). Plakoglobin is present in other types of cell–cell junctions with or without the classic cadherins (66). Plakophilin is related to plakoglobin and may bind directly to intermediate filaments. Desmoplakin also functions to anchor intermediate filaments to desmosomes. Other proteins present in the plaque on the cytoplasmic side of a desmosome include the bullous pemphigoid antigen (BPAG1), plectin, and IFAP300. Similar to focal adhesions, desmosomes may transduce signals (67) and are important in development of the lung (68,69). Perhaps more important to injury and repair, the function of desmosomes is modulated by several stimuli. Garrod et al. (67) have reviewed data that suggest that stimuli such as wounding can revert stable desmosomes from calcium independence to calcium dependence. The function of desmosomes is likely regulated by reversible protein phosphorylation. In this regard, it interests that activation of PKC is known to modulate desmosome function. As will be discussed later in this chapter, PKC activation is associated with increased mobility of epithelial cells. Modulation or disassembly of desmosomes may be necessary for cell migration.

Gap junctions are present on the lateral surfaces of airway epithelial cells and allow communication between the cells (70). Gap junctions have specific structural proteins called connexins. Some of the connexins are relatively specific for the lung (71–73). Gap junctions allow second messengers such as cAMP and Ca^{++} to diffuse from cell to cell which facilitates coordination of cilia beating and secretion amongst cells. Proinflammatory cytokines modulate gap junction-mediated communication (74).

C. Basement Membrane

The basement membrane is a special class of extracellular matrix that underlies many cell types including epithelial, endothelial, muscle, and nerve cells (75). The subepithelial basement membrane forms a barrier that separates epithelial cells from other cells and provides physical support. The basement membrane influences epithelial cell differentiation, polarization, and understanding its structure is key to understanding epithelial function. Some components of the basement membrane such as laminin 5 are involved in development and morphogenesis (69).

The basement membrane under the airway epithelium has not been subjected to detailed chemical analysis. Much of our understanding of basement membranes comes from studies of basement membranes where there is

sufficient material for analysis (75). When lung basement membranes are studied, it is usually by immunohistochemistry (76). The major components of basement membrane are type IV collagens, laminins, perlecan, and entactin/nidogen. Fibronectin is also present in many situations, particularly in disease states. The components are assembled into a meshwork of fine cords, visible by electron microscopy (77). Type IV collagen is genetically distinct from other collagens but has typical features including the triple-helical structure, hydroxyproline, and hydroxylysine. Type IV collagen is mostly a heteropolymer of two αl(IV) chains and one α2(IV) chain. Structurally, similar α3, α4, α5, and α6 chains have also been described and are used to a lesser extent. The noncollagenous domain of the α3 chain is the antigen target for the autoantibodies of Goodpasture's disease (78). Type IV collagens are organized in the basement membrane into lattices that provide structure (79). Laminins comprise a family of over 10 different laminin isoforms (80). Most laminins are made up of combinations of either $\beta 1\gamma 1$ or $\beta 2\gamma$l and one of five α chains. The laminins form noncovalent networks. Perlecan is a heparan sulfate proteoglycan that interacts with other basement membrane components (81,82). Perlecan also binds growth factors and may serve as a reservoir for growth factors in the basement membrane. Entactin/nidogen, name because of its independent discovery in two different laboratories, binds to a specific site on the γl chain of laminin and is found in equimolar amounts with laminin (83).

The basement membranes of lung tissues have been studied by immunohistochemical techniques, for the localization of specific proteins and proteoglycans (84,85), and by electron microscopy. Airway epithelium is different from many basement membranes by virtue of the more consistent presence of the lamina reticularis. The lamina reticularis is seen at both the light and electron microscopic levels as a lamina that is under the main body of the basement membrane, the lamina densa (86). The lamina reticularis contains a mixture of collagens, fibronectin, tenascin, and proteoglycans. Specific components play important roles in development (69,84). The lamina reticularis is thickened in asthma but, interestingly, not necessarily in chronic bronchitis (87,88). A subset of COPD patients with a thickened lamina reticularis has been described and they have more eosinophils, similar to asthma (89). The composition of the lamina reticularis may also be altered in disease states suggesting a potential pathogenic role for this structure in airway remodeling. The consequences of thickening of the lamina reticularis on airway function are not known.

III. NORMAL ACTIVITIES OF AIRWAY EPITHELIAL CELLS

The airway epithelium has important functions that are disrupted in COPD. Abnormal and disrupted functions may contribute to the pathogenesis of

COPD. For example, impaired mucociliary clearance likely contributes to susceptibility to pneumonia and bronchitis. The epithelium also contributes to airway inflammation and repair.

A. Mucociliary Clearance

The ability to remove any foreign material that arrives on the luminal surface of the airway as a consequence of inhaling large volumes of air is an important function of the airway epithelium. A coordinated system of mucus production and clearance of the mucus allows the airways to entrap and remove foreign material. The clearance of secretions is dependent on two mechanisms, ciliary beating, and cough. A third mechanism, phagocytosis by macrophages, can play a role if the first two mechanisms fail. Ciliated cells make up approximately 50% of the cells of the human trachea and the percentage falls off in more peripheral airways (90). Cilia contain the well-described array of microtubules called the axoneme (4,91). The axoneme is made up of nine microtubular doublets that have two rows of dynein arms. The movement of cilia is generated by sliding movements of the microtubules that are powered by adenosine triphosphate (ATP). There are a number of signaling systems that can influence ciliary beat frequency (92–95). Activation of cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), and PKC has all been associated with increases in ciliary beat frequency. Thus, any number of inflammatory mediators can potentially modulate ciliary function. A functional ciliary transport system is required for normal lung maintenance, cough alone is not enough. This is inferred from the observation that genetic abnormalities of ciliary function are associated with chronic lung disease (91).

A number of pathologic findings suggest that mucociliary clearance is impaired in smokers and chronic bronchitis. Ciliated cells are damaged in chronic bronchitis and COPD. The cilia themselves are shortened (5) and there are fewer ciliated cells (6). Enlargement of the bronchial glands, an increase in gland mass, and hyperplasia of mucous secreting goblet cells are important pathologic hallmarks of chronic bronchitis (8). In small airways, Clara cells are replaced by mucus-producing cells in smokers (21,22). Excess mucus production in the setting of damaged ciliary transport likely contributes to mucus plugging of airways.

B. Metabolic Functions

Given the exposure to inhaled toxins, it is not surprising that airway epithelial cells can metabolize important inhaled toxins and xenobiotics. Airway epithelial cells are capable of phase I metabolism by means of the cytochromes P-450 (96,97) and phase II, or conjugative enzymes such as

glutathione transferases and sulfotransferases (98,99). These metabolic pathways may also play important roles in airway inflammation. For example, sulfation is a predominant mechanism for the inactivation of catecholamines (100). Airway epithelial cells may be responsible in part for the metabolism of inhaled catecholamines. Studies by Beckmann et al. (98) demonstrated greatest expression of phenol sulfotransferases in the nonciliated secretory epithelial cells of the bronchioles with lower levels of expression in the larger airways. Phenol sulfotransferase expression is regulated in airway epithelial cells (101).

Airway epithelial cells may contribute to the metabolism and inactivation of inflammatory mediators such as the neuropeptides bradykinin, substance P, and neurokinin A. Airway epithelial cells express several peptidases including membrane-associated neutral endopeptidase, previously called enkephalinase, and angiotensin-converting enzyme (102). Neutral endopeptidase inhibitors augment airway responsiveness in several animal models (103). The loss of neutral endopeptidase activity, when epithelium is shed, has been hypothesized as a major mechanism of airway hyper-responsiveness and neurogenic inflammation in asthma (102–104). Angiotensin-converting enzyme also inactivates bradykinin and substance P. Angiotensin-converting enzyme inhibitors have been known to cause cough and airway hyper-reactivity in some patients and it has been suggested that the effect is mediated by a reduction in the metabolism of neuropeptides (105). Epithelial cells also participate in the degradation of histamine. Epithelial cells are a rich source of histamine *N*-methyltransferase (HMT) and histaminase (diamine oxidase, DAO) (106–108).

Bronchial epithelial cells express some metalloproteinases that are involved in activation or release of mediators. Recently, a TNF-α-converting enzyme (TACE) was described, which cleaves and releases the membrane-bound TNF-α. Expression of TACE follows that of TNF-α in bronchial epithelial cells (109). The TACE is also reported to cleave the precursor of TGF-α, with release of soluble mature TGF-α in various epithelial tissues (110).

IV. INFLAMMATION AND INJURY

Epithelial and luminal inflammation is a prominent feature of COPD (111). Alveolar macrophages and other leukocytes are well described, potent producers of proinflammatory cytokines in COPD patients and cytokines can be found in high concentration is sputum of COPD patients (112,113). It is now well accepted that pulmonary epithelial cells also participate in inflammatory cascades. It has been proposed that epithelial cells are key initiators of inflammation and are "sentinels" for the immune system (114–116). In this regard, epithelial cells may also participate in the innate immune system.

A. Inflammatory Mediators

The ability of airway epithelial cells to produce inflammatory mediators has been of interest in asthma and is receiving more interest in the study of chronic bronchitis and COPD (113,117,118). Airway epithelial cells produce a variety of proinflammatory mediators, including chemotactic cytokines, eicosanoids, growth factors, and nitric oxide (118).

The idea that epithelial cells function as a first line of defense is supported by the observations that epithelial cells are capable of releasing mediators relatively quickly after stimulation or damage. Koyama et al. (119) have shown that cultured bovine bronchial epithelial cells release arachidonate metabolites including LTB4 within 1 hr of exposure to endotoxin. Other chemotactic activities for neutrophils and monocytes were released at later time points (119). The release of arachidonate metabolites may be a rather general, early response of airway epithelial cells to injury as many stimuli will elicit LTB4 release from bovine bronchial epithelial cells in primary culture. Viral infection and ozone are just two of the described stimuli (120,121).

Epithelial cells also participate in more chronic inflammatory processes. Some hours after stimulation, airway epithelial cells release very potent chemotactic factors for neutrophils that likely play roles in the chronic attraction of leukocytes. The cytokine with perhaps the most potent chemotactic activity for neutrophils is IL-8 (122). The IL-8 is thought to have a relatively long half-life (123). The IL-8 is one member of a family of similarly sized (8–10 kDa) chemokines called the CXC chemokines. The release of CXC chemokines by airway epithelial cells is modulated by other cytokines. It has been shown that interleukin-17 (1–1000 ng/mL) increases the release of the CXC chemokines GCP-2, GRO-α, and IL-8 in a concentration-dependent manner (124,125). Epithelial cells also release chemokines of the C-C family, including RANTES, MCP-1, and eotaxin (126). These chemokines have more activity on macrophages (127–132) and their release is also modulated in inflammation (133). Epithelial cells release other cytokines such as IL-1, IL-6, and IL-18. Airway cells release GM-CSF in vitro (134–137), which is capable of promoting survival and activation of eosinophils, stimulating neutrophils, and stimulating-macrophage proliferation. Ohtoshi et al. (135) have demonstrated that epithelial cells from inflamed tissues release more GM-CSF than do cells from normal tissues. It is thought that epithelial cells interact with other cells to augment production of mediators such as GM-CSF (138). Thus, epithelial cells from inflamed tissues release factors that recruit, activate, and differentiate leukocytes.

Important to the theme of COPD is the observation that epithelial cells release inflammatory mediators in response to air pollutants. Nitrogen dioxide, ozone, and diesel exhaust particles have been studied (117,139). The magnitude of response of epithelial cells may be modified in COPD such

that there is some down regulation of the inflammatory response (117). The ability of epithelial cells to release IL-8 in response to air pollutants may be an important step in the damage, repair, and mucous hypersecretion of the epithelium. Cigarette smoke can prime epithelial cells for IL-8 release (140). Further, it has been suggested that the sequence of events leading to mucous hypersecretion is dependent on IL-8 recruitment of neutrophils, which then stimulate goblet cell hyperplasia and degranulation (141).

Epithelial cells are also capable of producing mediators which down-regulate inflammatory processes. Many cells are potential producers of transforming growth factor-β (TGF-β), including macrophages (142), epithelial cells (143–145), and fibroblasts (146,147). TGF-β is present in the epithelial lining fluid of the lung (148) and is present in the epithelium of damaged lung (149). TGF-β has also been demonstrated in the airways of subjects with COPD (150). TGF-β plays important roles in tissue repair and can function as a chemoattractant for leukocytes (151,152). Interestingly, TGF-β also has anti-inflammatory properties such as the ability to inhibit IL-2-dependent proliferation of T-lymphocytes (153). TGF-β also inhibits cytokine production by mononuclear cells (154). Among the arachidonic acid metabolites released by epithelial cells, PGE2 has a number of anti-inflammatory effects. PGE2 reduces neutrophil migration, for example Ref. 155. IL-6 is capable of reducing inflammation in several models of inflammation including an in vivo model of pulmonary inflammation. In this regard, like many other cytokines that may be "bifunctional," with differing activities dependent on the inflammatory setting, IL-6 also has well-documented proinflammatory effects.

An important aspect of inflammatory responses is thought to be the amplification of response through cytokine cascades. Epithelial cells likely play an important role in the cascade effect, especially in reference to IL-8 and MCP-1. The TNF-α is a potent stimulator of IL-8 expression in airway epithelial cells (156). It is thought that TNF-α released from macrophages followed by IL-8 and MCP-1 release constitutes an important cytokine network effect in the lung (156). It has also been reported that treatment of an airway epithelial cell line derived from a cystic fibrosis patient with IL-1β increases secretion of IL-6 and IL-8 (157). Other CXC chemokines are also released in response to cytokines. Interleukin-17 (1–1000 ng/mL) increases the release of the CXC chemokines GCP-2, GRO-α, and IL-8 (124,125). It has been shown that TH2 cytokines also influence airway epithelial cell production of cytokines (158,159).The IL-4 and IL-13 both can increase IL-8 secretion. The IL-4 but not IL-13 augments the effects of TNF-α on IL-8 secretion. The IL-4 and IL-13 inhibit the TNF-α-induced secretion of IL-6. Thus, epithelial cells participate in cycles of cytokine cascades that can accelerate, perpetuate, or inhibit inflammatory processes. Epithelial cells may produce the critical proinflammatory or anti-inflammatory cytokines that control the progress of inflammatory events. Denburg et al. (160) have

proposed that these structural cells play not only an amplifying role, but also rather a central role in creating a web of cytokines that control chronic inflammation in the tissue microenvironment.

B. Cell–Cell Interactions

Epithelial cells also participate in inflammatory processes by directly interacting with leukocytes. Epithelial cells can express MHC antigens (161–163). Increased expression of MHC antigens on bronchial epithelial cells has been described in chronic bronchitis and correlates with the amount of inflammation (164). Expression of MHC antigens allows cells to directly interact with lymphocytes and raises the possibility that epithelial cells can present antigen to lymphocytes. Both MHC class I (HLA-A,B,C in humans) and MHC class II (HLA-DR, DQ, DP in humans) antigen expression are regulated on a variety of epithelial cell types. The most potent stimulant for expression is likely γ-IFN.

Epithelial cells express cell surface molecules that enhance cell–cell adhesion with leukocytes. A number of investigations have emphasized the importance of the expression of intercellular adhesion molecule-1 (ICAM-1) by epithelial cells (170). The ICAM-1 is the ligand for LFA-1 on leukocytes (165). The interaction of ICAM-1 with LFA-1 is thought to strengthen the cell–cell adhesion mechanisms during activities such as antigen presentation, target recognition, and leukocyte transmigration (166–168). The expression of ICAM-1 is enhanced on airway epithelial cells by proinflammatory cytokines (169–171). The IL-1ß, TNF-α, γ-IFN, and IL-4 are cytokines that are implicated in the expression enhancing activity. The expression of ICAM-1 may be coordinated with local binding of IL-8, creating an area that facilitates leukocyte migration (172,173). The expression and function of adhesion molecules such as ICAM-1 is also modulated by viral infection (174,175). The ability of the cells to express ICAM-1 in the setting of a viral infection may be an important component of the defense mechanism to clear such infections. The ICAM-1 expression is increased in chronic bronchitis (164,176). So, in addition to producing neutrophil chemotactic factors, epithelial cells express an adhesion molecule that promotes neutrophil transmigration into the airway.

V. WOUND REPAIR AND REMODELING

A. Epithelial Repair

Inhalation of toxins and irritants, e.g., cigarette smoke, with damage to the epithelium is a major pathogenic process in COPD. Repair processes are initiated as part of the inflammatory response. If normal epithelial and tissue structure is restored or preserved, then normal tissue function should also be preserved. If normal tissue structure is not restored, then dysfunction

likely results. Consistent with this, changes in epithelial cells have been described in a number of studies of chronic bronchitis and smokers. Loss of epithelial cells is described (177,178) as well as squamous metaplasia (179,180). Abnormal or disordered repair may contribute to infection and inflammation since bacteria preferentially adhere to migrating epithelial cells (181). This may account for the tendency of the injured airway to become colonized with bacteria. Abnormal or disordered repair likely contributes to production of mediators that modulate airway remodeling. Fibrosis in bronchiolar walls has a strong correlation with airflow obstruction (177). The COPD may thus be seen as the result of inadequate or disordered repair processes (182,183). There are a number of mechanisms by which epithelial cells may be injured. Activated eosinophils and neutrophils release a considerable armamentarium of proteases, oxidants, and toxic molecules. The major constituent of eosinophil granules, major basic protein, is toxic to epithelial cells in culture (184,185). Neutrophil elastase is capable of causing epithelial cell detachment from the underlying matrix in in vitro assays (186). Acute inflammation is also associated with vasodilatation, leakage of fluid, and possible increases in hydrostatic forces that can damage epithelium (187,188).

Restoration of denuded epithelium has been extensively described at the electron microscopic level in both in vitro and in vivo models of repair. In models of intestinal, epidermal, or airway repair, cells at the edge of the wound migrate into the provisional matrix that forms in the wound (Fig. 3) (1,189–193). The initiation of migration requires the cells to decrease their attachment to their neighbor cells through shedding of the ectodomain of E-cadherin (57). The process can be very rapid (193). Once the epithelium is intact, the cells differentiate into the mature cell types. Interestingly, there are conflicting data on which of the cells of the normal bronchial airway are responsible for the migration into the wound. As noted above, basal cells are not necessarily the progenitors of all the other cell types. However, Shimizu et al. have shown that nearly every cell present in the early phases after airway injury expresses a basal cell marker, cytokeratin 14 (31). Cytokeratin 14 expression is lost over the next 2 weeks, during which columnar cells increasingly express markers associated with normal epithelial columnar cells. Although this observation is consistent with a basal cell origin for the cells present early in a wound, it is also consistent with dedifferentiation of the newly recruited cells and with the transient acquisition of cytokeratin 14 expression.

The stimuli for migration of epithelial cells into wounds have been described in several models. A number of potential attractants are likely present in wounds including the provisional matrix that forms in wounds (fibrin, fibronectin, vitronectin, collagens, various proteoglycans, and, likely, remnants of the basement membrane such as type IV collagen and laminin) (192,194). Fibronectin and collagens are potent stimulators

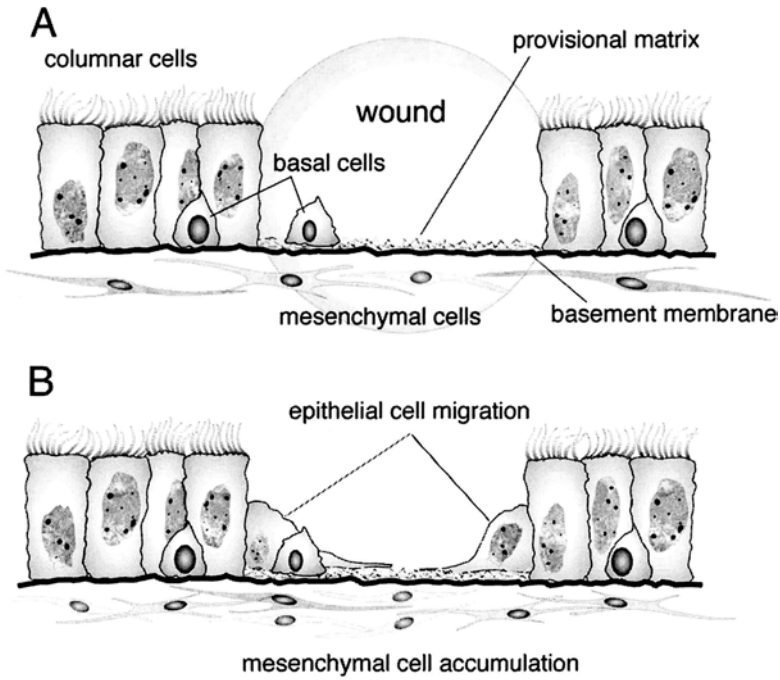

Figure 3 Schematic drawing of airway repair. (A) After a wound has been incurred, portions of the basement membrane become stripped of cells. A provisional matrix that has remnants of the basement membrane and plasma proteins including fibrin and fibronectin accumulates rapidly. (B) The provisional matrix as well as other stimuli induces epithelial cells to flatten and migrate across the provisional matrix, eventually restoring epithelial integrity. Mesenchymal cells may accumulate concurrently. Over the course of days to weeks, the newly recruited epithelial cells differentiate and restore epithelial architecture.

of bronchial epithelial cell migration in modified Boyden chamber assays (195). Laminin is less potent as an attractant. Bronchial epithelial cells are capable of producing fibronectin (196,197). Epithelial-derived fibronectin may represent a special form of the protein produced by either differential splicing of the gene or by post-translational modifications, which result in some biological differences compared to plasma-derived fibronectin (198). The cell-derived form of fibronectin is a more potent chemoattractant than is the plasma-derived form for fibroblasts (199). Mucin-associated TFF-peptides (formerly P-domain peptides or trefoil factors) enhance migration of human bronchial epithelial cells (200).

Inflammatory cytokines and growth factors can modulate the process of epithelial cell migration in wound healing. Certainly, leukocyte migration is modulated by inflammatory mediators and pharmacological

agents (201), and it appears that some of the same mechanisms of activation of migration may also act on epithelial cells. The TNF-α is able to stimulate bronchial epithelial cell motility and the effects are mediated by PKC (202,203). Other inflammatory mediators that have been shown to enhance bronchial epithelial cell migration are tachykinins and bombesin analogues (204,205). Inflammatory mediators such as combinations of IFN-gamma and IL-2 can drive repair activity of type II alveolar cells by stimulating migration and preventing programmed cell death (206). In contrast to mesenchymal cells, bronchial epithelial cell migration is enhanced by agents that activate protein kinase A (PKA) (207). TGF-β has been described as a chemoattractant for fibroblasts and bronchial epithelial cells (208,209). The effect of TGF-β in in vivo models is thought to be one of cellular accumulations, particularly the accumulation of fibroblasts (210). The effects of TGF-β maybe multiphasic, with initial effects on increasing cell accumulation in wounds, followed by later effects that alter morphology of the cells and increase cell attachment and wound strength. Bronchial epithelial cells also respond to growth factors including insulin, IGF-1, and EGF (211,212).

B. Airway Remodeling

Airway remodeling may be defined as the development of disrupted, abnormal airway morphology. Abnormal morphology influences function of the airway in COPD, contributing to airflow obstruction and bronchial hyper-responsiveness (177,213). Inflammation and injury of the epithelium likely contribute to airway wall remodeling in several ways. Epithelial cell metaplasia and increased mucin gene expression contribute to airflow obstruction. Epithelial cells produce matrix proteins that can be deposited in the airway wall and interact with fibroblasts to modulate fibroblast migration, proliferation, and contraction. The interactions of epithelial cells with fibroblasts and other mesenchymal cells are being increasingly recognized as part of a coordinated system that forms and maintains airway structure (214).

Mucus hypersecretion from hyperplastic airway goblet cells is a hallmark of COPD (215). A number of mediators are now known to modulate mucin gene expression and the formation of goblet cells. Activation of epidermal growth factor receptors is responsible for mucin production after inhalation of cigarette smoke in airways in vitro and in vivo (215,216). The TNF-α and neutrophil elastase augment mucin gene expression (110,217). The PKC and one of its substrates, MARCKS, have been reported to be involved in mucus hypersecretion (218–220).

Epithelial cells modulate the extracellular matrix in several ways. First, epithelial cells directly produce matrix proteins. Production of fibronectin

and collagen by bronchial epithelial cells is increased by TGF-β (196). The TGF-β is an important mediator of wound repair and has been the subject of recent reviews (221,222). Although not studied extensively, TGF-β expression is thought to be increased in bronchitis (223).

Epithelial cells have important interactions with mesenchymal cells. When bronchial epithelial cells were examined for ability to produce factors that subsequently modulated matrix production by fibroblasts in vitro, both stimulatory and inhibitory factors were found (199,224–226). The stimulatory factor is composed, in part, of TGF-β. The inhibitory activity produced by bronchial epithelial cells appeared to be PGE2, capable of inhibiting collagen production by fibroblasts. Thus, mediator "networking" is likely operating in the control of matrix production in epithelia. Epithelial cells also modulate fibroblast recruitment, proliferation, and contraction of matrix (227–229). Bronchial epithelial cell-conditioned medium stimulates fibroblast proliferation. The growth stimulatory activity in the conditioned medium is heterogeneous, including peptides, eicosanoids, and TGF-β (224). Some of the mediators that drive epithelial repair also drive fibroblast accumulation. Fibronectin is a wound component that attracts and supports epithelial cell survival (230). Fibronectin is also an attractant for fibroblasts (199). Epithelial cells may also influence peribronchial tissue through fibroblast-mediated contraction of collagenous matrix. Bronchial epithelial cells release mediators that enhance collagen gel contraction by fibroblasts in vitro (228). The importance of epithelial and mesenchymal interactions has lead to the development of in vitro models of the airway that include both cell types (224,231).

Both epithelial cells and fibroblasts express proteolytic enzymes that modulate extracellular matrix (232–234). Studies in several cell systems suggest that the underlying matrix proteins play crucial roles in directing this process of migration and differentiation (233,235). Recent studies are beginning to address the roles of matrix metalloproteinases in COPD (236–241). It appears that expression of certain metalloproteinases such as MMP-9 is increased in epithelium and may play a role in remodeling. Agents which modulate the function of metalloproteinases would be just one of many possible therapeutic approaches for peribronchial fibrosis.

So far, we have discussed repair in the context of airway remodeling but similar concepts likely apply to defective repair of alveolar walls in emphysema. The alveolar epithelial response to injury likely resembles that of the airway epithelium in many respects. Disrupted alveolar epithelial repair may contribute to the loss of alveolar walls in emphysema. Some of the same factors that promote bronchial epithelial repair also promote alveolar epithelial cell recruitment and proliferation following injury (242,243). Alveolar epithelial cells also communicate with mesenchymal cells. Fibroblasts can function as a bridge from endothelial cells to epithelial cells (244).

VI. SUMMARY

The studies reviewed above are consistent with the concept that the lung is a dynamic organ where tissue repair and turnover are required for the maintenance of normal structure. If repair processes are inadequate or disregulated, then remodeling and dysfunction occur. Epithelial cells could be considered the central players in many proposed pathogenic mechanisms for repair and remodeling in COPD. Restoration of a normal epithelium may be essential to the resolution of airway inflammation and remodeling in COPD. Improving our knowledge of the mediators controlling cellular migration, proliferation, differentiation, and matrix production should lead to insights into new therapeutic approaches.

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9

T-Lymphocytes

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I. INTRODUCTION

The global initiative workshop summary for chronic obstructive lung disease (GOLD) defines COPD as a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an *abnormal inflammatory response of the lungs to noxious particles or gases* (1). This definition is of interest because, for the first time, the importance of the inflammatory component in COPD is highlighted inviting a broader look at the possible pathogenic events in COPD, beyond the classical, and limiting, proteinase–antiproteinase paradigm.

The implication of an inflammatory response to the pathogenesis of emphysema, at least centrilobular emphysema, is not new. McLean in 1956 (2) and Leopold and Gough in 1957 (3) linked an inflammatory response in the lung parenchyma to the mechanism of lung destruction, but because their studies were based on autopsy specimens, frequently contaminated by terminal bronchopneumonia, this suggestion was interpreted conservatively. Later, in 1961, Anderson and Foraker (4) observed that the earliest pathological abnormality in centrilobular emphysema was hypercellularity within the alveolar wall and proposed a pathogenic role for the increased inflammatory cells in the pathogenesis of lung destruction.

The study of the inflammatory reaction in the lungs of smokers became possible shortly after with the advent of the relatively noninvasive fiber optic bronchoscope and BAL. Hunninghake and Crystal (5) and Martin et al. (6), utilizing the fiberoptic bronchoscope, showed that young

cigarette smokers have increased numbers of total cells and macrophages, but no neutrophils, while older smokers have increased numbers of both neutrophils and alveolar macrophages in the BAL, when compared with nonsmokers. From these data, originated the concept that the inflammatory reaction in the lungs of smokers consisted of neutrophils and macrophages. The connection between neutrophils and elastase, macrophages and proteinases, and the destruction of lung tissue by these cells and their potent proteinases was promptly made, and the proteinase–antiproteinase balance became the paradigm for the pathogenesis of COPD for over 40 years.

Most likely, there is an important link between the inflammatory cells, their proteinases, and the injury in smokers' lungs but, clearly, there are other inflammatory events not necessarily related to the proteinases that could be important and need to be explored. For the last 10 years, the investigation of the possible role of inflammation in the pathogenesis of COPD has switched to the T-cells after Finkelstein et al. (7) described a prominent T-cell infiltration in the lungs of patients with COPD that was strongly related to the extent of emphysema. Subsequent work by other authors (see later) confirmed these results and showed that the T-cells in the lung of these patients were predominantly CD8+ T-cells. These findings introduced possible new mechanisms for the pathogenesis of COPD and prompted us to suggest that, if T-cells were involved, COPD might be an autoimmune disease triggered by cigarette smoking (8,9).

The aim of this chapter is to review our present knowledge of T-cells in COPD, explore how they, through an adaptive immune inflammation (autoimmunity?), could act in the development of the disease, and to explain how the old and new paradigms could work together in the development of COPD.

II. INNATE IMMUNITY

A. Innate and Adaptive Immunity

The concept of inflammation has evolved substantially since the important observations made by Hunninghake and Crystal (5) and Martin et al. (6), and has been fully incorporated into the immunologist's domain. It is now recognized that an "early inflammatory" reaction—neutrophils, macrophages—represents the so-called *innate immune reaction*, a primitive function already found in prokaryotic beings (fruit fly). Intimately linked with the innate immunity, and only present in eukaryotic animals, is the *adaptive immune response* involving B- and T-cells. We should interpret the traditional inflammation in smokers—neutrophils, macrophages—as the innate immune response to cigarettes that, as we will see, could lead to an adaptive response with T-cells.

The physiological function of the immune system (inflammation) is defense against infectious microbes. However, even noninfectious foreign substances can elicit immune responses. Furthermore, mechanisms that normally protect individuals from infection and eliminate foreign substances are, in some situations, capable of causing tissue injury and disease. Therefore, a more inclusive definition of immunity is a reaction to foreign substances, including microbes, as well as to macromolecules, such as proteins and polysaccharides and other agents regardless of the physiological or pathological consequences of such a reaction (10).

It is important for the understanding of the immune reaction to cigarette-smoke exposure, to realize that innate and adaptive immune responses (which involve T-cells) are components of an integrated system of host defense in which numerous cells and molecules function co-operatively. Two important links exist between innate immunity and adaptive immunity. First, the innate immune response to microbes (or other offending molecules) stimulates adaptive immune responses and influences their nature. Second, adaptive immune responses use many of the effector mechanisms of innate immunity to eliminate microbes or other antigenic substances, and they often function by enhancing antimicrobial activities of the defense mechanisms of innate immunity (10).

It is not possible, therefore, to write about the role of the adaptive immunity (T-lymphocytes in COPD), without reviewing some of the features of the innate inflammatory responses induced by cigarette-smoke exposure, as the innate immune response will determine the development of the adaptive immune response involving CD4+ and CD8+ T-cells, as it is seen in COPD.

B. Innate Immune Reaction in COPD

The innate immune system consists of epithelial barriers, circulating cells [macrophages, neutrophils, eosinophils, mast cells, NK cells, $\gamma\delta$ T-cells, and dendritic cells (DCs)], and proteins (complement) that recognize microbes or substances produced by infections or other foreign harmful substances and initiate responses that eliminate the offending agent (10). It has been designed primarily to fight against offending micro-organisms, and most of the studies leading to the understanding of this complex system have been based on experiments involving micro-organisms and their products. However, cigarette smoke and other nonmicrobial pollutants (ozone, NO_2, and diesel fumes among others) produce a clear innate immune inflammation that is not driven by an infecting organism. Acute innate immune reactions in the lung can be seen after 24 hr of exposure to two cigarettes in mice (11) or after 6 hr of acid instillation into the trachea (12).

How does the respiratory system mount a reaction, designed as far as we know to fight microbial products, against cigarette smoke? As proposed

in 1989 by Janeway and Medzhitov (13), the innate immune recognition of microbes is based on the so-called "pattern-recognition receptors," the toll receptors, that recognize common structures in invading pathogens and start the inflammatory reaction characteristic of the innate immunity (stranger hypothesis of Janeway). However, as suggested by Matzinger (14), it is not the presence of a microbe per se, that is important, but whether it is dangerous. Matzinger proposed that during a microbial attack, it is the resulting cellular stress or tissue damage that alerts the immune system to respond. The previously described immune cell microdetectors (toll receptors) would still be operational, but instead of recognizing microbe parts, they would recognize signs of tissue distress, or molecules that are normally found only inside cells, unless released by damage. This is now recognized as the "danger hypothesis," and it has been shown that undefined cellular material including cytoplasm, "the intracellular molecular soup," (15) can activate the immune system. Intense research in this area is showing unexpected results. As an example, one of the components of this intracellular molecular soup, recently identified as an alarm for the immune system, is uric acid (16), a degradation product of nucleotides that is able to activate a central immune player, the DC. These findings provide compelling support for Matzinger's "danger theory" and a compelling explanation of how irritants, like cigarette smoke, could trigger a full-blown innate immune response.

The present evidence suggests that, by sending "danger" signals in response to cigarette smoke, the epithelium is responsible for the initiation and possibly maintenance of the innate immune response seen in smokers. Over 2000 different xenobiotic compounds have been identified in cigarette smoke, and it has been estimated that there are 10^{14} free radicals in each puff of cigarette smoke (17), a considerable xenobiotic and oxidant burden on the respiratory epithelium, which is the first line of defense to inhaled substances. Not surprisingly, the defense role comes at a price, as cigarette smoke can harm the epithelium. Sun et al. (18) exposed human bronchial epithelial cells in vitro to side stream cigarette smoke and found a 70% diminution in DNA synthesis, a 44% decrease in mitochondrion activity, and a 38% reduction of the surviving cell population. These experiments showed that cigarette smoke is cytotoxic to epithelial cells and also that the extent of injury was directly related to the concentration of smoke to which the cells were exposed (Fig. 1).

There is now ample evidence to show that once injured, the epithelium by increasing permeability and by the production of inflammatory mediators such as IL-8, IL-lβ, TNF-α, granulocyte-macrophage colony stimulating factor (GM-CSF), ICAM-1 could be a potent stimulator of an innate immune reaction (19,20). These epithelial changes and responses are likely to be of pathophysiological relevance as other established inflammatory stimuli (O_3, NO_2) (21), and also infectious stimuli like hemophilus influenza endotoxin, cause a similar release of proinflammation cytokines from these

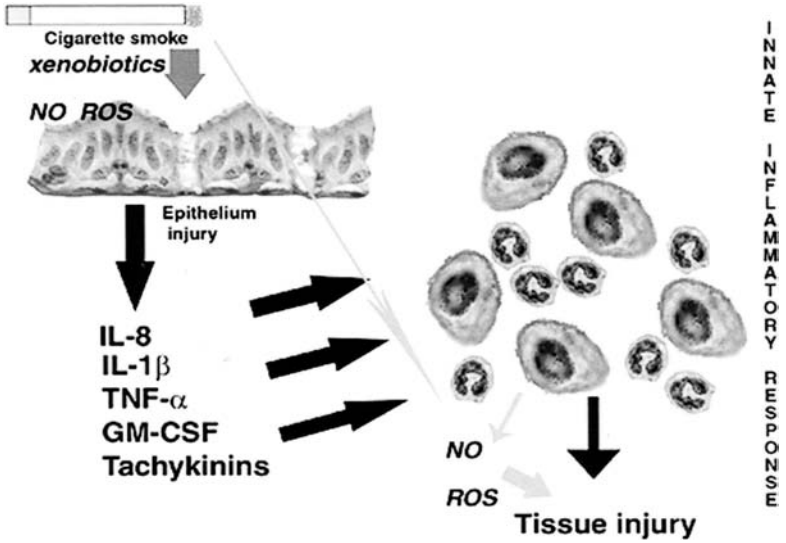

Figure 1 Epithelium in COPD. There is enough evidence to implicate the epithelium in the airways and alveolar wall as the initiator of the innate immune inflammatory response in smokers. Epithelial irritation, necrosis, and apoptosis induced by cigarette smoke would produce the necessary mediators, chemokines, and cytokines to attract the innate inflammatory cells. The persistence of this inflammatory reaction could be the key for the development of COPD.

cells (22). These findings indicate that infectious and irritant stimuli similarly affect epithelial cells toward the production of proinflammatory mediators and the induction of an innate immune response.

Another consequence of the injury of the epithelium by cigarette smoke and the resultant increase in epithelial permeability (21,23) is the production and release of tachykinins (substance P and neurokinin A). These neuropeptides are synthesized by sensory neurons and stored in the terminal parts of the axon collaterals found beneath and within the epithelium, around blood vessels, submucosal glands, and within muscle layer of the airways. The release of tachykinins from sensory nerves can be evoked by a variety of stimuli including cigarette smoke (24,25) and modulate a number of important immunological functions like T-cell proliferation (26) lymphocyte traffic (27), and cytokine production including IL-1, IL-3, IL-6, IL-10, IL-12, and TNF-α (28,29). Thus, the bronchial epithelium, in addition to acting as a physicochemical barrier, plays a crucial role in initiating pulmonary host defense mechanisms, both in health and in disease, by synthesizing and releasing a variety of mediators that can cause an innate immunity inflammatory cell differentiation, chemotaxis, and cell activation.

C. Consequences of the Innate Immune Reaction in the Lungs—Peptides, Apoptosis, and Necrosis

The sustained innate immune response found in the lungs of cigarette smokers (neutrophils, macrophages, eosinophils, mast cells, $\gamma\delta$ T-cells, DCs, and maybe NK cells), and their products (cytokines, oxygen radicals, and proteinases) are capable of producing matrix and cellular damage. Studies in mice have shown that after 24 hr of cigarette-smoke exposure, measurable increases in desmosine, a marker of elastin breakdown, and hydroxyproline, a marker of collagen breakdown, are found in BAL and their levels are correlated with the number of neutrophils in the BAL (11,30). Evidence of connective tissue breakdown in human smokers also exists. Increased plasma and urine levels of elastin-derived peptides and desmosine (31,32) have been found in COPD patients when compared with nonsmokers (33–36). However, the levels of elastin-breakdown products are also elevated in smokers without COPD (34,37–40). Smokers with rapid decline in lung function, likely to develop COPD, were found to excrete 36% more desmosine than low decliners (41,42). It could be concluded that proteases, probably derived from inflammation cells, induce the breakdown of the connective tissue network.

Despite the evidence, the concept of a protease–antiprotease imbalance as the main cause for the production of COPD does not explain why only some smokers develop COPD. It might be closer to reality to consider the connective tissue breakdown as one of the many consequences of the innate inflammatory reaction triggered by smoking. Furthermore, these breakdown products or peptides could be considered as "self-peptides" that could behave as self-antigens and be presented to T-cells, eventually triggering, in some smokers, a T-cell adaptive immune response against lung antigens (autoimmunity).

There is extensive literature investigating the possible role of infectious and environmental agents in the production of autoimmune reactions. A common conclusion, easily applicable to cigarette smoking, is that infectious and environmental agents have a great potential for altering self-proteins that could then be recognized as antigens by the adaptive immune system. Thus, a modified self-determinant could have the ability to elicit an autoimmune T-cell response while the self-determinant could not (42,43).

Among the important protein modifiers present in smokers are free radicals/oxidative stress. Both nitric oxide (NO)—by itself or combined with superoxide to form the potent oxidizing agent peroxynitrite—and other reactive oxygen species (ROS) can be strong protein modifiers and thus antigen producers. Nitric oxide and ROS may affect different cellular functions and result in cell-death, together with mitochondrial damage, DNA strand breaks, and structural/functional modification of proteins (43).

Oxidative modification of proteins has been implicated in the immune mechanism of various diseases like rheumatoid arthritis, multiple sclerosis, autoimmune antiphospholipid antibody syndrome, diabetes mellitus (42,43), and, lately, arteriosclerosis, in which epitopes generated in the process of atherogenesis, such as those produced by the oxidation of low-density lipoproteins, have been implicated as targets of autoimmunity (44,45). This is, to date, the most clear and unexpected example of how modified self-proteins can become antigenic and produce disease, a common disease.

Other external and xenobiotic agents have been implicated in the modification of self-proteins to be recognized as antigenic material. Importantly, xenobiotics, of which cigarette smoke is abundant, can interact with self-proteins and generate immunogenic determinants through various mechanisms, including covalent binding or noncovalent modifications (43). Induction of organ-specific autoimmune disease following tissue inflammation, and/or trauma has been frequently reported and likely occurs via tissue damage that results in the availability of previously isolated antigens, as is the case in ophthalmia following eye injury (46) or orchiditis following vasectomy (42). Infections with tissue tropic pathogens such as viruses may induce a similar autoimmune phenomenon, and also provide abundant cytokines and costimulatory molecules, the additional stimulus important in the perpetration of the immune response. This is exemplified in rodents and humans who develop diabetes after infection with Coxsackie B viruses and rheumatic fever after streptococcal infections (42,47).

Cigarette smoke could easily follow the same mechanisms. The tissue injury and potential modification of self-proteins could generate antigens and the associated innate immune inflammation associated with smoking could provide the necessary soluble mediators and co-stimulatory molecules for the initiation and perpetration of an adaptive immune response. It is important to recognize that in a disease like COPD, in which multiple mechanisms could induce different antigens (e.g., release of cryptic antigens, modified proteins, necrotic cells, apoptotic cells, etc.), more than one antigen could be involved in the generation of an immune response, and more than one might be responsible for the T-cell activation. This phenomenon has been described in insulin-dependent diabetes, where large numbers of autoantibodies and autoantigens have been found (48).

Besides the breakdown of elastin and collagen, cell injury mediated by proteinases could also be a source of antigenic material. Cellular survival seems to depend on signals from the integrin family of adhesion receptors continuously sensing the extracellular milieu (49,50), and a major signal for cellular apoptosis is loss of extracellular matrix contact that can be induced by proteases (51). Therefore, it is likely that lung injury in cigarette smokers involves apoptosis and/or necrosis of cells in the alveolar wall (epithelial and endothelial) following focal proteolytic damage to their underlying matrix (apoptosis) or directly to the cells themselves (necrosis)

(49). Apoptotic cells and/or their products are normally handled by antigen-presenting cells (APCs) and can act as antigens and stimulate an adaptive immune reaction mediated by the major histocompatibility complex (MHC) Class I molecules and priming CD8+ T-cells (see later). Increased apoptosis in emphysema in humans has been well documented (8).

Another direct consequence of exposure to cigarette smoke is necrosis of epithelial and endothelial cells. After exposure to tobacco smoke in vitro, a 38% reduction of the cell population in culture can be observed (18) probably as a result of necrosis. This was confirmed by a recent report showing that necrosis of epithelial and endothelial cells resulted after exposure to cigarette smoke condensates, probably as an effect of oxidative stress (52). Cellular necrosis releases the cytoplasmic contents, including the peptide fingerprint of the cell (including the antigenic fingerprint), and heat shock proteins (HSPs). The HSPs chaperone these peptides for presentation to DCs, where they are cross-presented to MHC Class I molecules and could activate CD8+ T-lymphocytes (see below).

An important requirement for the initiation of a successful adaptive immune reaction is the prevalence, and persistence, together with the dose response of the offending agent. In cigarette smoking, the insult prevails daily and persists for years largely predisposing to a high prevalence of disease.

It can be seen that the innate immune response, elicited by exposure to cigarette smoke, could produce a considerable amount of potential antigenic material (peptides, apoptotic, and necrotic debris) that ought to follow the rules set for the full immune reaction; i.e., innate immunity is followed by adaptive immunity, when it cannot handle the "pathogens" on its own. The difference between pathogens, the original reason for the evolution of the immune response, and exposure to pollutants (cigarette smoke, ozone, etc.) is that after pollutants trigger the innate immune reaction, the innate reaction itself becomes harmful, injures the lung, and has the potential to trigger an autoimmune reaction by "successfully" engaging the adaptive immune response. It would seem that the ability to suppress the development of an adaptive immune response would arrest this chain of events and could prevent the development of COPD (Fig. 2).

III. ADAPTIVE IMMUNITY

A. Dendritic Cells

We have seen that innate immunity specifically recognizes conserved molecules from pathogens, or danger signals from cells, through a family of receptors. This recognition by the immune system has two major effects. First, it triggers effector cells of inflammation, neutrophils, macrophages, and other cells, which represent an immediate defense at the sites of

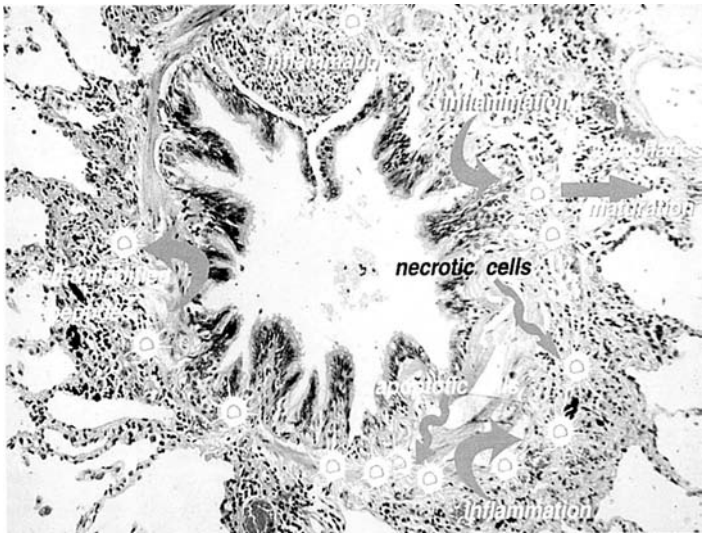

Figure 2 Consequences of the innate inflammatory response and cigarette smoke components on the lung (represented as an airway). Inflammation and smoke products could break down tissues, and induce cell apoptosis and necrosis. These products have potential antigenic determinants that will be taken up by the numerous dendritic cells (DCs). If the innate inflammation is "competent," it would create the proper microenvironment for the DCs to mature and migrate to the draining lymph nodes where they could present the antigens resulting from lung injury to T-cells.

pathogen entry. Second, the innate immune system can induce an adaptive immune response. Dendritic cells are the key cellular links between the innate and adaptive immunity playing a pivotal role as sensors of infection or injury for the initiation of adaptive immune responses (53).

It has been shown that recruitment of a wave of DCs into the respiratory tract mucosa is a universal feature of the acute cellular response to local challenge with bacterial, viral, and soluble protein antigens (54). This suggests that rapid amplification of specific antigen surveillance at peripheral challenge sites is an integral feature of the innate immune response, and serves as an "early warning system" to alert the adaptive immune system to incoming pathogens or body injury. It seems, therefore, that DCs may be evolution's answer to the problem posed by organisms that evade primitive (innate) first line defense systems (54). There is evidence in the literature that cigarette smoking is associated with an expansion in the DC population in the lower respiratory tract (55) and with a marked increase in the number of mature cells in the lung parenchyma (56). This is an indication that the lung response to cigarette-smoke exposure follows the established immune

response design, including innate immunity and readiness for an adaptive immune response, if necessary.

Dendritic cells respond to two types of signals: direct recognition of pathogens (through specific pattern-recognition receptors) and "danger signals," an indirect sensing of infection and/or injury (through inflammatory cytokines, internal cellular compounds, and ongoing specific immune responses) (57). In response to these signals, DCs are activated to enter an integrated developmental program called maturation, which transforms immature DC into efficient APCs and T-cell stimulators, and are, therefore, responsible for the development of an adaptive immune response. (see Reference 53 for a full review.)

The stimulation of a variety of surface receptors in the DC are known to trigger DC (53) maturation and antigen presentation: pathogenic compounds, inflammatory mediators such as TNF-α, IL-lβ, PGE-2, GM-CSF, immunoglobulins, T-cell derived signals mainly CD40L, and cell death, both necrotic and apoptotic (53,58–60). Heat shock proteins released by necrotic and injured cells are also important inducers of DC maturation (57). The innate immune reaction in smokers has been shown to be accompanied by many of the inflammatory mediators listed here, and could easily provide the necessary costimulation for DC maturation.

B. Heat Shock Proteins

The HSPs are reviewed here in some detail because they could have a primordial role in COPD, being responsible for the abundance of CD8+ T-cells in the disease, and the development of autoimmunity (61,62).

Genes that encoded HSPs were identified serendipitously in fruit flies that were inadvertently exposed to high temperature (63). These genes, and the proteins encoded by them, are present in all cells in all forms of life. The HSPs can be classified into 10 families with 1–5 closely related proteins in each. They constitute up to 5% of the total intracellular proteins; however, under stresses like exposure to high temperatures, toxins, oxidative conditions, etc., their intracellular levels can rise to 15% or more (57). Heat shock proteins are not normally present in the blood or other body fluids, nor are they expressed on the cell surface under normal conditions. Therefore, the presence of HSPs in the extracellular milieu would act as an excellent message that alerts the immune system to physical damage in the tissue, whether as a consequence of bacterial, chemical, mechanical, or oxidative injury. This chain of events would also explain the presence of a surface receptor for HSPs, which are normally intracellular, in APCs (57).

The immunological functions of HSPs began to emerge in the 1980s when it was observed that certain HSPs isolated from cancer cells elicited immunity to cancers where corresponding preparations from normal tissues did not (64). Immunogenicity of tumor-derived HSPs was the result of the

association of HSPs molecules with peptides that were generated by the degradation of proteins expressed by the cells from which the HSPs were purified (65–68). This suggests that the HSPs chaperone the peptide fingerprint, which includes the antigenic fingerprint, of the cells from which they are isolated and these complexes have the capability of eliciting MHC Class I restricted antigen-specific CD8+ T-cell responses, a phenomenon called cross-priming or cross-presentation (69).

It is now clearly established that the HSPs, as well as the major histocompatibility (MH) molecules, are peptide-binding proteins, with HSPs being more promiscuous than MHC, the difference in promiscuity having important physiological consequences. The difference in the stringency of requirements for peptide binding by the two make it possible for one (the HSPs) to bind to any available peptide and to render them presentable to the other (the MHC). The ability of the HSPs to scan the entire repertoire of intracellular proteins allows them to have a key role as informers of the MHC molecules (and therefore of the T-cells) with respect to intracellular pathogens (or pathogenic events). Thus, the HSPs carry peptides generated within cells, mediating transfer to the MHC Class I molecules of that cell (normal or ''intrinsic'' MHC I antigen presentation); if the cell is lysed, the HSPs within the cell are released and carry the chaperoned peptides, to the MHC molecules of the neighboring DCs: cross-presentation (or ''extrinsic'' presentation), to MHC I (57) (see below).

In addition to the targeting of chaperoned peptides for presentation, the interaction of HSPs (without peptides) with APCs leads to peptide-independent activation of innate immune mechanisms (61). Acting through toll receptors TLR2, TLR4, and CD14 (70–75), they can induce secretion of inflammatory cytokines TNF-α, IL-1β, IL-12, and GM-CSF by macrophages (70); induction of inducible nitric oxide synthase (iNOS) and production of NO by macrophages and DCs (76); secretion of chemokines such as monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), and RANTES by T-cells (77,78); maturation of dendritic cells, as measured by enhanced expression of MHC Class II, CD886 and CD40 (70,79–81); migration of DCs to draining lymph nodes (82) and translocation of nuclear factor-κB (NF-κB) into the nuclei of macrophage and DCs (70) perhaps the proximal event that mediates many of the events listed. In many of these activities, HSPs are strikingly reminiscent of bacterial lipopolysaccharides (LPS) or endotoxins, which exert powerful effects on APCs (57) (Fig. 3).

The presence of HSPs in the extracellular milieu would, therefore, act as an excellent message that alerts the DCs to physical damage of the surrounding cells, whether as a consequence of bacterial, viral, mechanical, or any other type of injury, and might therefore confer an immunological, and hence survival advantage to the organism. However, under special circumstances, HSPs have the potential to promote autoimmunity by serving

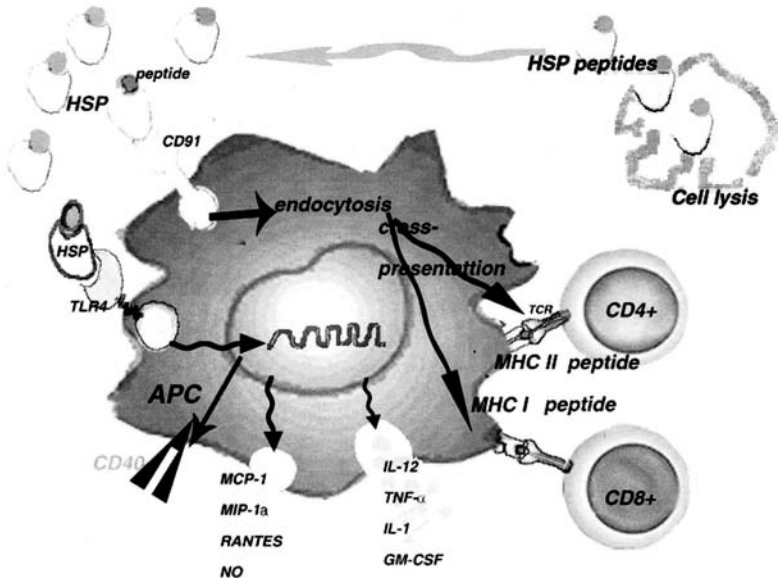

Figure 3 Role of HSP in peptide handling and presentation. HSP are promiscuous intracellular chaperones able to bind any peptides. They are only released after cell lysis and can carry the cell potential antigenic fingerprints. They have the ability to cross-present antigens using DC receptors MHC Class I-CD8+ and MHC II-CD4+ T-cells, thus both can be activated simultaneously. Through the toll receptor TLR4, they can activate the DC to produce the necessary costimulatory molecules for effective presentation and T-cell activation.

as endogenous signals for DCs maturation and having a role in the regulation of immunity versus tolerance (61,62).

C. The Adaptive Immune Reaction in COPD

In the early studies investigating the BAL of smokers, T-cells were found to be increased in the lungs of patients with COPD (6). However, the possible relevance of that finding was not explored at the time.

The study that initiated the present interest in the T-cell as a possible important cell in the pathogenesis of COPD was carried out by Finkelstein et al. in 1995 (7). These authors used immunochemistry, to identify the inflammatory cells infiltrating the alveolar wall, and morphometry, to define the extent of emphysema in smokers and nonsmokers undergoing lung resection. Their findings were surprising at the time, as they reported that the most prominent inflammatory cell in the lung parenchyma of smokers was the CD3+ T-lymphocyte that increased from a mean of 1546 cells/mm^3 in nonsmokers up to 10,000 cells/mm^3 in some smokers. Furthermore, a

clear correlation between the number of CD3+ T-cells and the extent of emphysema was found, certainly suggestive of the protagonism of the T-lymphocytes in the pathogenesis of emphysema in smokers (Fig. 4). Emphysema was also associated with the presence of increased numbers of alveolar macrophages, which significantly correlated with the numbers of T-cells, suggesting an interaction between these cells in the inflammatory process leading to emphysema, possibly mediated by a T-helper type 1 (Th-1) type of T-cell response (IFN-γ, TNF-α, IL-2 cytotoxine profile).

Abundant but variable numbers of T-cells (CD3+) together with other inflammatory cells are also found in the small airway of smokers (83). We studied the inflammatory infiltrates in a series of smokers and nonsmokers who had airway reactivity measured before undergoing lung resection (84). Smokers were divided according to the type of emphysema in the lung, CLE, or PLE. Due to the large variability in the numbers of inflammatory cells in the airways (from 0 to 500,000/mm^3), no statistical difference was found in the numbers of the different inflammatory cells in nonsmokers' and smokers' airways. However, the degree of airway reactivity correlated with the load of T-cells in the airways in smokers with CLE, but not in PLE or nonsmokers. Because similar total numbers of CD3+ T-cells were present in the three groups, we suggested that the T-cells in CLE were behaving differently possibly because they were of a different phenotype (Th-2 vs. Th-1). In support of this possibility is the finding that Th-2 type cytokines IL-4 and IL-5 mRNA (cytokines found in asthma) are abundantly expressed by

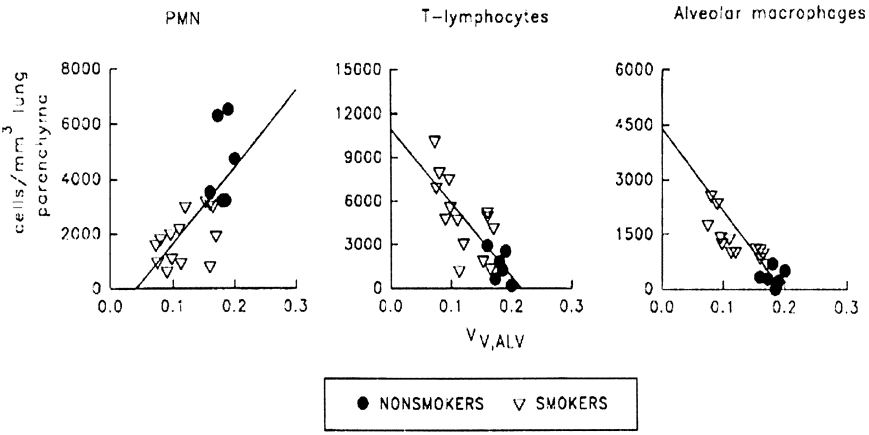

Figure 4 Correlation between the number of neutrophils (PMN), T-cells (CD3+), and alveolar macrophages (cells/mm^3) in the alveolar wall and emphysema (V_{V1} alv). V_v alv refers to the density of alveolar wall and was obtained by point counting. The lower the number, the less the alveolar wall density, and the larger the emphysema. All the correlations were significant with $p < 0.05$ or lower. (From Ref. 7.)

inflammatory cells in the wall of large airways in smokers with chronic bronchitis and COPD (85). These cytokines play an important role in the development of asthma, a disease characterized by an increase in airway reactivity; hence, it is possible that clones of T-cells in smokers with CLE could express a Th-2 cytokine profile that might induce airway reactivity in these cases. Furthermore, IL-4 has been shown to have profibrotic effects by stimulating fibroblasts to proliferate and secrete collagen (86) and this profibrotic role might be of relevance to the increased remodeling with fibrosis in the small airways and in the increased fibrosis in areas of emphysema found commonly in patients with CLE (84). Further characterization of the T-cell Th phenotype in the small airways of COPD would be necessary to confirm these possibilities.

Following the report by Finkelstein et al. (84) proposing a role for the T-cell in COPD, several authors identified the CD8+ T-cell as the predominant lymphocyte in the airways of smokers with COPD. O'Shaughnessy et al. (87), studying the large airways, and Saetta et al. (88), the small airways, showed that the only significant difference in the inflammatory cell infiltrate in asymptomatic smokers and smokers with COPD was the increase in CD8+ T-cells in patients with COPD. Furthermore, the number of CD8+ T-cells was negatively correlated with the degree of airflow obstruction, as measured by the FEV_1, again suggesting a possible role for these cells in the pathogenesis of the disease.

The CD4+ T-cells are also found, albeit in smaller numbers, in the airways of smokers with COPD and these cells express activated signal transducer and activator of transcription 4 (STAT-4), a transcription factor that is essential for activation and commitment of the Th-1 lineage. Not surprisingly, the number of CD4+ T-cells expressing activated STAT-4 was associated with the cells expressing IFN-γ, and activated STAT-4 lymphocytes correlated with the degree of airflow obstruction (89). These findings strongly support the idea that COPD is mediated by an active Th-1 immune reaction in the lung comprising both CD8+ and CD4+ T-cells.

Lams et al. (90,91) studying the small and large airways of smokers reported, as Costabel et al. (92) had years before in BAL, that the CD8+/CD3+ ratio in the airways increase as the amount smoked increased, and this ratio decreased after smoking cessation (90). These findings are very suggestive of a direct relationship between smoking and the recruitment and possible involvement of the CD8+ T-cell, and in consequence the adaptive immune system, in smokers.

Increased numbers of CD8+ T-cells can also be found in the adventitia of small pulmonary arteries of smokers when compared with nonsmokers, and this inflammation is greater in smokers with COPD (93,94). The significance of this finding is unclear, but it has been suggested that it might be related to the arterial changes seen in smokers.

The CD8+ T-cells are also the predominant T-cells infiltrating the alveolar wall in smokers with COPD (8,93), although CD4+ T-cells are also increased (8,95). Majo, studying lungs from nonsmokers and smokers obtained at surgery, found that, similar to the airways (88), the only measurable difference between smokers with and without COPD was a substantial increase in the number of total CD3+ and CD8+ T-cells in the alveolar wall of smokers with COPD (Fig. 5). Furthermore, the total number of T-cells, both CD8+ and CD4+, increased with the amount smoked in smokers with COPD, but not in healthy smokers, suggesting that the CD4+ T-cell is also involved in the inflammatory process in COPD. This is an expected finding, as CD4+ T-cell help is required for the cross-priming of CD8+ cytotoxic T-cell responses, for maintaining their memory and for ensuring their survival (see later).

Majo et al. (8), reasoning that as cytolytic CD8+ T-cells were involved apoptosis should be present, quantitated and found an increased number of apoptotic cells in the lungs of smokers with COPD which correlated with the numbers of CD8+ cytolitic T-cells in the alveolar wall. This and other reports, showing an increased number of structural lung cells undergoing apoptosis in emphysematous lungs (96–98), support the idea that CD8+

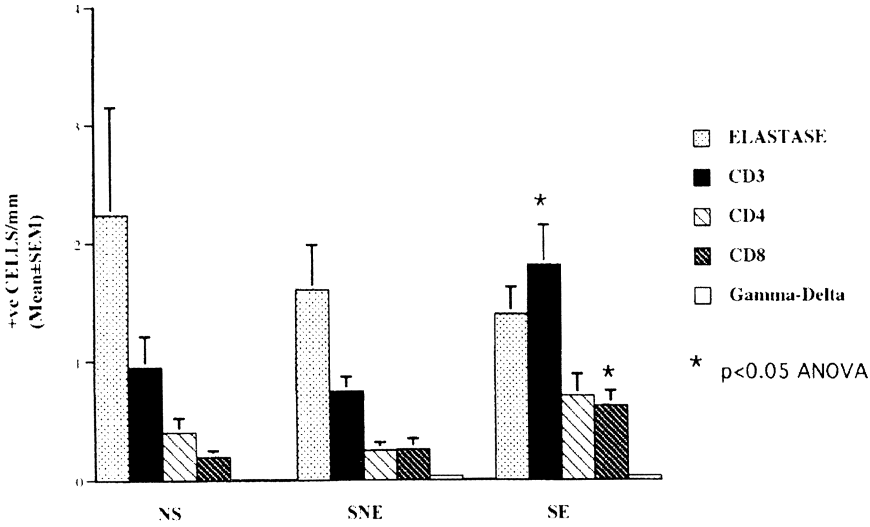

Figure 5 Inflammatory cells in the alveolar wall (cells/mm alveolar wall) in nonsmokers (NS), smokers without emphysema (SNE), and smokers with emphysema (SE). Immunostains were used to identify neutrophils (elastase) all CD3+(total total T-cells), CD4+, CD8+, and $\gamma\delta$ T-cells. The distribution of inflammatory cells differs from nonsmokers only in smokers with emphysema ($p < 0.05$ by ANOVA). (From Ref. 8.)

T-cells are inducing apoptosis of endothelial and epithelial cells in emphysema.

Majo et al. also reported, for the first time, the presence of $\gamma\delta$ T-cells in the lungs of smokers, with and without emphysema, a lymphocyte previously described in the mucosal surfaces of the gut. $\gamma\delta$ cells are preferentially associated with epithelial tissue and differ strikingly in function from $\alpha\beta$ T-cells. They recognize a large number of diverse antigens without clonal expansion, and it is believed that tissue-associated subsets of $\gamma\delta$ cells respond to tissue-specific "stress antigens" derived primarily from the epithelium. The role of these cells is complex, and they seem to have the ability to influence other immune cells and hence the course and outcome of a variety of inflammatory responses (99). Early in an inflammatory process, there is a large increase of (Vγ 4+) proinflammatory $\gamma\delta$ cells that express INF-γ and induce PMN accumulation in the lung. Later in an inflammatory process, the (Vγ 1+)$\gamma\delta$ cell can have an immunoregulatory role, express IL-4 and suppress inflammation (100). We have also found large numbers of $\gamma\delta$ cells in the lungs of mice exposed to cigarette smoke (101). The role of these cells in the development, or lack of, cigarette-induced disease in mice, and humans, might be very important, and could be investigated in the mouse model.

Recent studies looking at smokers with severe COPD demonstrated that the relatively mild infiltration with CD8+ and CD4+ T-cells found in patients with mild-to-moderate disease increases markedly in the lungs (95) and airways (102) of severely diseased patients. All inflammatory cells, except B-lymphocytes, were found to be increased in the lungs of patients with severe emphysema, even though these patients had not smoked more than the control subjects. By far, the more numerous cells were the CD4+ T-cell ($330 \pm 58 \times 10^{12}$) and the CD8+ T-cell ($250 \pm 51 \times 10^{12}$), but neutrophils, macrophages, and even eosinophils were also increased. A relationship between the degree of emphysema and the numbers of each cell type present in the tissue, strongest ($R^2 > 0.8$) for CD4+ CD8+ and alveolar macrophages, was found (93,95). These studies are of importance showing that, in COPD, inflammation with an abundance of T-lymphocytes and other inflammatory cells continues late into the disease process.

An important finding in one of these studies was that the average duration of smoking cessation in the severe COPD subjects was 9.2 years (103) indicating that the adaptive immune system continues to be involved after smoking cessation. Possibly the antigenic stimuli are still present (necrotic and apoptotic cells, extracellular matrix proteolytic fragments, etc.), and/or, as suggested by Retamales et al. (95), viral, and possibly bacterial, infections could act as costimulators [or by antigenic mimicry or polyclonal activators (42)], to keep the process going. We have recently reviewed how this phenomenon is typically found in other autoimmune diseases again emphasizing the role of autoimmunity in COPD (104).

Recently, we have shown that some mice strains exposed to cigarette smoke can develop emphysema and that the severity of emphysema, as assessed by the airspace enlargement and loss of recoil, seems to depend on the ability of the strain to sustain an innate immune response and to mount an adaptive immune response with CD4+ and CD8+ T-cells (101). The finding of animal models that follow the same pattern of disease as humans when exposed to cigarette smoke is important, as it would simplify the investigation of the pathogenic factors in general, and the role of the T-cells in particular, in the pathogenesis of COPD in humans.

There is, as we have seen, overwhelming evidence showing the presence of T-cells in the lungs in COPD and that these cells are activated. According to the present concepts of T-cells physiology (reviewed in this chapter), if the T-cells, alone or together with other inflammatory cells, were responsible for the lung injury and progression of COPD, it would be as a response to an antigenic stimulus originating in the lung. Hence, COPD would have to be considered an autoimmune disease triggered by smoking, as previously suggested (8,9,104).

IV. SYSTEMIC T-CELLS IN COPD

In an attempt to understand why only some smokers develop COPD, several authors have studied the T-cell population in the peripheral blood of smokers. In light-to-moderate smokers, the total number of T-cells and CD4+ lymphocytes appears to be increased. In heavy smokers, a relative decrease in CD4+ and an increase in CD8+ with a low CD4+/CD8+ ratio have been found by some authors (105,106), but not by others (107,108).

In one of these studies (107), CD8+ T-cells were increased only in smokers with COPD and normal diffusion capacity and the CD4+/CD8+ ratio in peripheral blood correlated with the FEV_1/FVC in these patients. These findings were not seen in patients with low diffusion capacity. The authors suggested that the group with normal diffusion capacity and airflow limitation might represent a group with severe small airway disease, and probably CLE, but without sufficient emphysema to decrease the surface area for diffusion. The group with low diffusion capacity might represent predominant emphysema, probably panlobular (PLE), smokers with less airway abnormalities, in which airflow limitation is mainly secondary to losses of elastic recoil (109). In favor of this possibility is the fact that the T-cell load in small airways of patients with COPD is an order of magnitude larger than in lung parenchyma. The mean T-cell number per mm^3 of airway wall is 53,672 with a median of 33,000 and a maximum of 348,000 cells in patients with CLE and these numbers are lower in PLE. In contrast, the mean T-cell number per mm^3 of alveolar wall was 5,000 with the highest count found in the patient with the most severe emphysema of 10,000 T-cells

per mm3 (7,84). The increase of CD8+ T-cells in the peripheral blood might be a reflection of the increase in the T-cells in the lung, that might "spill over" (107) or more likely to increase transit of the activated T-cells toward the lung. If that were the case patients with more cells in the lung would also have more cells in the peripheral blood.

The inconsistency in the finding of CD8+ increases in peripheral blood should not be surprising. These cells are usually expressed as percentages of total T-cells or CD4+/CD8+ ratios; as CD4+ T-cells, especially in patients with severe COPD, are also increased in the lung, they should follow the same path as CD8+ and also spill over or be found in transit in the peripheral blood. In favor of this possibility are the findings of Majori et al. (110). They reported that there was no difference between the percentage of T-cells in the peripheral blood in patients with COPD and controls. However, the percentage of CD4+ T-cells expressing intracellular IFN-γ was significantly higher in the COPD group, while the percentage of CD4+ T-cells expressing intracellular IL-4 was significantly lower. These findings indicate that the CD4+ T-cells in smokers, expressing IFN-γ are activated, and consequently increased in numbers in the lung and could spill over or, more likely, are in transit in the peripheral blood. This increase in the CD4+ T-cells in the peripheral blood, together with increases in CD8+, will keep the CD4+/CD8+ ratio unchanged.

It would be important to know if the CD4+ and CD8+ T-cells in the peripheral blood are just cells "in transit," homing to the lung, or activated T-cells with a "systemic mission" and capable of producing disease beyond the lung. The possible association of any of these findings with the development of the systemic manifestations in COPD would be an important field for investigation (111).

In contrast to the findings in active smokers, de Jong et al. (108)—and later Hodge et al. (112)—reported that ex-smokers with COPD clearly have an increase in CD8+ T-cells and decrease in CD4+/CD8+ ratio, while active smokers with COPD were no different than the controls. The excess CD8+ cells might represent a population of memory effector cells able to circulate in the peripheral blood. The number of central memory and memory effector cells is proportional to the number of effector cells generated after antigen presentation by DCs and as the number of CD8+ effectors are orders of magnitude larger than CD4+, it would not be surprising that CD8+ effector memory cells outnumber CD4+ in the peripheral blood. In favor of this interpretation are the findings of Hodge et al. (l12). These authors showed an increased propensity of peripheral blood T-cells (CD4+ and CD8+) in COPD to undergo apoptosis, most likely due to increased Fas, TNF-α, and TNF-R1 expression by these T-cells, an event most likely related to the state of activation of these cells, as the study of Majori et al. (110) above also showed.

V. T-CELLS AS EFFECTOR CELLS

A. T-Cell Traffic

Based on the present knowledge of the immune system (inflammation) and the interaction of the innate and adaptive immune systems towards fighting an attack to the host, the presence of T-cells in COPD would be an expected event. Furthermore, it would have been surprising if T-cells had not been part of the inflammatory component of the disease.

A role for T-cells in causing a particular immunological disease is suspected largely because of the demonstrations of T-cells in the diseased organ (10). The reasons for suspecting a T-cell-mediated injury when the number of these cells is increased in an affected organ are based on the principles that govern lymphocyte traffic homing and homeostasis. Most mature lymphocytes recirculate continuously going from blood to tissue and back to blood again as often as one to two times per day (113). Recirculation is not random but rather is targeted by active mechanisms of lymphocyte–endothelial cell recognition (114–117) that, together with the subsequent diapedesis across the vascular wall, direct lymphocyte homing from the blood (115–117) controlling the access of specialized lymphocyte subsets to particular tissues. Its specificity depends on developmental tissue and inflammation-specific specialization of vascular phenotypes and on developmental and microenvironmental regulation of lymphocyte homing and chemoattraction receptors (115–119).

There is an important dichotomy in lymphocyte trafficking between naïve (nonstimulated) and memory/effector (stimulated by antigen presentation) lymphocytes (115,117,120). Naïve lymphocytes are programmed to recirculate through secondary lymphoid tissues (lymph nodes, Peyer's patches, tonsils, and spleen) and cannot enter normal tissue (121). The secondary lymphoid tissues collect antigens from epithelial surfaces, somatic tissues, and blood and present it to naïve T- (and B-) cells in the context of specialized microenvironments that can drive their antigen-induced differentiation (122). Moreover, whereas the homing behavior of naïve T-cells is relatively homogeneous, the homing behavior of memory and effector lymphocytes is extremely heterogeneous, with distinct subsets displaying restricted, often tissue selective patterns of recirculation (115,117,120,123).

Among trafficking signals, chemokines and their receptors provide a central paradigm to understand the mechanisms regulating the tissue-specific recruitment of inflammatory cells. Although adhesion molecule expression is fundamental in the localization of leukocytes to inflamed tissue, adhesion molecules are only partially responsible for the preferential recruitment of specific leukocyte subsets during certain diseases. The migration of lymphocytes into tissues appears to depend on the expression of specific chemokines during the progression of the inflammatory disease (122).

When antigens are associated with an inflammatory stimulus, they trigger maturation and migration of DCs to tissue draining lymph nodes, where these cells produce IL-12 and induce development of Th-1 cells (124–126). IL-12-mediated induction of IFN-γ expression in T-cells is regulated by the transcription factor STAT-4 and T-box expressed in T-cells (T-bet) (127). Phosphorylation of STAT-4 is crucial for STAT-4 activation and translocation to the nucleus (128). Thus, IL-12/STAT-4 serves two essential functions in the development of activated Th-1 cells: as a growth signal-inducing survival and cell division and as a transactivator prolonging IFN-γ synthesis (129–131). It has recently been shown that CD4+ T-cells in smokers' lungs express phosphorylated STAT-4 and this was associated with the presence of IFN-γ in these cells indicating that they are activated effector CD4+ T-cells (89).

Homing receptor regulation during memory effector T-cell differentiation is analogous to (and temporally concomitant with) effector T-cell cytokine production (i.e., IFN-γ, IL-2 in the helper Th-1 subset) involving immunoregulatory cytokines, as well as the nature of antigenic and costimulatory signals (132). As lymphocytes must be positioned correctly to interact with other cells, the pattern of chemokine receptors, and the type and distribution of chemokines in tissues, will critically influence immune response (133–136). Imprinting, or selection, for tissue differential homing properties is determined by the local lymphoid organ microenvironment and begins almost immediately during the naïve-to-memory/effector T-cell transition, within one to two cell divisions in intestine-associated lymphoid tissues (137).

Recent studies have reported that T-cells trafficking to the human lung are distinct from either gut-homing or skin-homing T-cells and express chemokine receptors CXCR3, CCR5, and CCR4 (138). The production of chemokine receptors CXCR3 ligands IFN-γ-inducible protein-10 (IP-10), monokine induced by IFN-γ (Mig), and IFN-inducible T-cell chemoattractant (I-TAC) are specifically activated by IFN-γ and have critical roles in enhancing Th-1 T-cell recruitment and activation (139,140). Concomitant, preferential expression of the associated chemokine receptors on Th-1 T-cells has been demonstrated (141–143). Thus chemokine expression during a Th-1 response correlates directly with the specificity of the chemokine receptors expressed on Th-1 T-cells.

The function of these chemokines, induced in tissue by the T-cell activation, together with selectins and integrins, would be the extravasation and migration of leukocytes that express a large repertoire of chemokine receptors. These molecules exert most of their biological effects by binding to a large family of G-protein-coupled seven transmembrane receptors leading to activation of multiple intracellular signaling pathways (144). In addition to promoting leukocyte migration, chemokines are potent cellular activators (145,146).

The chemokine and chemokine receptor expression in asthma, a Th-2 T-cell-mediated disease, in sarcoidosis, a Th-1 disease, and in COPD have recently been described (138). In asthma, virtually all T-cells express IL-4 and CCR4, a receptor for CCR4-specific ligands macrophage-derived chemokine (MDC) and thymus and activation-regulated chemokine (TARC), which are strongly upregulated on airway epithelial cells. In sarcoidosis, IFN-γ, a Th-1 defining cytokine, was abundantly expressed together with CXCR3 chemokine receptors in the T-cells. No IL-4, CCR4, and CCR8 expression was detected in the T-cells of sarcoidosis lesion (138). These studies indicate that the recruitment of Th-2-activated and Th-1-activated T-cells into the lung uses very specific chemokines expressed at the moment of activation, after the T-cell develops a Th-2 or Th-1 commitment in different cytokine microenvironments.

Chemokines and T-cell receptors (TCRs) in smokers have been reported recently by Saetta et al. (147). All smokers, but mainly those with COPD, had significant expression of the CXCR3 receptor in the T-cells infiltrating the lung not found in nonsmokers. Furthermore, IFN-γ was coexpressed with CXCR3 in patients with COPD, and the interferon-induced protein-10 (IP-10), the ligand for the CXCR3 receptor, was strongly expressed in the airways and pulmonary arterioles in smokers with COPD, but not in smokers and nonsmoker controls. Importantly, the number of T-cells expressing CXCR3 in the lung, a chemokine receptor restricted to activated T-cells and NK cells (148,149), correlated inversely with the FEV_1/FVC ratio in smokers, suggesting that as the activated T-cells expressing CXCR3, and IFN-γ in the lung increase, there is an increase in lung damage and worsening of the lung function (147).

The T-cells in COPD did not express any of the chemokine receptors described in asthma (CCR4 and CCR8), indicating that, as with sarcoidosis, the infiltrating T-cells in COPD are activated, Th-1 committed, utilize Th-1 type of chemokines and receptors to home to the lung (138), and likely use Th-1 cytokines and functions (cytolysis) as effector tools to damage the lung tissue (Fig. 6).

These results are a strong indication that the T-cells in COPD which express phosphorylated STAT-4 and IFN-γ are effector cells, activated by antigenic peptides from the lung in the local lymphoid tissue, and homing back to the lung, the source of the antigens guided by Th-1 chemokines. The findings are another indication of an adaptive immune response taking place in the lung as a response to cigarette-smoke exposure and mediating tissue injury.

B. Activation of CD8+ T-Cells

The original role intended for the CD8+ cytotoxic T-lymphocytes (CTLs) was the killing of cells infected with intracellular pathogens such as viruses,

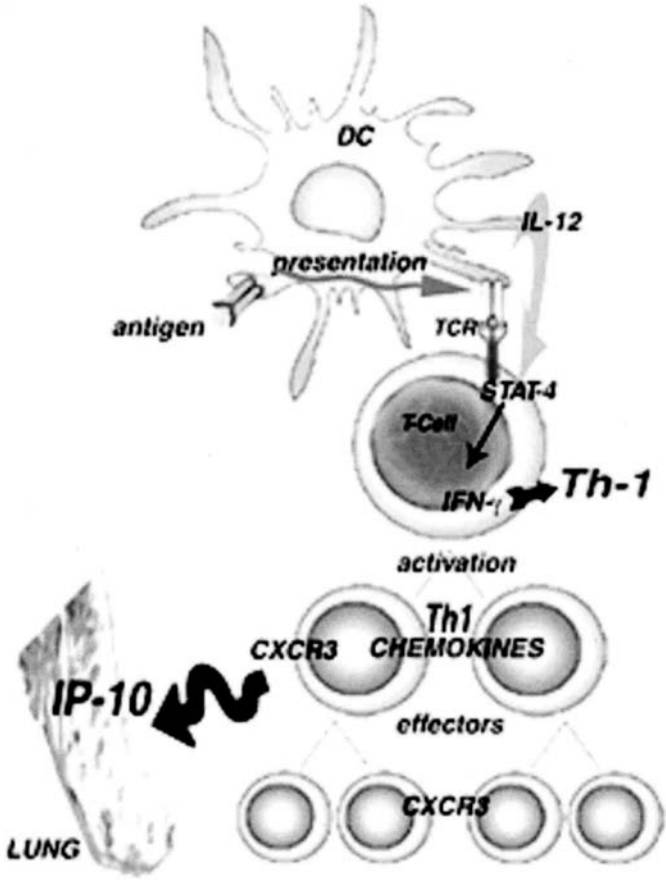

Figure 6 T-cell activation, Th-1 commitment and homing. During T-cell activation, the engagement of IL-12 produced by the DC or other APCs, with STAT-4 will induce the Th-1 phenotype in the T-cell. Once the Th-1 phenotype is defined, Th-1 chemokines and receptors are upregulated in tissues and cells directing the T-cell to the sites of inflammation: homing. Both these steps have been demonstrated in the lungs in COPD.

parasites, or bacteria. They recognize their target antigen as peptide fragments presented by products encoded within the MHC Class I loci by infected or injured cells. All nucleated cells can present Class I-associated peptides, derived from cytosolic protein antigens, to CD8+ CTLs because all nucleated cells express Class I MHC molecules. They are susceptible to viral infections and cancer causing mutations; therefore, it is important that the immune system be able to recognize cytosolic antigens harbored in any cell type (CTL-CD8).

The CD8+ CTLs recognize their target antigen as peptide fragments derived inside the cell and presented by the MHC Class I loci. Consequently, the MHC Class I-restricted presentation pathway is recognized as the "endogenous" pathway and this term distinguishes Class I presentation from the largely extracellular or "exogenous" MHC Class II-restricted presentation required for CD4+ T-helper (Th) cell recognition (10). Problems arise if the classical paradigm of "endogenous-only" Class I presentation is used to describe a CTL response to a virus, or other cell-derived antigen originating within a peripheral tissue like the lung. Naïve T-cells are not expected to enter nonlymphoid areas of the body, but rather recirculate between the secondary lymphoid organs (see above). To penetrate the lung harboring antigen-producing cells (like injured or virus-infected cells), CD8+ CTLs must be first primed in draining lymph nodes (CD8+ cannot be primed in the lung) and for this priming to take place antigen must make its way into draining lymph nodes (150). It is now evident that "exogenous" antigens can also access the MHC Class I processing pathway, and present antigens to the CD8+ T-cell, a phenomenon recognized as "cross-presentation" or "cross-priming" (151,152). By examining T-cell responses to antigens expressed exclusively in nonlymphoid compartments, in specially designed transgenic animals, it has been clearly shown that CD8+ T-cell activation occurs exclusively within the lymph nodes that drained the site of active antigen synthesis (150).

Cell-associated proteins (153,154) and particulate antigens (155,156) are much more effective at Class I-restricted CD8+ T-cells priming that are simple soluble molecules and it can be speculated that these antigenic forms mimic the tissue debris associated with viral infections. Cross-presentation might have been a mechanism primarily designed for peripheral viral infections to prime CTL responses in a central lymphoid compartment and for particulate antigens, in the form of damaged cells, to gain access to DCs (157).

There are a number of mechanisms that provide exogenous antigen access to the MHC Class I presentation pathway, the HSP pathways being the best described. Heat shock proteins (discussed above) are abundant soluble intracellular proteins that bind peptides including antigenic peptides generated within cells, and it has been conclusively shown (157) that HSP–peptide complexes elicit CD8+ T-cell responses in spite of exogenous administration, a route that typically would elicit CD4+ T-cell responses as they are normally presented through the "exogenous" MHC Class II pathway.

The ability of the HSP chaperoned peptides to cross-prime CD8+ T-cell responses is based on the ability of HSPs (gp96, HSP90, HSP70, and calreticulin) to interact with macrophages and DCs through common receptors, CD91 (158,159) and also TL-4 (159). This interaction leads to internalization of the complexes into a nonacidic endosomal compartment

followed by the transport of the complex to the cytosol (160–162), processing in the proteasomes, transporting to the ER, and then loading onto MHC Class I molecules and presenting to CD8+ T-cells (162). A relatively small proportion of the HSP–peptide complex internalized by the receptors enters an acidic compartment and is loaded into the MHC Class II molecules (64) leading to stimulation of CD4+ T-cell (163). This will allow for the simultaneous activation of CD4+ and CD8+ cells, an essential step in CD8+ activation, as we will see.

Heat shock proteins are released from cells undergoing lytic death, but not from cells dying of apoptosis (157,64) and necrotic cell lysates and also stressed cells have been reported to express cell surface HSP molecules (64,157) that could activate DCs directly. The HSP appear to be a universal mechanism for antigen capture and they permit a high-efficiency antigen uptake through a receptor-mediated mechanism.

Another newly described form of cross-presentation of antigenic material to the MHC Class I restricted pathway in DCs is by phagosomes (164,165). This finding extends the competence of phagosomes previously shown to be functional for the processing of MHC Class II complexes (166). Phagosomes are formed after the phagocytosis of microbes, apoptotic cells, and particulate matter by specialized cells: DCs, macrophages, and neutrophils. It has been found that soon after their formation phagosomes fuse with the endoplasmic reticulum (ER), site where MHC I molecules are synthesized. The ER–phagosome fusion defines a compartment that brings together all the MHC Class I processing and loading machinery, as well as exogenous antigens in a single subcellular organelle (165). By doing so DCs may focus all the cross-presentation machinery on the antigens relevant for the initiation of most immune responses, that is those acquired by phagocytosis.

Several important points arise from the mechanisms described. First, exogenous antigens derived from infectious agents, dying cells, proteins associated with inert particles (167,168), HSPs (84,169), and immune complexes (170,171) acquired in the lung can be cross-presented to the DCs in the draining lymph nodes and elicit a CD8+ response. Second, a mechanism exists to inform the DCs of two important events undergoing in the lung parenchyma: (a) cell injury and necrosis, which information could be carried by the HSPs; (b) apoptosis of parenchymal cells, which could be phagocytosed by DC and equally cross-presented (164,165) inducing a CD8+ response.

Another important fact about cross-presentation, mentioned before, is that the DC can present the same antigen through the MHC Class I pathway to CD8+ T-cells and through the MHC Class II pathway to the CD4+ T-cell (172,173). This is important because T-cell-dependent cytotoxic responses require the presentation of both Class II restricted and Class I restricted pathways by the same DC (174). This has led to the idea that Th

cells (CD4+) actively modify DC converting them into effective stimulators for induction of CTL from CD8+ naïve precursors (175,176). The CD4+ T-cells have been proposed to condition DCs for presentation of exogenous or endogenous antigens to CD8+ T-cells in a way that depends on ligation of the CD40 molecules on DCs (175,177,178) by the CD40 ligand expressed in CD4 T-cells. This cross-linking of CD40 molecules on DC leads to high levels of expression of Class II and costimulatory molecules and cytokine secretion by DC (179). The CD40-independent pathways have also been documented (Fig. 7) (180,181).

It has been reported that CD4+-independent antigenic stimulation of CD8+ T-cells, like some virus antigens, can prime CD8+ by achieving a

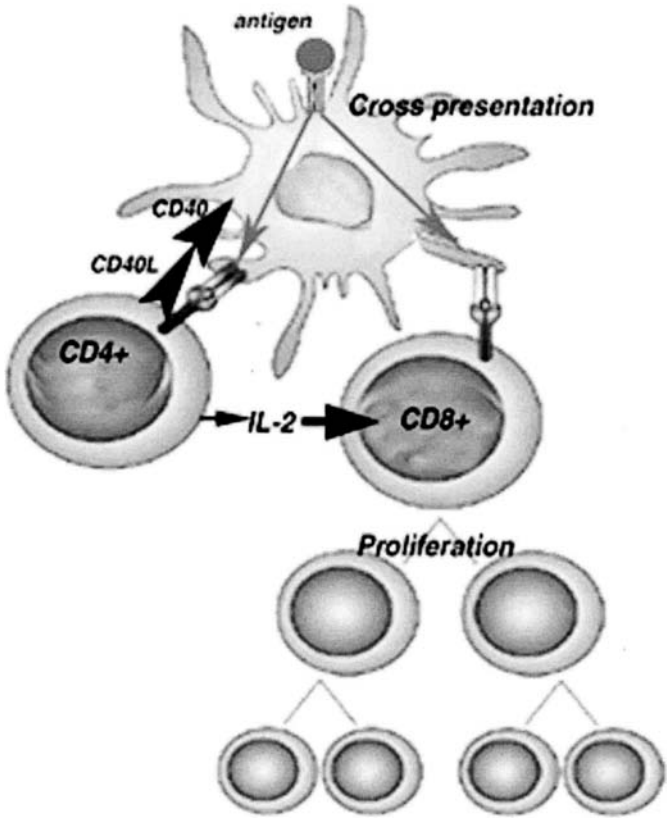

Figure 7 Ménage à trois. CD8+ T-cells need to be stimulated by CD4+ T-cells during antigen presentation from the DC, in order to become fully activated. This requires that the DCs present the same antigen to CD4+ and CD8+ T-cells, a step made possible by cross-presentation. This explains the presence of both CD8+ and CD4+ in the lungs of patients with COPD.

functionally equivalent degree of DC activation to that produced by CD4+ cells, either through direct infection or by provoking inflammatory host responses (176,182). However, even in those circumstances, secondary CD8+ (CTL) expansion (i.e., a secondary response upon re-encounter with antigen) is wholly dependent on the presence of Th CD4+ cells during, but not after, priming (183). Thus, it is now clear that T-cell help is required for the priming of cytotoxic T-cell responses, for maintaining CD8+ T-cell memory and for ensuring CD8+ T-cell survival (176,177,184,185).

As CD4+ T-cells are being activated by antigens, they would also undergo a clonal expansion with significant increases in numbers, albeit less than the CD8+, and this effector CD4+ would be found in increased numbers in the tissue source of the antigenic stimulus. It is not surprising that the CD8+ are the most numerous T-cells in the lung of patients with COPD, as once stimulated CD8+ T-cells undergo a much larger clonal expansion that the CD4+ T-cells (which can increase by several hundred fold). At the peak of a viral infection in mice, a 50,000- to 100, 000-fold increase in the number of antigen-specific CD8+ T-cells may be detected. Similarly, in humans infected with Epstein–Barr virus, up to 10% of the circulating CD8+ T-cells are specific for the virus (10). The remarkable clonal expansion of antigen-specific T-cells, with virtually no expansion of bystander T-cells (those not specific for the antigen) suggests tightly controlled activating signals and cellular responses.

It follows that it would not be totally correct to assume that COPD is solely a CD8+ T-cell-mediated abnormality, as CD4+ T-cells should also be present and increased, and once activated, they most likely exercise their effector functions along the CD8+ T-cell. At least three reports (8,9,102) have described increased numbers of CD4+ T-cells in the lungs of patients with COPD (reviewed above).

These findings, together with the previously described mechanism of CD8+ T-cell activation, suggest that the innate immunity triggered by cigarette smoking causes substantial damage to the cellular component of the lung with production of antigenic substances together with production of chemokines, cytokines, and other mediators. These antigens (self, sequestered, modified proteins, necrotic debris, and apoptotic cells) are taken by DCs which, under sufficient costimulators and mediators provided by the innate immune inflammation, undergo maturation and migration to the local lymphatic tissues. If the conditions are "propitious," these DCs will cross-present these "extrinsic" antigens by the MHC Class I pathway to CD8+ T-cells, and also to the CD4+ T-cells by the MHC Class II pathway. This "ménage à trois," CD4+, CD8+, and DC, would be able to stimulate each other producing important clonal expansions of CD8+ T-cells, the largest, and CD4+ T-cells to a lesser degree. Subsequently both cells would then "home" to the lung, the site of antigen source, where they will exercise their effector potential and damage the lung. This process would be

perpetrated as long as the offending agent, cigarettes, continues to originate an innate immune inflammation and antigenic products and sometimes, for reasons that are not clear, after smoking cessation.

C. Effector Functions of CD8+ and CD4+ T-Cells

The designed role for CD8+ T-cells is cytotoxicity. Naïve CD8+ CTL T-cell precursors have no cytotoxic activity and must undergo an activation process (described above) requiring 1–3 days for maximal activity. This process requires a TCR stimulated induction, with the co-operation of CD4+ T-cells, of cytokine receptors (e.g., IL-2 and IL-6). These cytokines then induce the expression of a large array of granule components (186), including (a) membrane-perturbing proteins—perforin and granulysin, (b) granule serine proteases—granzymes A, B and also C, and others, (c) perforing inhibitors—calveticulin and cathepsin b, and (d) stored T-cell effector molecules—Fas ligand and possibly β-chemokines (187). These granules then reside in the cytoplasm of the cell, where they await further instructions, i.e., their next encounter with MHC Class I antigen bearing target cells (in the tissue where antigens are originating).

After a cytolitic attack, target cells may die by necrosis, under the effects of perforin and probably granulysin (188), and/or apoptosis. Apoptosis can be mediated by granzymes A, B in a caspase dependent, and also independent manner targeting mitochondria and DNA degradation directly (187).

As most, if not all, nucleated cells can express MHC Class I molecules, any cell that, for some reason, presents antigens (virus infected, tumor, and damaged cell) can be the target for a CD8+ CTL, as long as the CD8+ T-cell has been primed by DCs for the antigen being presented. When granules are present, the killer cell orients its granules to the region of receptor activation (TCR–MHC-1 binding site) and releases the granule components into the region of contact between the killer and the target. This region of contact is initiated by the receptor TCR, and then organized into an ''immunological synapse'' formed by adhesion molecules (e.g., LFA-1 and ICAM-1 TCD-2) (187,188). The CD8+ CTLs can kill multiple cells by reorienting their granules to another region of contact, in contrast to natural killer cells (NKCs), the innate immunity lymphocyte killers, which must rearm themselves in response to IL-2 before they are effective against new targets (189). As reviewed before both necrotic debris and apoptotic cells are powerful sources of antigenic material that could reach the DC (or macrophages) by HSPs or phagosomes and perpetrate the T-cell response.

The T-cells (CD8+ and CD4+) can also kill their targets by the Fas-mediated pathways. Fas-(CD95) is a member of the TNF receptor family that is expressed on the surface of T-cells and many other cells, and initiates a signaling cascade leading to apoptotic cell death. The death pathway is

initiated when Fas binds to Fas-ligand, expressed on activated T-cells (190). This pathway appears to be active in all killer cell lineages but most important for CD4+ T-cells, especially those of the Th-1 phenotype (191). In vivo transplantation experiments suggest that the perforin/granzyme pathway dominates the MHC Class I elimination pathway (CD8+ CTLs) and that Fas/FasL dominates the MHC Class II (CD4+) elimination pathway (192,193). It has been proposed that the Fas pathway may have primarily an immunoregulatory role and to a lesser extent, an immune effector role (194,195).

In addition to killing infected, injured, or stressed cells directly, CD8+ T-cells also produce a number of cytokines including TNF-α lymphotoxin and IFN-γ, a so-called Tc-1 phenotype (Tc for cytolytic T-cell) and can behave as CD4+ Th-1 (for T-helper) lymphocytes. There is evidence that CD8+ in the lungs of COPD patients expresses IFN-γ (10,147), but interestingly no perforin expression has been documented in the lungs of patients with COPD. All these cytokines would enhance the inflammatory reaction in the lung besides the direct killing by CD8+ CTL.

There is experimental evidence of the unique role of CD8+ T-cells in causing lung damage and their capacity to recruit other inflammatory cells to the lung. Enelow et al. (196) examined the specific impact of autoantigen recognition by CTLs on pulmonary structure and function using a mouse model, expressing a transgene for influenza virus hemagglutining (HA) in the lung. Adoptive transfer of HA-specific CD8+ CTLs lead to the development of lethal injury in the transgenic mice and the injury was restricted to the lung. The earliest finding in the lung was a loss of alveolar epithelial cells, a result of direct cytolysis by CD8+ CTLs, followed by the accumulation of a mononuclear inflammatory infiltrate in the lung parenchyma, comprised mainly of alveolar macrophages, but also other inflammatory cells (197,198). It has been shown that the process of apoptosis, including that induced by CTLs, can be an important stimulus in the release of proinflammatory mediators and accounts for the important inflammatory infiltrate found in these studies (187,199).

The effector functions of the CD4+ T-cell are mainly mediated by cytokines, Th-1 in our discussion. Essentially, once T-cells (CD4+ and CD8+) are activated and home to the lung (in our case) source of the antigens, they stimulate much greater leukocyte migration, the so-called "immune inflammation" (to indicate the role of the T-cells in the process). Endothelial cells and alveolar macrophages are probably the two MHC Class II endowed cells that would attract CD4+ into the lung (10).

Once in the lung and activated, Th-1 CD4+ (and Tc-1 CD8+) T-cells will express TNF-α and chemokines, and induce ligands for leukocyte adhesion molecules promoting their attachment to the endothelium, while the chemokines promote the transendothelial migration into the extravascular tissue. Activated T-cells provide a very sustained source of these cytokines.

The TNF-α will also induce production of vasodilatory substances (VEGF, postacyclin) and coagulation factors that would facilitate the entry of leukocytes to the site of injury (10).

One of the main functions of the effector Th-1 T-cells (and Tc-1) is the activation of alveolar macrophages. This is mediated by IFN-γ and the expression of CD40 ligand, CD40L, will engage CD40 in macrophages that are presenting antigens to the T-cells. Once activated, macrophages will increase production of reactive oxygen intermediates, nitric oxide, and lysosomal enzymes and will increase secretion of many cytokines, TNF-α, IL-1, IL-2, IL-18 among others (10). Activated macrophages are aimed at the more efficient killing of organisms and promote further inflammation mainly by TNF-α, IL-1, and short-lived lipid mediators.

Activated macrophages can also induce the formation of repair tissue by secreting growth factors that stimulate fibroblasts proliferation (platelet-derived growth factor), collagen synthesis (TGF-β), and new vessel formation. In addition to their effector functions, activated macrophages become more efficient APCs by increasing the MHC Class II expression and stimulation of T-cell proliferation and differentiation such as IL-12 and IL-18 (10).

It can be seen that the inflammatory process leading to disease in COPD cannot be focused in one single cell. Each cell would have their role, or roles, to play in the process, but there is a necessary and important co-operation among all the cells involved and this can be best orchestrated by the T-cells, as we have seen. The rest of the inflammatory cells, besides being effector arms under the direction of the T-cells, enhance and maintain the T-cell function by providing the necessary inflammatory milieu for the maintenance of T-cell activation and costimulation.

A complex inflammatory process is a perfectly designed instrument to fight infection that has gone wrong producing disease; a link of events well described in autoimmune diseases, and most likely operating in COPD.

VI. WHY ONLY SOME SMOKERS DEVELOP COPD: THE IMMUNOLOGICAL PARADIGM

We have, so far, reviewed the potential role that T-lymphocytes could play in the development of COPD, and how the increase of T-cells in the lungs of smokers could be prompted by the presence of antigenic peptides: self or modified self-antigens, in the lung. This proposed new paradigm for the pathogenesis of COPD, autoimmunity, could also explain why only a relatively small proportion of smokers develop COPD, a fact that the proteinase–antiproteinase paradigm could not easily explain.

We could assume that all smokers would initially develop airway and parenchymal inflammation, an innate immune response, with tissue damage

and consequent production of antigenic molecules that would reach the DC by the mechanisms reviewed before. However, not all smokers seem to react to these antigens and develop the disease, and even when disease develops the severity varies for the same amount of smoking. A careful review of the published data on inflammatory cell infiltration in the lung shows a large variability in the number of T-cells and other inflammatory cells in smokers' lungs, probably accounting for the variable degree of lung damage. Possible reasons for these differences could be that the T-cell repertoire of those patients who develop COPD includes autoreactive clones that have escaped thymic deletion to some lung proteins not expressed in the thymus (not all proteins are), or that a break in immunological tolerance has occurred.

Tolerance, the "immunologist's Holy Grail" (197), is a poorly understood, ever changing, and continuously evolving concept, comprising central and peripheral tolerance. In today's concept of central tolerance, immature T-cells randomly rearrange their TCR genes and proliferate at high rates, eventually migrating to the medulla of the thymus, where they are exposed to autoantigens in the presence of self-MHC molecules. Those cells with very low or very high affinity to self-antigens undergo negative selection succumbing to apoptosis. However, cells with receptors with intermediate affinity to such complexes undergo a positive selection and migrate to the periphery (10,200).

Today's paradigm is that a low level of autoreactivity (i.e., reactivity to self-antigens) is physiologic (201), that autoantigens help to form the repertoire of mature lymphocytes, and that the survival of naïve T-cells in the periphery requires continuous exposure to autoantigens (202). Thus, the lymphocyte evolved not to distinguish itself from a foreign substance, as it was believed, but to respond to antigen only in certain microenvironments (203). As autoreactivity is physiological, the challenge is to understand how it becomes a pathological process.

One important point that has emerged in the studies of tolerance is that tolerance is not an all or nothing phenomenon—various degrees of tolerance, thus disease can occur (204). If COPD was due to different degrees of failure in tolerance to self or modified self-antigens, it could explain the variable responses of the lung to cigarette exposure, the variability in the number of T-cells in the lungs of smokers, the wide range of FEV_1 found in smokers and the correlation found between the level of FEV_1 and extent of emphysema with the number of T-cells in the airways and lung parenchyma (7,88).

The emphasis of immunological tolerance has recently switched from central to peripheral, and the interactions of T-cells with DCs might be the most important factor in determining peripheral tolerance (205). Antigen stimulation can lead to divergent T-cell responses that range from the deletion of antigen-specific lymphocytes and tolerance to the generation of a large number of effector cells followed by establishment of immunological

memory. The mechanisms that account for these divergent responses remain uncertain, but are probably mostly related to the amount of signal T-cells received by interacting with DCs (205). These responses are determined by: (a) the concentration of peptide–MHC complexes, which determines the rate of TCR triggering (206); (b) the concentration of costimulatory molecules, which determines the extent of signal amplification (207); and (c) the duration of the interaction between T-cells and DCs, which determines for how long the signal is accumulated (205).

Hierarchical thresholds of stimulation have been defined for cell proliferation, differentiation, and death (208–210). These thresholds depend on the density of antigen-presenting DCs in the local lymphoid tissue, which determines the frequency and duration of T-cell stimulation and hence the signal strength and the T-cell fate. If a DC presents low levels of antigen, expresses low levels of costimulatory molecules, does not produce cytokines, and consequently can deliver only a low level of stimulation, the T-cell would be eliminated by apoptosis (205). In contrast, when peripheral tissues are inflamed, the large numbers of DCs recruited are activated, mature and migrate to the lymph nodes. There they could present high levels of antigen and costimulatory molecules and produce IL-12, so providing optimal conditions for strong and sustained T-cell stimulation.

However, because the DC–T-cell interactions are stochastic, it is conceivable that not all T-cells receive the same level of stimulation, even in the same proliferating clone (205). The T-cells that have received high signal strength acquire tissue-homing capacity can enter inflamed tissues, and exert effector function (211) for extended periods of time (212), either because they are long lived or are continuously replaced, but they do not seem to divide in situ. At low signal strength, naïve T-cells proliferate, but do not acquire effector function and retain lymph node homing capacity or become regulatory CD4+ CD25+ suppressor cells (213).

It can be seen that the highly variable stimulatory conditions found in lymphoid organs and the stochastic nature of DC–T-cell interactions would lead to the generation of many T-cell fates. Clearly the balance of these resulting T-cell populations will determine the fitness and overall number of cells homing to the inflamed tissue, their survival and ultimately, their ability to clear a pathogen (205), or in our case produce lung damage (Fig. 8).

Another emerging topic in peripheral tolerance is the role of CD4+ CD25+ suppressor cells. These cells seem to be generated when T-cells are partially activated by encounter with antigen by the DCs, in the absence of or insufficient costimulatory signals or contact with DC, not "fully loaded" with antigens (see above). These lymphocytes have been considered anergic, however, it is becoming increasingly clear that they are likely to have a regulatory function. These are cells that protect against pathological or clinical undesirable inflammatory immune responses, having the ability to

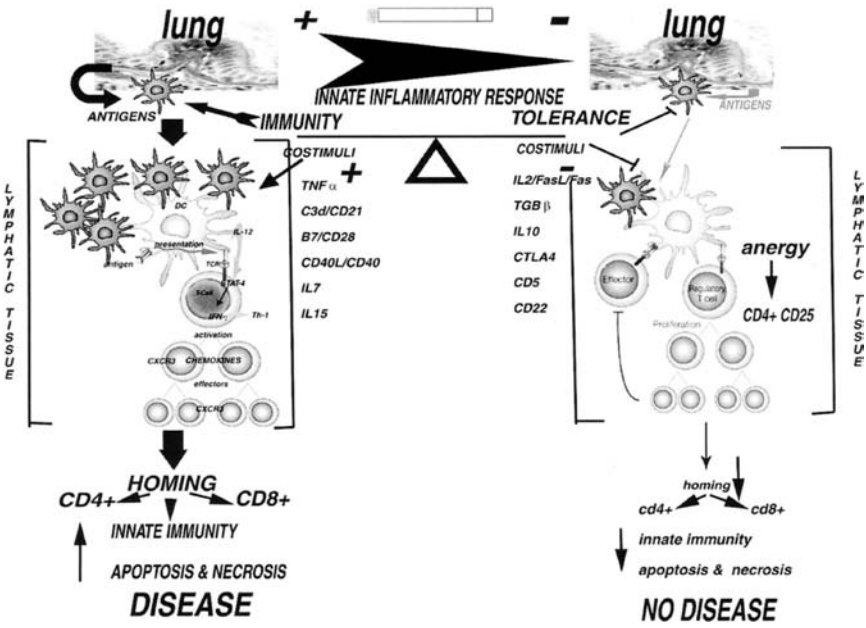

Figure 8 Role of immune tolerance in the development of autoimmune disease and possibly COPD. The left panel represents zero tolerance and the full-blown disease. The right panel represents 100% tolerance and no disease. Since tolerance is not an all or nothing phenomenon, different degrees of tolerance/immunity would produce different disease severity (between left and right panels). The degree and persistence of the innate immunity which conditions the costimuli, and thus the microenvironment of the DCs could be the most important determinant of the immunity/tolerance balance.

inhibit other immune cell functions, either directly through cell–cell contact or indirectly through the secretion of anti-inflammatory mediators such as IL-10, TGF-β, or IL-4 (213). The importance of these cells in the prevention of Type I diabetes development in predisposed individuals has recently been demonstrated (214).

A recent report by Barceló et al. (215) showed that smokers who develop COPD have a deficient response of CD4+ CD25+ regulatory T-cells in the BAL and the peripheral blood. This observation opens up an important area of research in the immunology of COPD, and we await, with interest, further developments in this field.

Genetic factors might ultimately be responsible for the disturbance in the natural balance between immunogenic and tolerogenic stimuli that can give rise to autoimmune disease. The predisposition to autoimmune disease represents the net effect of enhancing and protective genes (216,217), and as

each susceptibility gene confers its own level of risk, the predisposition to autoimmunity depends on which combination of susceptibility and protective genes is present, not solely on the number of each. Genes also control the vulnerability of target organs and the accessibility of antigens in target organs (218–220).

Among all the genes that are associated with autoimmunity, the strongest associations are with MHC genes, especially Class II. It is believed that MHC molecules influence the development of autoimmunity by controlling T-cell selection and activation. However, expression of a particular HLA (or MHC) gene is not, by itself, the cause of any autoimmune disease, but it may be one of several factors contributing to autoimmunity (218).

Tolerance, or different degrees of failure in tolerance, could be the basis for the different outcomes in smokers. However, how these concepts apply to smokers, and which of the factors reviewed would induce tolerance, and hence none, or minimal disease, would have to be defined.

A very important determining factor for the development of an adaptive immune response is the level of cytokines, and other mediators in the DCs' microenvironment determined by the strength of the innate immune response. It would then follow that variability in the innate immune response could be an important factor determining the outcome. Recently, Lazarus et al. (221) provided unexpected evidence of genomic variability in the genes encoding innate immunity. The authors suggested that the naturally occurring variation in the innate immunity genes could have an important role in human susceptibility to a variety of diseases that relate to the immune system including COPD (221).

Of interest in this regard are our findings in the studies of the genetic basis for resistance and susceptibility to emphysema in mice exposed to cigarette smoke. It seems that the strain resistant to the development of emphysema prevents its development by downregulating a large number of inflammatory genes, and suppressing the innate immune response. In contrast, the susceptible strain allows for a fluid and persistent innate immune response, and consequently develops an adaptive response, T-cell inflammation, and eventually emphysema (222).

These studies emphasize again the importance of considering inflammation as a complex interaction of cellular functions working towards a single goal and involving all the cells in the repertoire.

VII. SUMMARY AND CONCLUSIONS

Smokers, after the first few cigarettes, develop a prominent, but probably variable, innate immune inflammation, likely orchestrated by the epithelium. Neutrophils, macrophages, eosinophils, mast cells, $\gamma\delta$ cells, and DCs, cells with an innate immune mission to fight infections and other

challenges, are found in the lungs of smokers. If persistent, this inflammation, together with all xenobiotic and oxidants present in the smoke, will damage the lung matrix and the parenchyma, and produce potentially antigenic determinants that would be taken up by DCs. The innate inflammation, together with their cytokines and mediators, would create a propitious microenvironment for the maturation and consequent migration of DCs to the local lymphatic tissue, where they would present these antigens to T-cells.

Various, and not well understood, factors governing the interaction of DCs and T-cells will determine the degree of tolerance the T-cells show to these antigens. Tolerance will essentially eliminate the autoreactive T-cells and the disease will not develop. Different degrees of tolerance would result in variable degrees of disease, from mild to severe.

There is strong evidence to support the fact that the T-cells in the lungs of smokers with COPD are activated CD8+ and CD4+ T-cells that have returned to the lung, the source of antigenic material, attracted by the expression of Th-1 chemokines and chemokine receptors. These T-cells express IFN-γ indicating that COPD would be a Th-1-mediated disease, in which CD8+ CTL and CD4+ T-cells would be able to cause tissue injury by inducing apoptosis and necrosis (CD8+ CTL) and by recruiting large numbers of innate immune cells. These cells under the powerful direction and activation of the T-cells would continue to exercise their effector functions (proteases, mediators, oxidation) which produce more injury and more antigenic substances that would perpetuate and aggravate the disease. Why the disease continues to progress after smoking cessation is not clear, but this is a feature of many autoimmune diseases and COPD could be no different.

Viral and other infections, together with exposure to pollutants, may play a role in all phases of the disease, by providing important costimulatory mediators that could induce maturation of DCs, by producing mimicry of antigens for the disease and by polyclonal stimulation of the adaptive immunity. It could also explain why a simple "cold" might produce symptoms in patients with severe COPD often lasting for a long time after the "cold" is gone.

The mounting evidence implicating the T-cells as an important component of the inflammation and the disease in smokers is overwhelming. If we accept that the T-cells are part of the inflammatory component, we have to accept the reason why T-cells are in the lung, i.e., they are responding to an antigen challenge originating in the lung. If this is the case, we cannot escape the conclusion that COPD is a disease produced by antigens (self or modified-self) from the lung (autoimmune) secondary to smoking, as suggested before. Of course, none of this could be possible without a significant, and persistent, innate immune inflammation, comprising of neutrophils, macrophages, and perhaps $\gamma\delta$ T-cells.

It would be important to accept or at least explore this possibility, as it might lead us to a better understanding, and therefore new, and perhaps more effective, therapeutic approaches to the disease.

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10

Inflammatory Mediators

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I. INTRODUCTION

While many inflammatory mediators have been identified in asthma (1,2), there is much less information about the production and role of mediators in COPD. The COPD is a complex inflammatory disease that involves many different types of inflammatory and structural cells, all of which have the capacity to release multiple inflammatory mediators (Fig. 1). This suggests that mediator antagonists may have some potential as new therapies for COPD. of the redundant effects of many inflammatory mediators it, unlikely that antagonists of a single mediator, will provide major clinical benefit, as is the case in asthma. There is much less information available about the mediators of COPD than those of asthma. Unlike asthma, some patients with COPD also have systemic features of the disease and these are also likely to be mediated via inflammatory mediators.

II. LIPID MEDIATORS

As in asthma, lipid mediators derived from arachidonic acid may play an important role in the pathophysiology of COPD.

A. Prostaglandins

There is an increase in the concentration of prostaglandin(PG) E_2 in exhaled breath of COPD patients (3) (Fig. 2). This is likely to be derived from cyclooxygenase-2 (COX-2), which is expressed in alveolar macrophages

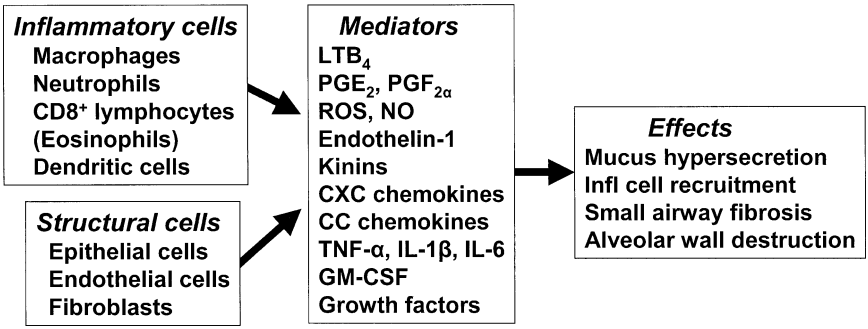

Figure 1 Multiple inflammatory cells and mediators are involved in the pathophysiology of COPD. LTB_4, leukotriene B_4, PG, prostaglandin, IL, interleukin, GM-CSF, granulocyte-macrophage colony stimulating factor, ROS, reactive oxygen species, NO, nitric oxide; Infl, Inflammatory.

(4). There is an increased COX-2 expression in alveolar macrophages from patients with COPD compared to normal control subjects (5). This is presumably as a result of induction by inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, which activate the transcription factor nuclear factor κB (NF-κB), the key regulator of COX-2 (6). Inflammatory cytokines may also activate sphingomyelinase in the cell membrane to generate ceramide which may also up-regulate COX-2 independently of NF-κB (7).

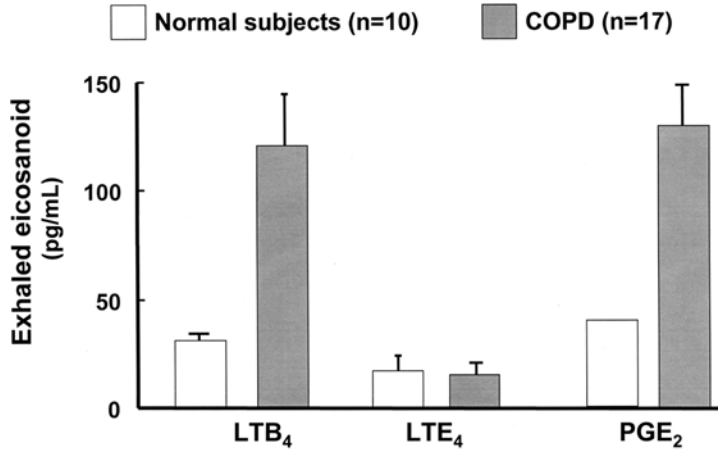

Figure 2 Increased concentrations of leukotriene (LT)B_4, prostaglandin (PG) E_2 but not the cysteinyl-leukotriene LTE_4 in exhaled breath condensate of patients with COPD.

The PGE_2 is a bronchodilator of human airways (8), inhibits the release of proinflammatory cytokines from monocytes (9) and acetylcholine release from airway cholinergic nerves (via EP_3 receptors) (10), suggesting that it may have beneficial effects in COPD airways. Furthermore, PGE_2 markedly enhances the anti-inflammatory actions of phosphodiesterase-4 (PDE4) inhibitors which are in clinical development as anti-inflammatory therapy for COPD (11). However, PGE_2 also has potentially detrimental effects in stimulating mucus secretion and expression of mucin genes (MUC5AC, MUCB) (12) and in sensitizing and activating airway sensory nerves to enhance coughing (13,14). Inhalation of the nonselective COX inhibitor indomethacin is reported to reduce mucus hypersecretion in patients with COPD (15), but long-term trials of COX inhibitors (and in particular COX2 inhibitors) have not yet been undertaken.

The $PGF_{2\alpha}$ is also increased in exhaled breath condensate of COPD patients (3). The $PGF_{2\alpha}$ is a bronchoconstrictor and also activates airway sensory nerves to produce cough (16).

B. Leukotrienes

Human alveolar macrophages express cytosolic phospholipase A_2 and release leukotriene (LT)B_4 and platelet-activating factor on activation (17). The LTB_4 is increased in exhaled breath condensate of patients with stable COPD (3) (Fig. 2) and is further increased during exacerbations (18). The LTB_4 is also increased in the sputum of patients with COPD, particularly during exacerbations (19,20). Plasma concentrations of LTB_4 are also reported to be increased in COPD patients (21). The cellular source of LTB_4 in COPD is likely to be from alveolar macrophages, airway epithelial cells, and neutrophils.

The LTB_4 is a potent chemoattractant of neutrophils through the activation of BLT_1 receptors that are expressed predominantly on neutrophils. The BLT_2 receptors are expressed on T-lymphocytes (22).The BLT_1 antagonists, such as LY29311, have now been developed for the treatment of neutrophilic inflammation (23). The BLT_1-receptor antagonists inhibit the neutrophil chemotactic activity of sputum from COPD patients, indicating the potential clinical value of such drugs (20,24), but only give about 25% inhibition indicating that other neutrophil chemotactic factors are also involved. The LTB_4 antagonists have also been shown to reverse lipopolysaccharide-induced survival of neutrophils from COPD patients (25).

Cysteinyl-leukotrienes are increased in asthma and largely derived from mast cells, but there is no evidence that they are increased in COPD. Thus, exhaled breath condensate shows an increase in concentration of cys-LTs in adults and children with asthma, but not in patients with COPD (3,26,27).

III. REACTIVE OXYGEN AND NITROGEN SPECIES

Oxidative and nitrative stresses are an important feature of COPD and there is increasing evidence that they are involved in its pathophysiology.

A. Reactive Oxygen Species

There is compelling evidence for increased oxidative stress in patients with COPD (28,29) (see Chapter 12). Cigarette smoke contains a high concentration of reactive oxygen species (ROS) (10^{17} moles/puff) and inflammatory cells, such as activated macrophages and neutrophils, also contribute. Evidence for increased oxidative stress in COPD is provided by demonstration of increased concentrations of hydrogen peroxide (H_2O_2) in expired condensates (30), increased 8-isoprostane levels in expired condensate (31) and ethane, a product of lipid peroxidation, in expired air (32). These markers of oxidative stress are further increased during exacerbations (18,30).

The increased oxidative stress in COPD may have several deleterious effects; oxidation of antiproteases, such as α_1-antitrypsin (α_1-AT) and secretory leukoprotease inhibitor (SLPI), may reduce the antiprotease shield, and may directly activate matrix metalloproteinases (MMP), resulting in increased proteolysis. H_2O_2 directly constricts airway smooth muscle in vitro (33) and hydroxyl radicals (OH^-) potently induce plasma exudation in airways (34). Oxidants also activate NF-κB, which orchestrates the expression of multiple inflammatory genes, including IL-8, TNF-α, and MMP-9. The ROS are normally counteracted by endogenous (glutathione, uric acid, bilirubin) and exogenous (vitamin C and vitamin E from diet) antioxidants. There is evidence for a reduction in antioxidant defenses in patients with COPD which may further enhance oxidative stress (28,29).

B. Nitric Oxide

Nitric oxide (NO) is generated in COPD from the enzyme inducible NO synthase (iNOS), which is expressed in macrophages and lung parenchyma of patients with COPD, particularly in patients with severe disease (35,36). The NO is markedly increased in exhaled breath of patients with mild asthma reflecting the inflammatory process in the airways, but in patients with COPD exhaled NO levels are little raised above normal (37–39), but are more clearly increased during exacerbations (37,40).

C. Peroxynitrite

This may be because exhaled NO levels are depressed by cigarette smoking and oxidative stress, as NO combines avidly with ROS to form peroxynitrite. This is supported by the fact that nitrate concentrations, formed by metabolism of peroxynitrite, are increased in breath condensate and sputum of cigarette smokers and patients with COPD (41,42). There is also a

reduction in an undefined "peroxynitrite inhibitory activity" in sputum of COPD patients (42). Peroxynitrite reacts with tyrosine residues in certain proteins to form 3-nitrotyrosine, which may be detected immunologically. There is an increased 3-nitrotyrosine immunoreactivity in sputum macrophages from patients with COPD (35). Oxidative stress and peroxynitrite may also reduce histone deacetylase-2 levels in macrophages, thereby inducing resistance to the anti-inflammatory actions of corticosteroids (43).

IV. PEPTIDE MEDIATORS

A. Endothelins

There is an increased concentration of endothelin-1 (ET-1) in induced sputum of patients with COPD (44), particularly during exacerbations (45). Plasma ET-1 concetnrations are also elevated in COPD patients, particularly in patients who develop nocturnal hypoxemia during the night (46,47). This may reflect the release of ET-1 by hypoxemia. The ET-1 is a potent vasoconstrictor and also causes vascular smooth muscle hyperplasia. There is increased expression of ET-1 in pulmonary endothelial cells of patients with COPD who have secondary pulmonary hypertension (48), suggesting that ET-1 may contribute to the vascular remodeling associated with hypoxic pulmonary hypertension.

B. Bradykinin

Although the role of bradykinin and related kinins in asthma has been extensively explored (49), there is very little information about kinins in COPD. It is likely that kinins are generated in the airway of COPD patients as plasma exudation occurs. Furthermore, proinflammatory cytokines that are found in COPD airways increase the expression of bradykinin B_1- and B_2-receptors in pulmonary cells (50–52). Bradykinin is a potent bronchoconstrictor of human airways, particularly small airways (53), stimulates mucus secretion (54), and potently potentiates cough by an effect on unmyelinated sensory nerve endings in the airways (55).

C. Tachykinins

Increased substance P concentrations have been reported in induced sputum of patients with chronic bronchitis (56); this is presumably derived form sensory nerve endings, although non-neuronal sources of tachykinins are now recognized. For example, sputum macrophages express substance P in response to endotoxin stimulation (57). Tachykinins are potent stimulants of submucosal gland and goblet cell secretion. The effects of cigarette smoke on mucus secretion is also blocked by tachykinin antagonists in experimental animals, indicating that tachykinin release from sensory nerves mediates

these effects (58). Substance P stimulates secretion from human airways in vitro and this effect is mediated via NK_1-receptors (59). In porcine airways tachykinins elicit submucosal gland section via NK_1 receptors in gland cells, but also via NK_3 receptors on cholinergic nerve terminal (60).

V. CHEMOKINES

Over 50 different chemokines are now recognized and they activate up to 20 different surface receptors (61). Chemokine receptors belong to the 7 transmembrane receptor superfamily of G-protein-coupled receptors and this makes it possible to find small molecule inhibitors, which have not yet been possible for classical cytokine receptors (62). Some chemokines appear to be selective for single chemokine receptors, whereas others are promiscuous and mediate the effects of several related chemokines. Four different families of chemokines are now differentiated, based on differences in the position of critical cysteine residues; CC, CXC, C, and CX_3C chemokines are recognized. Each chemokine molecule binds to a single or several receptors expressed on target inflammatory cells, resulting in the activation of signal transduction pathways that then result in chemotaxis or other cellular activities that include proliferation, differentiation, and survival. Chemokines appear to act in sequence in determining the final inflammatory response and so inhibitors may be more or less effective depending on the kinetics of the response (63).

Chemokines play a critical role in orchestrating inflammatory and immune responses by regulating the trafficking of inflammatory and immune cells to target organs (64). Several chemokines are involved in the recruitment of inflammatory cells in COPD (65). There is considerable interest in identifying chemokines in COPD as small molecule chemokine receptor inhibitors are now in development for COPD (66,67).

A. Interleukin-8

The CXC chemokine IL-8 (CXCL8) is a potent chemoattractant of neutrophils and it is not surprising that it has been implicated in COPD. The IL-8 levels are markedly increased in induced sputum of patients with COPD and correlate with the increased proportion of neutrophils (68,69). The concentrations of IL-8 are even more elevated in patients with emphysema due to αl-antitrypisn deficiency (70). The concentrations of IL-8 in induced sputum are further increased during acute exacerbations, which presumably contribute to the increased numbers of neutrophils and the increased purulence of the sputum (24,71,72). There is a correlation between IL-8 concentrations and the bacterial colony count in sputum, indicating that bacterial infection may induce neutrophilic inflammation, at least in part, via induction of IL-8 release in the airways (73,74). The IL-8 is also increased in BAL fluid of

patients with COPD and correlates with numbers of neutrophils (75,76). The concentrations of IL-8 are significantly higher in smokers with emphysema than in matched smokers without airflow limitation, whereas the concentrations of other CXC chemokines in BAL do not appear to discriminate between these groups (77).

The cellular source of IL-8 in COPD is not completely certain. Airway epithelial cells secrete IL-8 in response to several stimuli, including TNF-α and cigarette smoke extract (78–80). The IL-8 protein and mRNA are increased in bronchiolar epithelial cells of patients with COPD (81) and there is increased basal release of IL-8 from airway epithelial cells of patients with COPD (82,83). Alveolar macrophages also secrete IL-8 in response to the same stimuli and cells derived from patients with COPD secrete more IL-8 than those from normal smokers who in turn secrete more than macrophages from normal nonsmokers (84). Neutrophils themselves also release IL-8, and attract more neutrophils, so that a self-perpetuating inflammatory state may be established (85). The secretion of IL-8 is regulated transcriptionally by several transcription factors, amongst which NF-κB is predominant. NF-κB is activated in alveolar macrophages of patients with COPD and is further activated during exacerbations (86,87).

Neutralization of IL-8 with a blocking antibody significantly reduces the neutrophil chemotactic activity of sputum from patients with COPD (20,24). The reduction in neutrophil chemotactic activity is only of the order of $\sim$30%, however, indicating that other neutrophil chemotactic factors are also involved and that blocking IL-8 alone may be insufficient as a therapeutic strategy to reduce neutrophil inflammation in the respiratory tract.

The IL-8 acts via two receptors: CXCRl, which is a low affinity receptor that is specific for IL-8, and CXCR2, which has high affinity and is shared by other CXC chemokines (Fig. 3). It is likely that CXCR2 mediates the chemotactic response of neutrophils and monocytes to IL-8, whereas CXCRl may mediate the effects of IL-8 on release of mediators and proteases. There is a marked up-regulation of CXCR2 in airway epithelial cells during acute exacerbations of COPD and this is correlated with the increased numbers of neutrophils in the airway (88).

B. Other CXC Chemokines

Growth-related oncogene-α (GRO-α, CXCL1) is another CXC chemokine that is likely to be involved in COPD. The GRO-α activates neutrophils, monocytes, basophils, and T lymphocytes via CXCR2 (89) (Fig. 4). The concentrations of GRO-α markedly elevated in induced sputum and BAL of patients with COPD compared to normal smokers and nonsmokers (90) (Fig. 5). The GRO-α selectively activates CXCR2 and is chemotactic for neutrophils and monocytes. There is an increase in the monocytes chemotactic response to GRO-α in COPD patients and this may be related

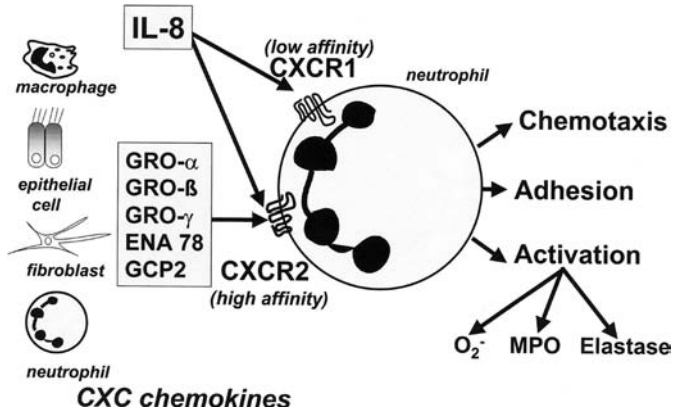

Figure 3 The CXC chemokine receptors on neutrophils. The IL-8 binds with low affinity to CXCR1, resulting in adhesion and activation and to CXCR2 with high affinity resulting in chemotaxis. The CXCR2 is also activated by other CXC chemokines, including growth-related oncogene (GRO)-α, -β and -γ, epithelial cell-derived neutrophil-activating peptide of 78 kD (ENA-78) and granulocyte chemotactic protein (GCP)-2.

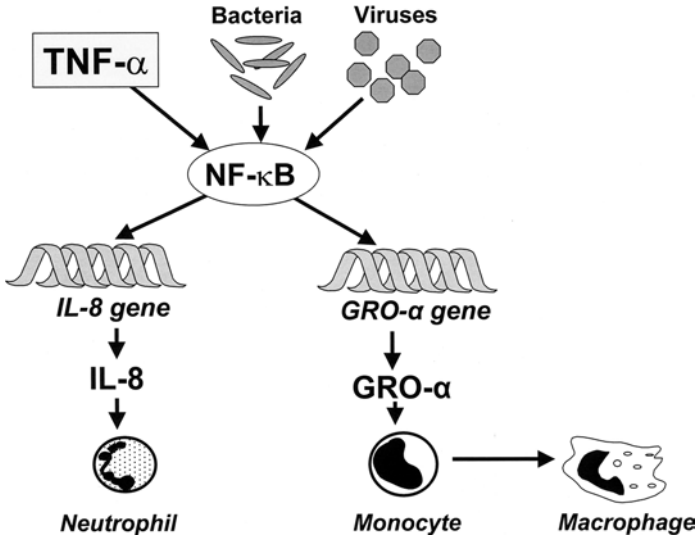

Figure 4 Activation of the CXC chemokines IL-8 and growth-related oncogene (GRO)-α via NF-κB after stimulating with TNF-α, bacteria and viruses. This results in chemotaxis of neutrophils and monocytes that differentiate into alveolar macrophages.

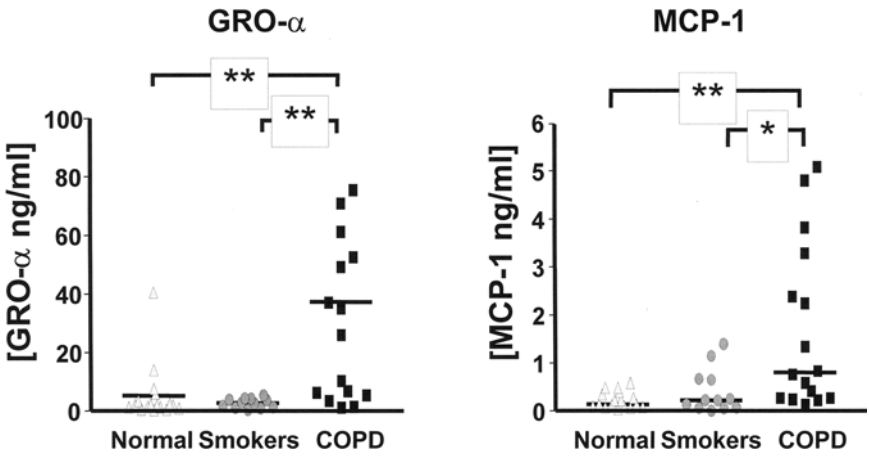

Figure 5 Elevated concentrations of growth-related oncogene(GRO)-α and MCP-1 in induced sputum of patients with COPD. (From Ref. 90.)

to increased turnover of CXCR2 on monocytes of COPD patients (91). It is possible that the increased chemotactic response of monocytes to GRO-α is one of the mechanisms leading to increased numbers of alveolar macrophages in the lungs of patients with COPD (92) (Fig. 3) and could be one of the molecular mechanisms of susceptibility to cigarette smoking.

Epithelial cell-derived neutrophil-activating peptide-78 (ENA-78, CXCL5) is derived predominantly from epithelial cells and also activates CXCR2 (93), although monocytes do not appear to show an increased chemotactic response to this chemokine as they do to GRO-α (91). The ENA-78 is increased in BAL fluid of COPD patients compared to normal subjects, but there is no difference between patients with emphysema and normal smokers (77). A marked increase in expression of ENA-78 has been reported in epithelial cells during exacerbations of COPD (88).

The mechanisms by which $CD8^+$, and to a lesser extend $CD4^+$ cells accumulate in the airways and lungs of patients with COPD is not yet understood. However, homing of T cells to the lung must depend upon some initial activation then adhesion and selective chemotaxis. T cells in peripheral airways of COPD patients show increased expression of CXCR3, a receptor activated by interferon-γ inducible protein of 10 kDa (IP-10, CXCL10), monokine induced by interferon-γ (INF-γ) (Mig, CXCL9) and interferon-inducible T cell-α chemoattractant (I-TAC, CXCL11). All three chemokines activate CXCR3, although I-TAC has the highest affinity (94). The CXCR3 is expressed on T-lymphocytes, particularly of the $CD8^+$ subtype. There is increased expression of IP-10 by bronchiolar epithelial cells and airway smooth muscle cells and this could therefore contribute to the accumulation of $CD8^+$ cells, which preferentially express CXCR3

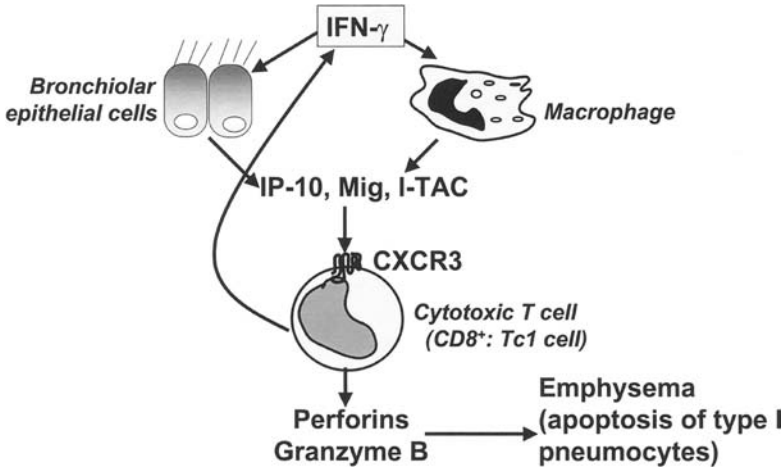

Figure 6 Chemotaxis of cytotoxic ($CD8^+$) T-lymphocytes via activation of CXCR3 by the CXC chemokines INF-γ inducible protein of 10 kDa (IP-10), monokine induced by INF-γ (Mig) and interferon-inducible I-TAC. The $CD8^+$ cells may release performs and granzyme B which may induce apoptosis in alveolar cells and release IFN-γ which in turn activates the release of these chemokines.

(95–97). It is of interest that INF-γ stimulates dendritic cells to produce IP-10 and Mig which then enhances their ability to attract $CD8^+$ cells (98). Alveolar macrophages also have the capacity to produce IP-10 and Mig and result in attraction of $CD8^+$ T cells (99). Since $CD8^+$ T cells produce INF-γ, this provides a potential feed-forward amplification loop. The role of $CD8^+$ T cells in COPD is not yet certain, but as they have the capacity to produce performs and granzyme B they might induce apoptosis in alveolar epithelial and endothelial cells, thereby contributing to emphysema (100,101) (Fig. 6).

C. CC Chemokines

Monocyte chemotactic protein-1 (MCP-1, CCL2) is a CC-chemokine that activates CCR2 on monocytes and T lymphocytes. The CCR2 may play a role in COPD, as MCP-1 levels are increased in sputum, bronchoalveolar lavage, and lungs of patients with COPD (Fig. 5) and MCP-1 is expressed in alveolar macrophages and epithelial cells (81,90,102). The MCP-1 is a potent chemoattractant of monocytes and may therefore be involved in the recruitment of macrophages in COPD. Indeed the chemoattractant effect of induced sputum from patients with COPD is abrogated by an antibody to CCR2. Since macrophages appear to play a critical role in COPD as a source of elastases and neutrophil chemoattractants, blocking CCR2 may

be a therapeutic strategy in COPD and small molecule inhibitors are in development.

The CCR3 are predominantly expressed on eosinophils and therefore play an important role in asthma. In COPD, there is a small increase in eosinophils and eosinophil basic proteins in induced sputum and bronchoalveolar lavage fluid and an increase in eosinophils has been described in exacerbations of chronic bronchitis (68,103,104). This suggests that eosinophil chemoattractants may play some role. The RANTES (released by activated normal T cells expressed and secreted, CCL5) activates CCR3 and is strongly expressed in airway epithelial cells of patients with chronic bronchitis exacerbations (105). Eotaxin (CCL11) and CCR3 show increased expression in the bronchi of patients with exacerbations of chronic bronchitis and are correlated with increased numbers of eosinophils (106).

The CCR4 and CCR8 are selectively expressed on Th2 cells and are activated by the chemokines macrophage-derived chemokine (MDC, CCL22) and thymus and activation-dependent chemokine (TARC, CCL17) (107). However, Th2 cells are not prominent in COPD so it is unlikely that these receptors are relevant.

The CCR7 plays a role in the migration of dendritic cells to regional lymph nodes and therefore blocking this receptor might suppress antigen presentation (108). There is an increase in the number of dendritic cells in rat lungs exposed to cigarette smoke (109,110) and in the airways and alveolar walls of smokers (111,112), but the chemotactic factors involved have not yet been determined. The MIP-3α (CCL20) which acts on CCR6 that is expressed by immature dendritic cells is potent chemoattractant of dendritic cells and is expressed by airway epithelial cells in response to interferon-γ (IFN-γ) (113).

D. CX_3C Chemokines

The unique CX_3C chemokine fractalkine, which is tethered to cell surfaces, shows increased expression in human airway epithelial cells after stimulation with IFN-γ and may be involved in recruitment and adhesion of monocytes, T lymphocytes, and natural killer cells to epithelial surfaces (114). Whether fractalkine or its receptor CX_3CR1 is increased in COPD is not yet known.

VI. CYTOKINES

Since chronic inflammation is a prominent feature of COPD, it is not surprising that cytokines play a key role in its pathophysiology.

A. Tumor Necrosis Factor-α

The TNF-α is present in high concentration in the sputum of COPD patients (68), particularly during exacerbations (71). There is also an increase in

soluble TNF receptors in sputum (115). Polymorphisms of the promoter of the TNF-α gene have been associated with increased susceptibility of COPD in some studies (116–118), but not in others (119–121). This may be related to the type of COPD studied and has been associated with more severe disease (122).

The TNF-α activates NF-κB, which switches on the transcription of inflammatory genes, including chemokines and proteases, in epithelial cells and macrophages. In experimental models of emphysema, TNF-α appears to mediate cigarette-induced connective tissue breakdown in the lungs (a precursor of emphysema) through the release of matrix metalloproteinase-12 (MMP-12) from macrophages, which then release TNF-α from these cells (123).

Serum concentrations of TNF-α and stimulated TNF-α production from peripheral blood monocytes are increased in weight-loosing COPD patients, suggesting that it may play a role in the cachexia of severe COPD (124–126). The TNF-α inhibits the expression of skeletal muscle proteins via activation of NF-κB (127). This suggests that inhibitors of TNF-α might be useful in reversing the skeletal wasting seen in COPD as well as reducing the airway inflammatory response (128). Plasma concentrations of TNF-α are increased slightly in COPD patients compared to normal controls during exercise but this is not associated with any increase in TNF-α expression in skeletal muscle (129).

B. Interleukin-1β

The IL-1β has similar actions to TNF-α and is a potent activator of alveolar macrophages from COPD patients (130). Bronchial epithelial cells in culture release more IL-1β than cells form normal subjects after stimulation with cigarette smoke (131). However, elevated levels of IL-1 in COPD have not yet been reported. The IL-1 receptor antagonist (IL-1RA) is an endogenous inhibitor of IL-1 effects and has been reported to be reduced in asthma. In COPD macrophages, a reduced secretion of IL-1RA compared to normal macrophages in response to *Chlamydia* infection has been described (132).

C. Interleukin-6

The IL-6 concentrations are increased in induced sputum, bronchoalveolar lavage, and exhaled breath condensate of COPD patients, particularly during exacerbations (133–135). The IL-6 is also increased in the plasma of COPD patients, especially during exacerbations (136,137). The IL-6 is a marker of inflammation, as it is activated by NF-κB, but its role in inflammation is uncertain as it has both anti-inflammatory and proinflammatory actions and its effects may be determined by the presence of other cytokines.

D. GM-CSF

The concentrations of granulocyte-macrophage colony stimulating factor (GM-CSF) in BAL fluid are increased in stable COPD but markedly elevated during exacerbations (138). The GM-CSF is important for neutrophil survival and may play an enhancing role in neutrophilic inflammation. Like other proinflammatory cytokines, it is predominantly regulated by NF-κB.

E. Interleukin-10

The IL-10 is a potent anti-inflammatory cytokine that is released from monocytes and alveolar macrophages in response to inflammatory stimuli. The IL-10 secretion is markedly reduced in alveolar macrophages from patients with asthma (139) and its concentrations are reduced in sputum of patients with asthma and COPD, suggesting that a similar abnormality may apply on COPD (140). The IL-10 production appears to be increased in macrophages from normal smokers (141), but it is not certain whether macrophages from COPD patients show a relatively reduced production, as in asthma, which may help amplify inflammation.

F. Other Cytokines

Overexpression of IL-13 and also interferon-γ in murine lungs unexpectedly results in emphysema that is mediated by increased expression of MMPs and cathepsins (142,143). There is also an association between a promoter polymorphism in the IL-13 gene and COPD, as also seen for asthma (144). The relevance of IL-13 cytokines in the pathogenesis of human emphysema has not yet been elucidated, however. Another Th2 cytokine IL-4 is a classical Th2 cytokine which plays a key role in asthma, but it has been found to unexpectedly increased in chronic bronchitis, particularly in $CD8^+$ T lymphocytes around submucosal glands, although this was not observed in COPD (145). A third Th2 cytokine IL-9 is also increased in airway T lymphocytes of patients with COPD (96).

The IL-17 is a cytokine that releases CXC chemokines from airway epithelial cells (146), but is not increased in sputum of COPD patients (147).

VII. GROWTH FACTORS

Marked structural changes are found in small airways and lung parenchyma, presumably as a result of chronic inflammation and the release of cytokines, particularly growth factors that induce fibrosis and cell metaplasia.

A. Transforming Growth Factors

Transforming growth factor (TGF)-β1 shows increased expression in small airway epithelial cells and alveolar macrophages of patients with COPD and

might play a role in the characteristic fibrosis in small airways (148,149). Increased secretion of TGF-β is also reported in peripheral blood monocytes form COPD patients (150). Increased TGF-β expression in peripheral lung tissue of COPD patients has been correlated with immunoreactivity for 4-hydroxy-4-nonenal, a marker of oxidative stress (151).The TGF-β induces the release of collagen tissue growth factor (CTGF), which mediates the fibrosis response to TGF-β (152). In a human epithelial cell line, latent adenovirus infection (which has been associated with COPD) induces increased expression of both TGF-β and CTGF (153).

The TGF-β potently down-regulates β_2-adrenergic receptors by inhibiting gene transcription in human cell lines (154,155) and markedly reduces the bronchodilator response to β-agonists in airway smooth muscle in vitro (156).

Alveolar macrophages produce TGF-α in much greater amounts than TGF-β (157) and this may be a major endogenous activator of epidermal growth factor (EGF) receptors that play a key role in regulating mucus secretion in response to many stimuli, including cigarette smoke.

B. Epidermal Growth Factor

The EGF and TGFα activate EGF receptors which appear to play a key role in the regulation of mucus secretion, the expression of mucin (MUC) genes and differentiation of mucus secreting cells (158). The EGF receptors are involved in the increased mucus secretory response to oxidative stress and cigarette smoke (155,155,159,160).

C. Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is a major regulator of vascular growth and is likely to be involved in the pulmonary vascular remodeling that occurs as a result of hypoxic pulmonary vasoconstriction in severe COPD. There is an increased expression of VEGF in pulmonary vascular smooth muscle of patients with mild and moderate COPD, but paradoxically a reduction in expression in severe COPD with emphysema (161). Inhibition of VEGF receptors in rats using a selective inhibitor induces apoptosis of alveolar endothelial cells resulting in emphysema (162) and this appears to be associated with oxidative stress (163). Interestingly, the concentration of VEGF is increased in induced sputum of patients with asthma and chronic bronchitis but is significantly reduced in patients with COPD with emphysema (164).

D. Fibroblast Growth Factors

Fibroblast growth factor (FGF)-1 and FGF-2 and FGF receptors are abnormally expressed in airway and pulmonary vascular smooth muscle

and airway epithelial cells in peripheral lung of patients with COPD (165). The increased expression of FGF is particularly correlated with vascular remodeling.

VIII. CONCLUSIONS

It is clear that many inflammatory mediators are involved in the chronic inflammation and structural changes that occur in COPD. These mediators are derived not only from activated inflammatory cells, such as alveolar macrophages, neutrophils, and T lymphocytes that are recruited to the airways and lungs, but also from structural cells in the respiratory tract, including epithelial and endothelial cells and fibroblasts, which transform into mediator producing cells. These mediators have complex effects in the airways, resulting in recruitment of inflammatory cells form the circulation, bronchoconstriction, vascular changes, mucus secretion and structural changes in the airways, and lung parenchyma. These mediators may also spill over into the systemic circulation to produce the systemic changes such as cachexia and muscle wasting seen in severe disease. The therapeutic implications are that blocking the generation or receptors for these mediators will have a beneficial clinical effect and several mediator antagonists are now in development (67). However, blocking a single mediator when so many are involved and with redundant effects, it is unlikely that this approach will produce major clinical benefit unless the mediator plays a pivotal or unique role and is high in a cascade of events. The only way to determine the importance of a mediator is to study the effect of a specific inhibitor in the disease and this will require careful and prolonged clinical studies.

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11

Oxidative Stress

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I. INTRODUCTION

Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$) and the hydroxyl radical ($^{\bullet}OH$) are highly unstable species with unpaired electrons, capable of initiating oxidation. Biological systems are continuously exposed to oxidants, either generated endogenously by metabolic reactions (e.g., from mitochondrial electron transport during respiration or during activation of phagocytes) or exogenously, such as air pollutants or cigarette smoke. The lung exists in a high-oxygen environment and, together with its large surface area and blood supply, is susceptible to injury mediated by ROS. Production of ROS has been directly linked to oxidation of proteins, DNA, and lipids, which may cause direct lung injury or induce a variety of cellular responses, through the generation of secondary metabolic reactive species. ROS may alter remodeling of extracellular matrix and blood vessels, stimulate mucus secretion, inactivate antiproteases, cause apoptosis, and regulate cell proliferation (1,2) (Fig. 1). Alveolar repair responses and immune modulation in the lung may also be influenced by ROS. Furthermore, increased levels of ROS have been implicated in initiating inflammatory responses in the lungs through the activation of transcription factors such as nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1), signal transduction, chromatin remodeling and gene expression of pro-inflammatory mediators (3). It is proposed that ROS produced by phagocytes that have been recruited to the sites of inflammation are a major

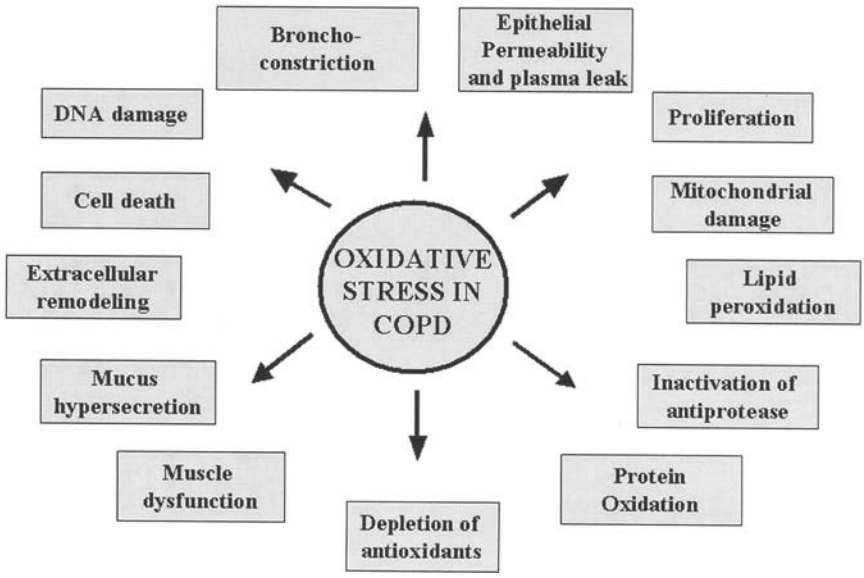

Figure 1 ROS-mediated cellular responses.

cause of cell and tissue damage associated with many chronic inflammatory lung diseases including COPD (1).

This chapter will review the evidence for the role of ROS in the pathogenesis of COPD, discuss the cellular and molecular mechanisms, and pathophysiological consequences of increased ROS release in COPD. These include increased sequestration of neutrophils in the pulmonary microvasculature, oxidative inactivation of antiproteases and surfactants, mucus hypersecretion, membrane lipid peroxidation, muscle dysfunction, mitochondrial respiration, alveolar epithelial injury/permeability, breakdown/remodeling of extracellular matrix, and apoptosis. Increased levels of ROS produced in the airways are reflected by increased markers of oxidative stress in the airspaces, sputum, breath, lungs, and blood in patients with COPD. The rationale for antioxidant therapeutic intervention in the light of oxidative stress will also be discussed.

II. CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

COPD is a slow progressive condition characterized by airflow limitation, which is largely irreversible (4,5). Cigarette smoking is the major aetiological factor in this condition. More than 90% of patients with COPD are smokers, but not all smokers develop COPD (6). However, 15–20% of cigarette smokers appear to be susceptible to its effects and show a rapid decline in forced

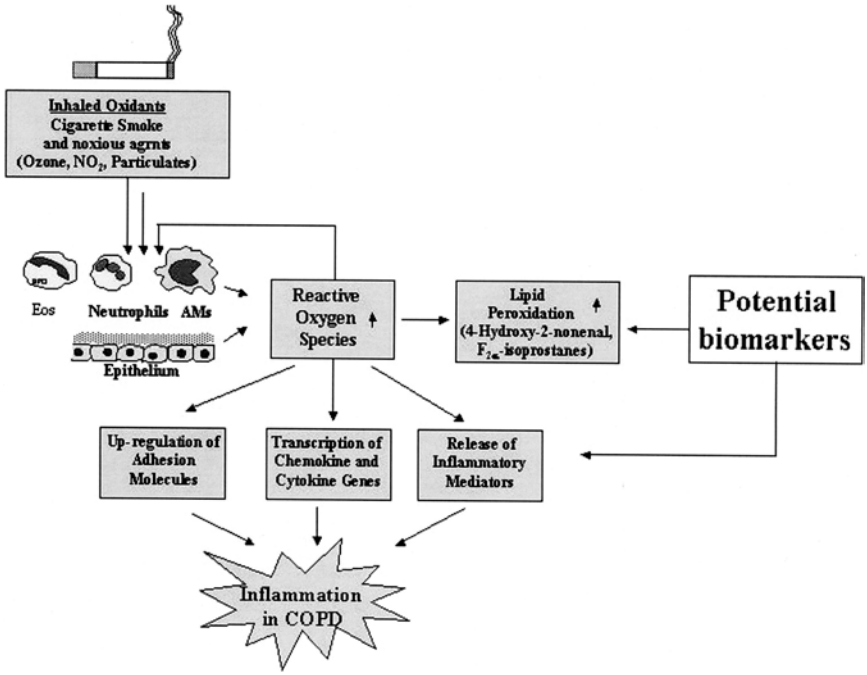

Figure 2 Mechanisms of ROS-mediated lung inflammation in COPD and generation of potential biomarkers. Inflammatory response is mediated by oxidants either inhaled and/or released by the activated neutrophils, alveolar macrophages, eosinophils, and epithelial cells leading to production of ROS and membrane lipid peroxidation. Activation of transcription of the pro-inflammatory cytokine and chemokine genes, upregulation of adhesion molecules and increased release of pro-inflammatory mediators which are involved in the inflammatory responses in patients with COPD.

expiratory volume in one second (FEV_1) an develop the disease (6). An increased oxidant burden in smokers derives from the fact that cigarette smoke contains more than 10^{15} oxidant/free radical molecules per puff. Many of these molecules are relatively long-lived such as tar-semiquinone which can generate $^{\bullet}OH$ and hydrogen peroxide (H_2O_2) by the Fenton reaction (7,8). Other factors, such as air pollutants, infections, and occupational dusts that may exacerbate COPD, also have the potential to produce oxidative stress (9) (Fig. 2).

III. CELL-DERIVED ROS

A common feature of COPD is the development of an inflammatory response, characterized by activation of epithelial cells, and resident macrophages, and the recruitment and activation of neutrophils, eosinophils,

monocytes, and lymphocytes. Inflammatory cells, once recruited in the airspace become activated and generate ROS in response to inflammatory mediators. The activation of macrophages, neutrophils, and eosinophils generates $O_2^{\bullet-}$, which is rapidly converted to H_2O_2 under the influence of superoxide dismutase (SOD), and $^{\bullet}OH$ is formed non-enzymatically in the presence of Fe^{2+} as a secondary reaction. ROS and reactive nitrogen species (RNS) can also be generated intracellularly from several sources such as mitochondrial respiration, the NADPH oxidase system, and xanthine/xanthine oxidase (Fig. 3). However, the primary ROS-generating enzyme is NADPH oxidase, a complex enzyme system that is present in phagocytes and epithelial cells.

In addition to NADPH oxidase, phagocytes employ other enzymes to produce ROS, which involves the activity of heme peroxidases (myeloperoxidase, MPO) or eosinophil peroxidase (EPO). Activation of EPO results in the formation of the potent oxidant hypochlorous acid (HOCl) and hypobromous acid (HOBr) from H_2O_2 in the presence of chloride (Cl^-) and bromide (Br^-) ions, respectively (Fig. 4). It is believed that the oxidant burden produced by eosinophils is substantial because these cells possess several times greater capacity to generate $O_2^{\bullet-}$ and H_2O_2 than neutrophils, and the content of EPO in eosinophils is 3–10 times higher than the amount of MPO present in neutrophils (10).

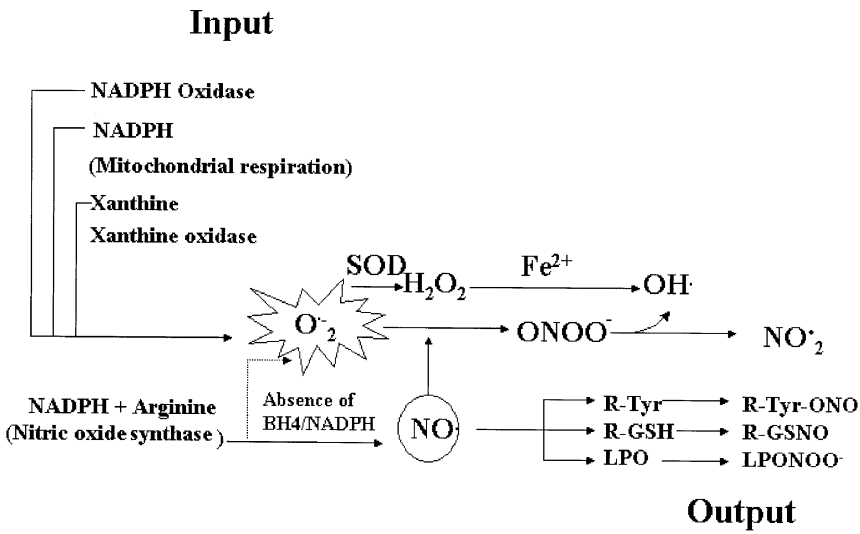

Figure 3 Cellular generation of NO-derived ROS and RNS. Intracellular reactive oxygen species O_2^- = superoxide anion, NO = nitric oxide, H_2O_2 = hydrogen peroxide, $OH^{\bullet}$ = hydroxyl radical, NO_2 = nitrogen dioxide, $ONOO^-$ = peroxynitrite.

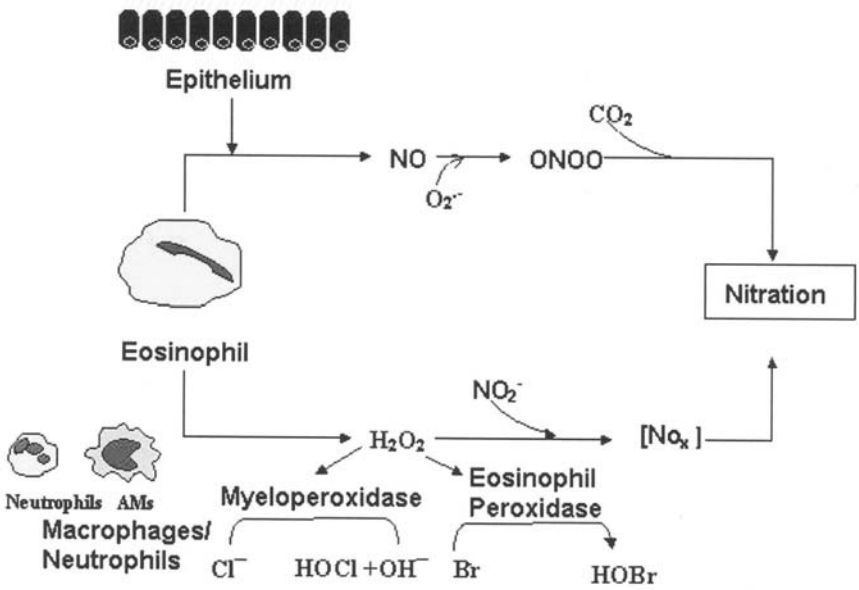

Figure 4 Model of potential pathways used by circulating cells for the generation of NO-derived reactive oxygen species and reactive halogen species.

The physiological consequences of EPO-dependent formation of brominated oxidants such as HOBr in vivo are unknown. HOBr reacts rapidly with a variety of nucleophilic targets such as thiols, thiol ethers, amines, unsaturated groups, and aromatic compounds.

Several transition metal salts react with H_2O_2 to form $^{\bullet}OH$. Most attention in vivo for the generation of $^{\bullet}OH$ has focused on the role of iron. Iron is a critical element in many oxidative reactions. Free iron in the ferrous form catalyzes the Fenton reaction and the superoxide driven Haber–Weiss reaction, which generate the $^{\bullet}OH$, an ROS which damages tissues, particularly cell membranes by lipid peroxidation (Fig. 5).

IV. INHALED OXIDANTS AND CIGARETTE SMOKE

Cigarette smoking, inhalation of airborne pollutants, either oxidant gases [such as ozone, nitrogen dioxide (NO_2), sulfur dioxide SO_2] or particulate air pollution results in direct lung damage as well as in the activation of inflammatory responses in the lungs. These environmental noxious agents are implicated in the pathogenesis and exacerbations of COPD. Cigarette smoke is a complex mixture of over 4700 chemical compounds, including high concentrations of oxidants/free radicals ($> 10^{15}$ molecules/puff) (7). Short-lived oxidants such as $O_2^{\bullet-}$ and nitric oxide (NO) are predominantly

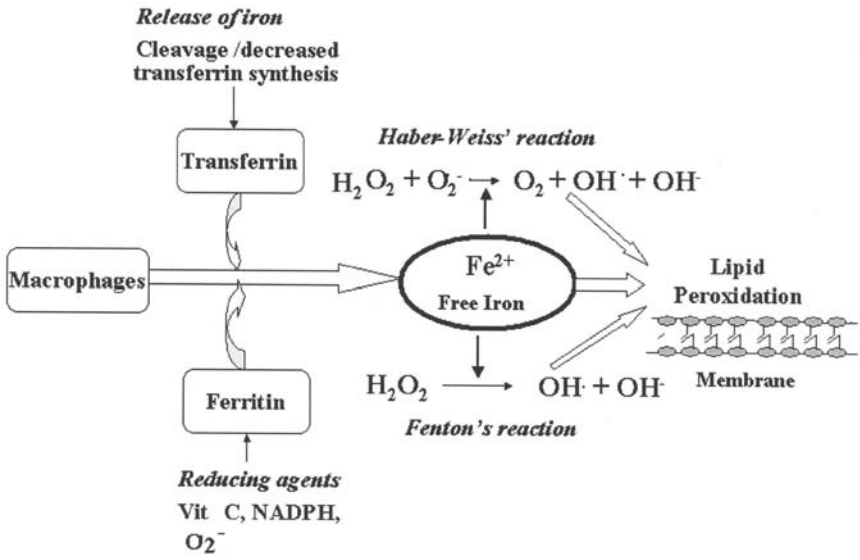

Figure 5 Free iron catalyzed membrane lipid peroxidation via Fenton and Haber–Weiss reaction.

found in the gas phase. NO and $O_2^{\bullet-}$ immediately react to form highly reactive peroxynitrite ($ONOO^-$) molecule. The radicals in the tar phase of cigarette smoke are organic in nature, such as long-lived semiquinone radicals, which can react with $O_2^{\bullet-}$ to form $^{\bullet}OH$ and H_2O_2 (8). During redox cycling, the semiquinone intermediate reduces molecular oxygen to the superoxide anion. The cycle can be sustained by biological reducing equivalents [ascorbate, NAD(P) H, glutathione, etc.] which reduce the oxidized quinoid substances back to their reduced states, enabling them to produce again the superoxide radical (Fig. 6). The aqueous phase of cigarette smoke condensate may undergo redox recycling for a considerable period of time in the epithelial lining fluid (ELF) of smokers (8). The tar phase is also an effective metal chelator and can bind iron to produce tar-semiquinone + tar-Fe^{2+}, which can generate H_2O_2 continuously (8). Furthermore, since both cigarette tar and lung epithelial lining fluid contain metal ions, such as iron, Fenton chemistry will result in the production of the $^{\bullet}OH$ which is a highly reactive and potent ROS. Sidestream cigarette smoke contains more than 10^{17} reactive organic compounds per puff, such as carbon monoxide, nicotine, ammonia, formaldehyde, acetaldehyde, crotonaldehyde, acrolein, *N*-nitrosamines, benzo(*a*) pyrene, benzene, isoprene, ethane, pentane and other genotoxic and carcinogenic organic compounds. The concentrations of these reactive compounds present in the epithelial lining fluid (ELF) following inhalation of cigarette smoke have been previously calculated (11) (Table 1).

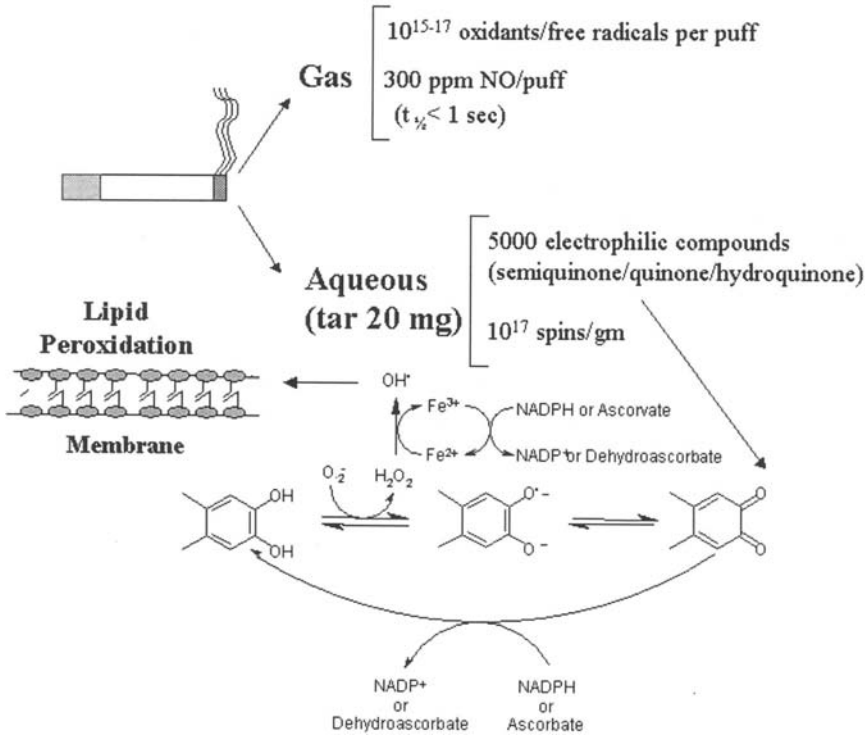

Figure 6 Cigarette smoke-derived gas and tar phase showing oxidative stress. During redox cycling, the cigarette smoke containing semiquinone intermediate reduces molecular oxygen to the superoxide anion. The cycle can be sustained by biological reducing equivalents [ascorbate, NAD(P)H, glutathione, etc.] which reduce the oxidized quinoid substances back to their reduced states, enabling them to produce again the superoxide radical.

V. OXIDATIVE STRESS IN THE ALVEOLAR SPACE

The oxidant burden in the lungs is enhanced in smokers by the release of ROS from macrophages and neutrophils. Oxidants present in cigarette smoke can stimulate alveolar macrophages to produce ROS and to release a host of mediators, some of which attract neutrophils and other inflammatory cells into the lungs. Both neutrophils and macrophages, which are known to migrate in increased numbers into the lungs of cigarette smokers, compared with non-smokers, can generate ROS via the NADPH oxidase system. Moreover, the lungs of smokers with airway obstruction have more neutrophils than smokers without airway obstruction. Circulating neutrophils from cigarette smokers and patients with exacerbations of COPD release more $O_2^{\bullet-}$ (12). Cigarette smoking is associated with increased

Table 1 Calculated Concentrations of Some Major Components in Gas-Phase Cigarette Smoke and Their Levels When Deposited in Respiratory Tract Lining Fluid (RTLF)

Component	In cigarette (μmol/cig)	In RTLF (μM)
Aldehydes		
Acetaldehyde	20	2000
Formaldehyde	1.5	150
Acrolein	0.8	80
Crotonaldehyde	0.2	20
Nitrogen oxide		
Nitric oxide	12	1200
Nitrogen dioxide	1.0	100
Organic free radicals (alkyl, alkoxyl, peroxyl)	0.02	2.0

$\sim 1 \times 10^{17}$ radicals/cig = 0.02 μmol radicals/cigarette (11), assuming complete deposition in respiratory tract lining fluid.

content of MPO in neutrophils, which correlates with the degree of pulmonary dysfunction (13). MPO activity also has a negative correlation with FEV_1 in patients with COPD, suggesting that neutrophil MPO-mediated oxidative stress may play a role in the pathogenesis of COPD (13).

Alveolar macrophages obtained by bronchoalveolar lavage (BAL) fluid from the lungs of smokers are more activated compared with those obtained from non-smokers. One manifestation of this is the release of increased amounts of ROS such as $O_2^{\bullet -}$ and H_2O_2 (1,14). Exposure to cigarette smoke in vitro has also been shown to increase the oxidative metabolism of alveolar macrophages. Subpopulations of alveolar macrophages with a higher granular density appear to be more prevalent in the lungs of smokers and are responsible for the increased $O_2^{\bullet -}$ production by smoker's macrophages (1).

The generation of ROS in epithelial lining fluid may be further enhanced by the presence of increased amounts of free iron in the airspaces in smokers. This is relevant to COPD since the intracellular iron content of alveolar macrophages is increased in cigarette smokers and is increased further in those who develop chronic bronchitis, compared with non-smokers (15). In addition, macrophages obtained from smokers release more free iron in vitro than those from non-smokers (16). The lungs of smokers contain darkly pigmented areas of high ESR spin heme iron, non-heme iron, and carbon-centered radicals (7). This mechanism may be responsible for the excess iron that accumulates in the alveolar macrophages of smokers.

Eosinophils have been shown to be prominent in the airway walls of both in stable and mild exacerbations of bronchitis. Bronchoalveolar lavage

(BAL) from patients with COPD has also been shown to contain increased levels of eosinophilic cationic protein. Furthermore, peripheral blood eosinophilia is also considered to be a risk factor for the development of airway obstruction in patients with chronic bronchitis and is an adverse prognostic sign. However, despite the presence of increased number of eosinophils, EPO-mediated generation of specific 3-bromotyrosine has not been detected in COPD patients. This does not provide support for a role of brominating oxidants in eosinophil-mediated ROS damage COPD.

VI. ALTERATIONS IN LUNG TISSUE

An important link between oxidative stress and the pathogenesis of COPD would come from the demonstration of the reaction of ROS with target lung molecules and the presence of these oxidatively modified molecules in increased amounts in the lungs of smokers, particularly those who develop COPD. ROS such as $O_2^{\bullet-}$ and $^{\bullet}OH$, generated and released by activated immune and inflammatory cells, are highly reactive, and when generated close to cell membranes oxidize membrane phospholipids (lipid peroxidation), a process which may continue as a chain reaction (Fig. 7). Thus, a single $^{\bullet}OH$ can result in the formation of many molecules of lipid hydroperoxides in the cell membrane. Many of the effects of ROS in airways may be mediated by the secondary release of inflammatory lipid mediators such as 4-hydroxy-2-nonenal (4-HNE), a footprint of oxidative stress/lipid peroxidation. 4-HNE, a highly reactive diffusible end product of lipid

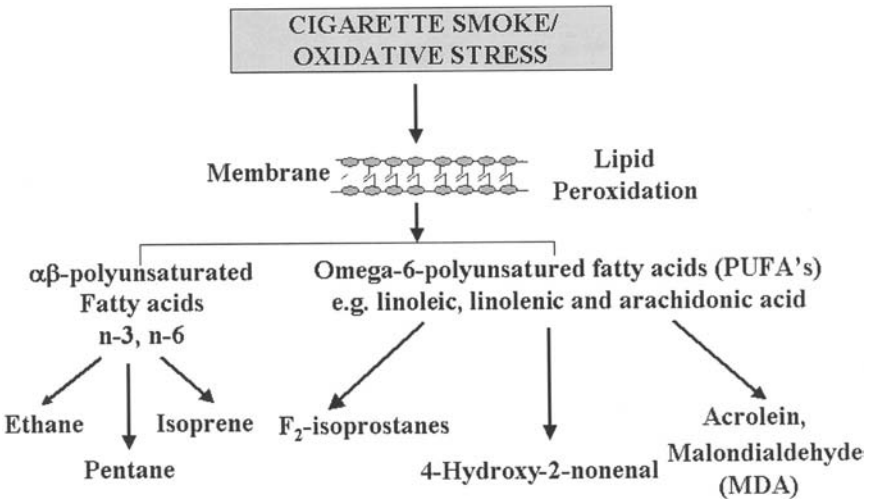

Figure 7 Membrane lipid peroxidation of polyunsaturated fatty acids leading to generation of various aldehydes.

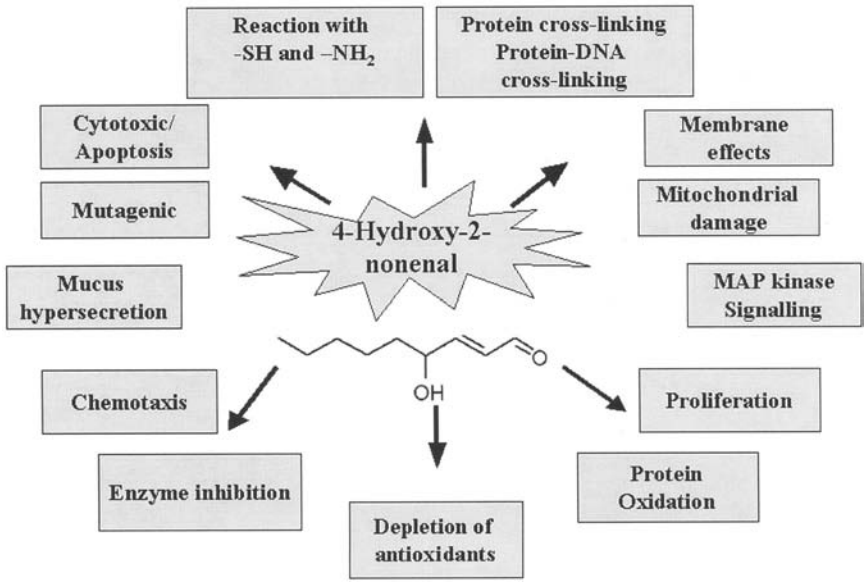

Figure 8 Cellular and molecular responses of 4-hydroxy-2-nonenal.

peroxidation, is known to induce/regulate various cellular events, such as proliferation, apoptosis, and activation of MAP kinase signaling pathways (17,18) (Fig. 8). 4-HNE has a high affinity towards cysteine, histidine, and lysine residues. It forms adducts with proteins, altering their function. It also acts as a chemoattractant for neutrophils in vitro and in vivo (18). Recent data indicate increased 4-HNE-modified protein levels in airway and alveolar epithelial cells, endothelial cells, and neutrophils in subjects with airway obstruction, compared to subjects without airway obstruction (19) (Fig. 9a and b). Levels of 4-HNE-adducts in alveolar and airway epithelium have been shown to be inversely related to the FEV_1, suggesting a role for 4-HNE in the pathogenesis of COPD. Products of lipid peroxidation have been found in increased amounts in the lungs of cigarette smokers, and the levels relate to the length of the smoking history. Increased levels of 4-HNE adducts in lungs of COPD patients are further confirmed by Aoshiba et al.(20) who have recently showed that cigarette smoking enhanced the levels of 4-HNE adducts in bronchiolar epithelial and alveolar type cells in mice. Thus evidence is accumulating that cigarette smoke triggers oxidative stress leading to lipid peroxidation in lungs of smokers and patients with COPD.

VII. SYSTEMIC OXIDATIVE STRESS

COPD is now considered to have not only local lung, but systemic manifestations. One manifestation of a systemic effect is the presence of markers of

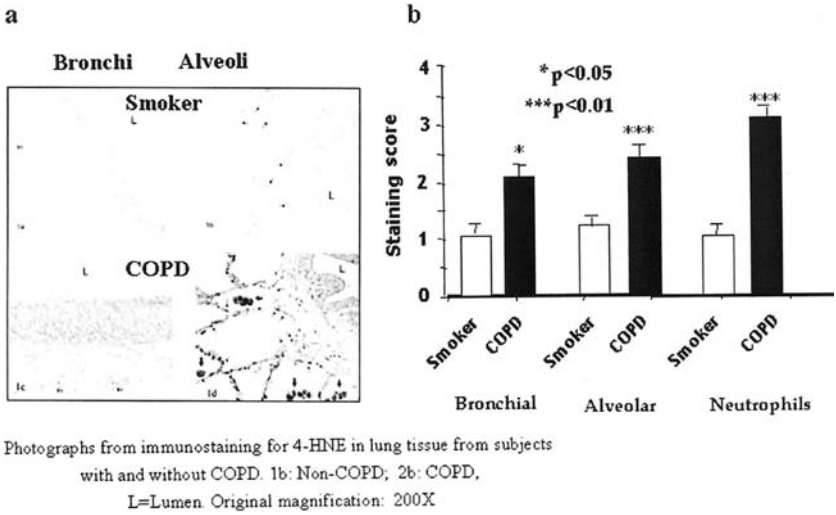

Figure 9 (a) Photographs from immunostaining for 4-HNE in lung tissue from subjects with and without COPD. 4-HNE adducts were localized predominantly in bronchial, bronchiolar, and alveolar epithelial cells, in endothelial cells, neutrophils, and CD68+ cells (macrophages) (19). The levels of 4-HNE-modified proteins were higher in bronchial and alveolar epithelial cells, and bronchial endothelial cells and neutrophils of subjects with COPD compared to subjects without COPD 1A: Non-COPD, bronchial; IB: Non-COPD, alveolar; 2A: COPD bronchial; 2B: COPD alveolar. L = lumen. Original magnification: 200×. (b) Individual immunostaining scores for 4-HNE-adducts in bronchial (br) and alveolar (alv) lung tissue in epithelial cells, endothelial cells, and neutrophils. The mean is indicated, as well significance levels (p) for differences between the indicated groups.

oxidative stress in the blood in patients with COPD. The neutrophil appears to be a critical cell in the pathogenesis of COPD (1). Epidemiological studies have shown a relationship between circulating neutrophil numbers and the FEV_1. Moreover, a relationship has also been shown between the change in peripheral blood neutrophil count and the change in airflow limitation over time. Similarly, a correlation between $O_2^{\bullet-}$ release by peripheral blood neutrophils and bronchial hyperreactivity in patients with COPD has been shown, suggesting a role for systemic ROS in the pathogenesis of the airway abnormalities in COPD (21).

Various studies have demonstrated increased production of $O_2^{\bullet-}$ from peripheral blood neutrophils obtained from patients during acute exacerbations of COPD, which returned to normal when the patients were clinically stable (12,22). Other studies have shown that circulating neutrophils from patients with COPD show upregulation of their surface adhesion molecules, which may also be an oxidant-mediated effect (12,23). Activation may be even more pronounced in neutrophils which are sequestered in the

pulmonary microcirculation in smokers and in patients with COPD, since neutrophils which are sequestered in the pulmonary microcirculation in animal models of lung inflammation release more ROS than circulating neutrophils (1).

Superoxide anion and H_2O_2 can be generated by the xanthine/xanthine oxidase (XO) reaction. XO activity has been shown to be increased in cell free BAL fluid and plasma from COPD patients, compared to normal subjects; and this has been associated with increased $O_2^{\bullet-}$ and lipid peroxide levels (24).

VIII. REACTIVE OXYGEN AND REACTIVE NITROGEN SPECIES AS SURROGATE MARKERS IN PLASMA AND EXHALED BREATH CONDENSATE

Interest is growing in identifying biomarkers for diseases in which oxidative stress is involved. Direct measurements of oxidative stress are difficult since ROS/free radicals are highly reactive and also short lived. An alternative has been to measure markers of the effects of radicals on lung biomolecules such as lipids, protein or DNA, or to measure the stress responses to an increased oxidant burden. Various invasive and semi-invasive means of assessing these oxidative biomarkers are available. Recent studies have focused on applying non-invasive techniques to evaluate oxidative stress in COPD. Measurements of these surrogate markers have been made in blood, urine, breath or breath condensate or in induced or spontaneously produced sputum of smokers and patients with COPD. The assessment of oxidative stress biomarkers in exhaled breath condensate (EBC) is emerging as a promising area of future research in COPD (25–27). The relative concentrations of various oxidant/antioxidant biomarkers detected in EBC are listed in Table 2 (reviewed in Refs. 25–27). There is, however, a limitation of ROS/RNS biomarkers at present due to the lack of correlation with disease severity or outcome. Furthermore, the validity of EBC as a tool for the assessment of airway oxidative stress is still questionable owing to limitations in the reproducibility of analyzed oxidative biomarkers, with respect to intra- and inter-individual variability, sampling time and variability in dilution of respiratory droplets by water vapor, sensitivity, and specificity of the assays used. Other confounding factors contributing to variation include smoking, consumption of caffeine, alcohol, and intake of diet rich in antioxidants. Identification of specific and reproducible biomarkers of oxidative stress and inflammation in EBC would be of great value for non-invasive investigations of the natural history and epidemiology of COPD, and for phenotyping in genetic studies.

A. ROS

Hydrogen peroxide, measured in exhaled breath condensate (EBC), is a direct measurement of oxidant burden in the airspaces. The source of

Table 2 Oxidant/Antioxidant Biomarkers in Exhaled Breath Condensate and Their Measurements

Biomarker	Analysis	Values in EBC		
		Non-smoker	Smoker	COPD
Reactive oxygen species				
Hydrogen peroxide, μM	Spectroscopy, fluorometry, chemiluminescence	0.01–0.09	0.10–0.75	0.2–2.6
Reactive nitrogen species				
Nitrite, μM	Electrometry	0.64	0.44–2.4	2.6
Nitrite and nitrate, μM	Spectroscopy	20.2	20.2–29.2	—
Nitrotyrosine, ng/mL	ELISA	0.66–6.3	7.2	—
Nitrosothiols, μM	GC/MS, HPLC	—	0.1–0.46	0.24
Lipid peroxidation products				
TBARS, μM	Colorimetry, ELISA	—	—	0.48
Malondialdehydes, nmol/L	Enzyme immunoassay (EIA), GC/MS, HPLC	17.2–19.4 nmol/L by LC/MS	—	57.2 by LC/MS
F_2-Isoprostanes, pg/mL	Liquid chromatography–tandem mass spectrometry (LC/MS)	3.9–15.8 by EIA and 7 ± 4 by GC/MS	24.0	42.5
Antioxidant				
Glutathione, nM	Spectrophotometry, HPLC, liquid chromatography–tandem mass spectrometry	14.1	—	—

H_2O_2 in EBC is unknown but may in part derive from the release of $O_2^{\bullet-}$ from alveolar macrophages. Smokers and patients with COPD have higher levels of exhaled H_2O_2 than non-smokers, and levels are even higher during exacerbations of COPD (25–27). However, H_2O_2 levels vary considerably in healthy young non-smokers and smokers from 0.01 to 0.09 μM and 0.10 to 0.75 μM, respectively (28). This variation (60–80%) in H_2O_2 concentrations may be attributed to different storage conditions and/or analytical techniques used for EBC H_2O_2 assay. Thus the variability of the measurement of exhaled H_2O_2 due to its highly volatile nature, along with the presence of other confounding factors, e.g., increased generation of ROS by cigarette

smoke-mediated redox cycling, has lead to concerns over its reproducibility as a marker for oxidative stress in smokers and in patients with COPD (28). The level of oxidative biomarkers in EBC can also be affected by diurnal variation and age (25–27).

B. Reactive Nitrogen Species and Carbon Monoxide

Exhaled NO has been used as a marker of airway inflammation and indirectly as a measure of oxidative stress. There have been some reports of increased levels of NO in exhaled breath in patients with COPD, but not to the extent reported in asthmatics (25,29). Although another study failed to confirm this result (30), smoking increases NO levels in breath and the reaction of NO with $O_2^{\bullet-}$ (which forms $ONOO^-$) limits the usefulness of this marker in COPD, except perhaps to differentiate from asthma.

Carbon monoxide (CO) is another biomarker of oxidative stress which is generated by the induction of the stress responsive protein heme oxygenase-1 (HO-1). HO-1 breaks heme to biliverdin which is converted into bilirubin by biliverdin reductase with the release of CO and Fe. Bilirubin has antioxidant properties whereas CO has been shown to be cytoprotective. The release of CO can be measured in exhaled breath and has been shown to be elevated in patients with COPD (31). Cigarette smoking increases the formation of RNS and results in nitration and oxidation of plasma proteins. The levels of nitrated proteins (fibrinogen, transferrin, plasminogen, and ceruloplasmin) were higher in smokers (32) compared to non-smokers. Nitric oxide and ONOO-mediated formation of 3-nitrotyrosine in plasma and free catalytic iron (Fe^{2+}) levels in epithelial lining fluid (ELF) are elevated in chronic smokers (33–35). Furthermore, the levels of nitrotyrosine and inducible nitric oxide synthase (iNOS) were higher in airway inflammatory cells obtained by induced sputum from patients with COPD, compared to those with asthma (35). The levels of nitrotyrosine were negatively correlated with the FEV_1%. A recent study by Kanazawa et al. (34) has shown that increased levels of nitrogen oxides and reduced peroxynitrite inhibitory activity were present in induced sputum from patients with COPD. Similarly, Ichinose et al. (33) have shown increased immunostaining of nitrotyrosine and iNOS in airway inflammatory cells obtained from induced sputum in patients with COPD without any change in exhaled NO. They also showed a significant negative correction between the FEV_1 and the amount of nitrotyrosine formation in subjects with COPD. The increased level of RNS was inhibited following steroid therapy in patients with COPD, and the reduction in nitrotyrosine and iNOS immunoreactivity in sputum cells was correlated with the improvement in FEV_1 and airway responsiveness to histamine (35). These direct and indirect studies indicate that an increased RNS- and ROS-mediated protein nitration and lipid peroxidation, respectively, may play a role in the inflammatory response which occurs in COPD.

C. Lipid Peroxidation Products

There is increasing evidence that aldehydes, generated endogenously during the process of lipid peroxidation, are involved in many of the pathophysiological effects associated with oxidative stress in cells and tissues. Compared with free radicals, lipid peroxidation aldehydes are generally stable, can diffuse within, or even escape from the cell and attack targets far from the site of the original free radical event. In addition to their cytotoxic properties, lipid peroxides are increasingly recognized as being important in signal transduction for a number of important events in the inflammatory response.

Isoprostanes are products of non-enzymatic lipid peroxidation and have therefore been used as markers of oxidative stress. The isoprostanes are ROS catalyzed isomers of arachidonic acid and are stable lipid peroxidation products, which circulate in plasma and are excreted in the urine (36). The levels of 8-iso-prostglandin $F_2\alpha$ (8-isoprostane) in EBC are elevated in healthy smokers and more markedly in patients with COPD reflecting the degree of oxidative stress (25,37). We have recently shown that plasma and BALF levels of 8-isoprostanes were increased in chronic smokers and patients with stable COPD than in healthy, age-matched non-smoking subjects (Fig. 10). The plasma levels of F_2-isoprostanes were even higher in patients with acute exacerbations of COPD, than in patients with stable COPD, indicating that increased oxidative stress occurs in exacerbations. Furthermore, the increased levels of these markers of lipid peroxidation products have been correlated with airway obstruction. Urinary levels of

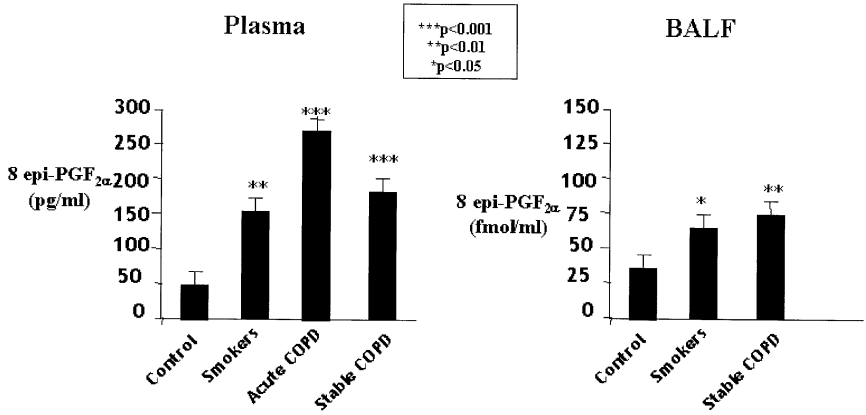

Figure 10 Plasma and BALF levels of F_2-isoprostanes in smokers with and without COPD measured by gas chromatography/negative ion chemical ionization mass spectrometry with an internal standard. Each bar is mean ± SE of 12–18 subjects/patients. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$.

F_2-isoprostane have been shown to be elevated in patients with COPD compared with control subjects and are even more elevated during exacerbations of COPD (36). Thus, 8-isoprostane is currently one of the reliable oxidative stress biomarkers that have been shown to be elevated in plasma, EBC, BALF, and urine of patients with COPD. However, it is not known whether the increased level of lipid peroxidation products found in this condition is the result of primary lung-associated processes (inflammatory responses), such as alveolar macrophage activation, neutrophil activity, or caused by the ongoing lipid peroxidative chain reaction in the alveoli, parenchyma, or airways, which are induced by inhaled oxidants/cigarette smoke.

Indirect and non-specific measurements of lipid peroxidation products, such as thiobarbituric acid reactive substances (TBARS), have also been shown to be elevated in breath condensate and in lungs of patients with stable COPD (39,40). The levels of plasma lipid peroxides (TBARS) have been shown to be elevated in COPD, and negatively correlated with the FEV_1. Oxidative stress, measured as lipid peroxidation products in plasma, has also been shown to correlate inversely with the % predicted FEV_1 in a population study, suggesting that in patients with COPD lipid peroxidation may play a role in the progression of the disease. Similarly, hydrocarbons such as ethane and pentane in exhaled air are increased in smokers and in patients with COPD (25,38). Other specific products of lipid peroxidation, such as malondialdehyde (MDA) and 4-HNE, derivitized immediately after sampling, can be measured successfully by a more reliable HPLC method. Using liquid chromatography–tandem mass spectrometry technique, Corradi et al. (41) have recently shown the higher concentrations of MDA in the EBC of COPD. Further research of longitudinal and cross-sectional studies of well-characterized smokers with and without COPD are needed to evaluate the correlation of a broad array of putative oxidative markers of COPD to susceptibility, severity, exacerbations, and progression of the disease.

IX. SYSTEMIC AND LOCAL DEPLETION OF ANTIOXIDANTS

The ELF is the interface between the airspace epithelium and the external environment and forms a critical defense mechanism against inhaled oxidants or those produced by cells in the airspaces. Antioxidants in ELF include low molecular weight antioxidants, metal binding proteins, antioxidant enzymes, sacrificial reactive proteins, and unsaturated lipids. The concentrations of non-enzymatic antioxidants vary in ELF (Table 3). Some of these antioxidants, such as glutathione and ascorbate are concentrated in ELF compared to plasma, which may indicate their relative importance in ELF. The major antioxidants in ELF include mucin, reduced glutathione, uric acid, protein (largely albumin), ceruloplasmin, and ascorbic acid.

Table 3 Antioxidant Constituents of Plasma and Epithelial Lining Fluid (ELF)

Antioxidant	Plasma (μM)	ELF (μM)
Ascorbic acid	40	100
Glutathione	1.5	100
Uric acid	300	90
Albumin-SH	500	70
Alpha-tocopherol	25	2.5
β-Carotene	0.4	—

Smoking and exacerbations of COPD result in decreased antioxidant capacity in plasma in association with depleted protein sulfydryls in the plasma (12,22). The decrease in antioxidant capacity in smokers occurred transiently during smoking and resolved rapidly after smoking cessation (Fig. 11). In exacerbations of COPD, however, the decrease in antioxidant capacity remained low for several days after the onset of the exacerbation, tending to return towards normal values at the time of recovery from the exacerbation (22). The depletion of antioxidant capacity could in part be explained by the increased release of ROS from peripheral blood neutrophils, as shown by a significant negative correlation between neutrophil superoxide anion release

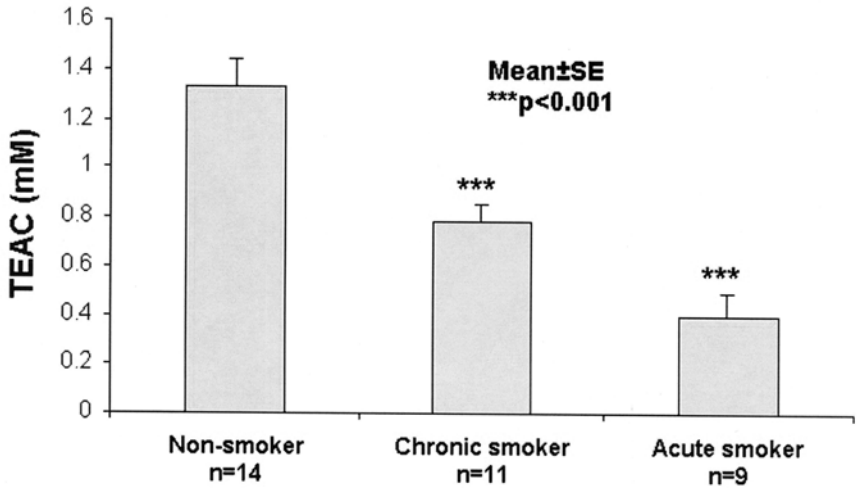

Figure 11 Plasma antioxidant capacity measured as Trolox Equivalent Antioxidant Capacity (TEAC) in healthy non-smokers and healthy chronic smokers who have not smoked for 12 hr and smokers who smoked two cigarettes one hour prior to measurement (acute smoker). $^{***}p < 0.001$ compared with non-smokers. Data are Mean ± SEM.

and plasma antioxidant capacity (12). Thus there is clear evidence that oxidants in cigarette smoke markedly decrease plasma antioxidants.

Studies showing depletion of total antioxidant capacity in smokers are associated with depletion of the major plasma antioxidants, i.e., ascorbic acid, vitamin E, β-carotene, and selenium in the serum of chronic smokers (42). Moreover, decreased vitamin E and vitamin C levels were reported in leukocytes from smokers (1). Ascorbate appears to be a particularly important antioxidant in the plasma (Table 3). Cigarette smoke-induced lipid peroxidation of plasma in vitro is decreased by ascorbate (1). These studies have suggested that cigarette smoking depletes a variety of multiple antioxidants that are needed to quench an array of free radicals present in cigarette smoke and to inhibit the inflammatory response induced by cigarette smoking. Changes in other antioxidants and antioxidant enzymes in response to cigarette smoke have been variable. Reduced levels of vitamin E in the BAL fluid of smokers and a marginal increase in vitamin C in BALF of smokers, compared to non-smokers, have been shown (1). Similarly, alveolar macrophages from smokers have both increased levels of ascorbic acid and augmented uptake of ascorbate (1). Increased activity of antioxidant enzymes (SOD and catalase) in alveolar macrophages from young smokers has also been reported (1). However, increased superoxide generation by alveolar macrophages in elderly smokers was associated with decreased antioxidant enzyme activities when compared with non-smokers. The activities of CuZnSOD, glutathione-*S*-transferase (GST), and glutathione peroxidase (GP) are all decreased in alveolar macrophages from elderly smokers. These direct and indirect studies indicate that an increased systemic and local pulmonary oxidant burden occurs in smokers, and in patients with COPD (1).

X. DEPLETION OF LUNG GLUTATHIONE

Several studies have suggested that GSH homeostasis may play a central role in the maintenance of the integrity of the lung airspace epithelial barrier. Decreasing the levels of GSH in epithelial cells leads to loss of barrier function and increased permeability (14,43,44).

There is limited information on the respiratory epithelial antioxidant defenses in smokers, and less for patients with COPD. Several studies have shown that GSH is elevated in BALF in chronic smokers (14,45). However, this increase is not present immediately after acute cigarette smoking (14).

However, Harju et al. (46) have found that the γ-glutamylcysteine synthetase, γ-GCS (now called as γ-glutamylcysteine ligase) immunoreactivity was decreased (GSH levels were not measured) in the airways of smokers compared to non-smokers suggesting that cigarette smoke predisposes lung cells to ongoing oxidant stress. This suggests the twofold increase in BALF

GSH in chronic smokers may not be sufficient to deal with the excessive oxidant burden during smoking, when acute depletion of GSH may occur (44). Neurohr et al. (47) recently showed that decreased GSH levels in BALF cells of chronic smokers were associated with a decreased expression of γ-GCS-light subunit without a change in γ-GCS-heavy subunit expression. This highlighted the fact that increased GSH levels in the ELF of chronic smokers were not associated with increased GSH levels in alveolar macrophages. The acute effects of cigarette smoke condensate (CSC) on GSH metabolism have been studied in a human alveolar epithelial cell line in vitro, and in rat lungs in vivo after intra-tracheal CSC instillation, CSC produced a dose and time-dependent depletion of intracellular GSH, concomitant to the formation of GSH-conjugates. Similar results were shown in animal lungs in vivo (2,44). Thus, further studies are needed to investigate the regulation of GSH levels in the lungs of smokers and patients with COPD to devise appropriate GSH therapy.

XI. AIRSPACE EPITHELIAL INJURY/PERMEABILITY

By virtue of its direct contact with the environment, the airspace epithelial surface of the lungs is particularly vulnerable to the effects of oxidative stress produced by cigarette smoke. Cigarette smoke-induced oxidant injury to the respiratory tract epithelial cells may result from several processes e.g., (a) a direct toxic interaction with constituents of cigarette smoke (including free radicals) which have penetrated the protective antioxidant shield of the ELF; (b) damage to the cells by toxic reactive products generated by interaction between cigarette smoke and ELFs; and (c) reactions occurring subsequent to activation of inflammatory-immune processes initiated by (a) and/or (b).

Oxidants in cigarette smoke can produce direct oxidative damage to components of the lung matrix, such as elastin and collagen. Elastin synthesis and repair can also be impaired by cigarette smoke, which can augment proteolytic damage to matrix components and thus enhance the development of emphysema.

Airspace epithelial injury is an important early consequence of the inflammation produced by cigarette smoke and results in an increase in airspace epithelial permeability. Human studies have shown increased epithelial permeability in chronic smokers compared with non-smokers, as measured by increased ^{99m}technetium-diethylenetriaminepentacetate (^{99m}Tc-DTPA) lung clearance, with a further increase in ^{99m}Tc-DTPA clearance following acute smoking (14). The injurious effect of both the whole and vapor phases of cigarette smoke on human alveolar epithelial cell monolayers is shown by increased epithelial cell detachment, decreased cell adherence, and increased cell lysis (2). These effects were in part oxidant-mediated since they were

abrogated by the antioxidant glutathione (GSH) in concentrations (500 μM), which are present in the epithelial lining fluid. Extra- and intra-cellular glutathione appears to be critical to the maintenance of epithelial integrity following exposure to cigarette smoke. Studies have demonstrated that the increased epithelial permeability of epithelial cell monolayers in vitro and in rat lungs in vivo following exposure to cigarette smoke condensate was associated with profound changes in the antioxidant glutathione (2,44). Furthermore, depletion of lung GSH alone, by treatment with the glutathione synthesis inhibitor buthionine sulfoximine, induces increased airspace epithelial permeability both in vitro and in vivo (2).

XII. NEUTROPHIL SEQUESTRATION AND MIGRATION IN THE LUNGS

The inflammatory response in the airspaces in COPD is characterized by the influx of leukocytes of which polymorphonuclear leukocytes are the prominent cells. The recruitment of neutrophils to the airspaces is initiated by the sequestration of these cells in the lung microcirculation. Sequestration of neutrophils in the pulmonary capillaries permits cell interaction with the pulmonary capillary endothelium, resulting in their adherence to the endothelium and thereafter their transmigration across the alveolar capillary membrane to the interstitium and airspaces of the lungs in response to inflammation or infection.

Neutrophils can be activated while in transit in the pulmonary microcirculation by a number of mediators, including cytokines released from resident lung cells, alveolar macrophages, epithelial and endothelial cells. Inhaled oxidants such as those contained in cigarette smoke and other air pollutants could influence the transit of cells in the pulmonary capillary bed by decreasing the neutrophil deformability (in order neutrophil to pass the smaller capillary segments). Studies in man using radio-labeled neutrophils and red cells show a transient increase in neutrophil sequestration and decreased neutrophil deformability in the lungs during smoking/oxidative stress (1), which returns to normal upon cessation of smoking. A similar decrease in deformability can be demonstrated in vivo for neutrophils from the blood of subjects who are actively smoking (1). Support for oxidative stress-mediated decreased neutrophil dormability comes from in vitro studies, which show that the decreased neutrophil deformability induced by cigarette smoke exposure is abolished by antioxidants, such as glutathione (21).

Neutrophils sequestered in the pulmonary microcirculation will subsequently respond to chemotactic components in cigarette smoke and become more adhesive to pulmonary vascular endothelial cells, in preparation for migration into the airspaces. Smoke exposure in humans results in increased levels of chemotactic factors in the airspaces. Studies in animal models of

smoke exposure have shown increased neutrophil sequestration in the pulmonary microcirculation, associated with upregulation of adhesion molecules on the surface of these cells (1,21). Activation of neutrophils sequestered in the pulmonary microvasculature could also induce the release of ROS and proteases within the microenvironment with limited access for free radical scavengers and antiproteases. Thus, destruction of the alveolar wall, as occurs in emphysema, could result from a proteolytic or oxidant insult from the intra-vascular space, without the need for the neutrophils to migrate into the airspaces. Increased sequestration of neutrophils also occurs in the pulmonary microcirculation during exacerbations of COPD (1,21).

XIII. CONSEQUENCES OF OXIDANT/ANTIOXIDANT IMBALANCE

A. Protease/Antiprotease Imbalance

The development of a protease/antiprotease imbalance in the lungs is a central hypothesis in the pathogenesis of emphysema in smokers. This theory was developed from studies of early onset emphysema in antitrypsin (α_1-AT) deficient subjects. In the case of smokers with normal levels of α_1-AT, the elastase burden may be increased as a result of increased recruitment of leukocytes to the lungs and there may be a functional deficiency of α_1-AT, due to oxidative inactivation of α_1-AT in the lungs.

It is clear that an imbalance between an increased elastase burden in the lungs and a functional "deficiency" of α_1-AT due to its inactivation by oxidants is an over simplification since other proteinases and antiproteinases are also likely to have a role. Early studies showed that the function of α_1-1-antitrypsin in bronchoalveolar lavage was decreased by around 40% in smokers, compared with non-smokers (48). This "functional α_1-l-AT deficiency" is thought to be due to inactivation of the α_1-1-AT by oxidation of the methionine residue at its active site by oxidants in cigarette smoke. Secretory leukoprotease inhibitor (SLPI), another major inhibitor of neutrophil elastase (NE), can also be inactivated by oxidants (49).

In vitro studies have also shown loss of α_1-l-AT inhibitory capacity when treated with oxidants including cigarette smoke. In addition, oxidation of the methionine residue in α_1-l-AT was confirmed in the lungs of healthy smokers (50). Furthermore, neutrophilic inactivation of α_1-l-PI may be caused by hypochlorous acid or peroxynitrite. It has been shown that stimulated alveolar type II epithelial cells and alveolar macrophages (but not fibroblasts) from guinea pigs inactivated α-l-PI in the presence of myeloperoxidase (1,51). These studies supported the concept of inactivation of α_1-l-AT by oxidation of the active site of the protein. As already discussed, macrophages from the lungs of smokers release increased amounts

of ROS, which could also inactivate α_1-l-AT in vitro (21). However, most of the α_1-1-AT in the airspaces in cigarette smokers remains active and is therefore still capable of protecting against the increased protease burden. In addition, the original observation that oxidation of α_1-1-AT occurs in bronchoalveolar lavage in smokers has not been confirmed (52).

B. Mucus Hypersecretion

Chronic bronchitis is associated with hyperplasia of both epithelial goblet cells and submucosal glands in the airways. Mucins, which are complex glycoproteins that provide the viscoelastic properties of mucus, are an essential protective mechanism in the upper airways. The regulation of mucins is altered in the lungs of COPD patients. The airways of smokers contain more goblet cells than do those of non-smokers and goblet-cell activation results in mucus hypersecretion leading to airway plugging. Cigarette smoke can activate epidermal growth factor (EGF) receptors by tyrosine phosphorylation, resulting in the induction of mucin (MUC5AC gene expression) synthesis in epithelial cells and in vivo in lungs (53). Selective EGFR typrosine kinase inhibitors and antioxidants inhibited the cigarette smoke-mediated MUC5AC gene expression suggesting the role of ROS in the gene expression (53). 4-HNE have been shown to cause the release of mucus from airway epithelial cells and fibroblast by the activation of ERK1/2 (54). It has also been shown that elastase released from neutrophils impairs mucociliary clearance and stimulates goblet cell metaplasia and mucin production. Neutrophil elastase increases the expression of MUC5AC, by enhancing mRNA stability (55). Understanding of the EGF receptor signaling pathway in cigarette smoke-mediated upregulation of mucin gene expression could lead to targeted inhibition of mucus hyper-production in epithelial cells.

C. Remodeling of Extracellular Matrix

Oxidative stress has been shown to be involved in the remodeling of extracellular matrix in lung injury. Treatment of cigarette smoke to alveolar macrophages obtained from patients with COPD release increased amounts of MMP-9 compared to that of smokers suggesting the role of oxidative component of CS in increased elastolytic enzyme by alveolar macrophages of only patients with COPD (56). It has been shown that the oxidative stress caused by ozone and lipid peroxides induce matrix protein type I collagen and MMP-1 gene expression (57). Other forms of oxidative stress derived from *tert*-butyl hydroperoxide and iron can also modify collagen synthesis, by a mechanism presumably involving redox sensor/receptor. Recent study by Wang et al. (58) has shown that cigarette smoke produces airway wall remodeling in rat tracheal explants by induction of procollagen and

NF-κB activation via an ROS-dependent mechanism. They suggested that transactivation of EGFR, rather than MAPK activation, was involved in airway remodeling. Furthermore, the cigarette smoke-mediated activation of EGFR was oxidant-dependent.

D. Apoptosis

Apoptosis, or programmed cell death, is a physiological program for removal of harmful or unwanted cells in vivo leading to resolution of inflammation. Recently, it has been proposed that apoptosis of alveolar wall cells occurs in response to cigarette smoking, resulting in progressive cell loss and emphysema. Oxidative stress has been implicated in mediating apoptotic processes, particularly in airway epithelial cells. Recent evidence from both in vitro and in vivo studies in animals and in man has shown that apoptosis occurs in smoke exposed macrophages, airway epithelial, dendritic cells, and lung fibroblasts (59,60). This may be due to activation of caspases and/or apoptosis signal-regulating kinase-1 which is held in an inactive form by thioredoxin and when oxidized by ROS this triggers apoptotic pathways (61). The cigarette smoke-mediated apoptosis was inhibited by antioxidants such as glutathione and *N*-acetyl-l-cysteine treatments (59,60). Recent reports have shown both in vivo and in vitro that cigarette smoke exposure produces endothelial cell apoptosis (62–65) and that pulmonary vascular endothelial cell apoptosis is present in emphysematous lungs (62). Recently, Tuder et al. (66) have shown that inhibition of KDR leads to increased oxidative stress which is mediated by reactive carbonyls and aldehydes leading to emphysema. They have also showed that both a caspase inhibitor (Z-Asp-CH2) and a superoxide dismutase mimetic (M40419) blocked the development of emphysema and significantly reduced lung markers of oxidative stress and apoptosis (65). In addition, signaling and survival pathways involving AP-1, NF-κB, and Akt and the downregulation of the vascular endothelial growth factor receptor KDR (VEGF-KDR) have been proposed as part of the mechanism (62,63). However, using a variety of cellular models, it is proposed that cigarette smoke induces necrosis by inhibition of caspase activation and hence it prevents apoptosis (67). Aoshiba et al. (68) have shown that single intratracheal injection of active caspase-3 resulted in epithelial apoptosis and enhanced elastolytic activity in mice. Further confirmation of the role of apoptosis in emphysematous changes in murine model was described by intratracheal injection of nodularin, a proapoptotic serine/threonine kinase inhibitor. Surprisingly, this approach did not cause inflammation or other forms of lung pathology, a hallmark of emphysema. Nevertheless, it is clear that lung structural cells undergo apoptosis by cigarette smoke but the scenario may be different for inflammatory cells. It is likely that cigarette smoke and its constituents through their

interaction with ECM may inhibit inflammatory cell apoptosis thereby promoting the inflammatory response in the lungs. Further studies are required to understand the mechanism of cigarette smoke-induced cell death using both in vitro and in vivo models so as to define strategy for safe resolution of inflammation or inhibit the alveolar wall destruction.

E. Muscle Dysfunction

Dysfunction of the respiratory and of peripheral skeletal muscles is known to occur in patients with severe COPD. Weight loss, skeletal muscle dysfunction, and exercise limitation are increasingly recognized as important features of systemic effects of COPD. Oxidative stress occurs in skeletal muscle during skeletal muscle fatigue, weakness, and sepsis induced muscle dysfunction, accompanied by an increased load imposed on the diaphragm in patients with severe COPD (69). This may be due to hypoxia, impaired mitochondrial metabolism, and increased cytochrome C oxidase activity in skeletal muscle in patients with COPD (70). Ribera et al. (71) have recently shown that mitochondrial electron transport chain function is enhanced in inspiratory muscles of patients with COPD. This was associated with an increase in functional demand on the muscles to endurance training-like effect leading to increased oxidative stress. Engelen et al. (70) have found reduced muscle glutamate (a precursor of glutathione) levels associated with increased muscle glycolytic metabolism in patients with severe COPD. Lowered levels of glutamate were associated with decreased GSH levels, suggesting that oxidant/antioxidant imbalance is involved in skeletal muscle dysfunction in patients with COPD (72). A causal relationship between abnormally low muscle redox potential at rest and the alterations of protein metabolism observed in patients with emphysema has been suggested. This is supported by Rabinovich et al. (69) who showed decreased muscle redox capacity probably due to lower ability to synthesize GSH during endurance training in patients with COPD. Moreover, this was clearly evident in patients who had low body mass index. Recently Agusti et al. (73) have shown that apoptotic pathways may be involved in skeletal muscle atrophy in patients with COPD. They hypothesize that this might be related to ''cachexia'' and accelerated ageing. Thus, oxidative stress-mediated muscle atrophy may involve the loss of skeletal myocytes/myofibers through an apoptosis in patients with COPD. However, it remains to be determined whether oxidative stress and/or poor nutrition (alterations in calorie intake or lowered basal metabolic rate) play a central role in mediating muscle mass wasting/apoptosis, particularly in susceptible subsets of patients with COPD. The other unanswered question is whether the susceptibility of subgroups of COPD patients to oxidative stress and injury in muscle wasting is due to their inability to boost endogenous protective defense, and/or defective repair.

XIV. OXIDATIVE STRESS AND THE DEVELOPMENT OF AIRWAYS OBSTRUCTION

The neutrophil appears to be a critical cell in the pathogenesis of COPD. Previous epidemiological studies have shown a relationship between circulating neutrophil numbers and the FEV_1 (1,21). Moreover, a relationship has also been shown between the change in peripheral blood neutrophil count and the change in airflow limitation over time. A relationship between peripheral blood neutrophil luminol enhanced chemiluminescence, as a measure of the release of ROS and measurements of airflow limitation has been shown in young cigarette smokers. Oxidative stress, measured as lipid peroxidation products in plasma, has also been shown to correlate inversely with the % predicted FEV_1 in a population study (74).

It is possible that inter-individual differences in antioxidant capacity may contribute to the differences in the susceptibility against cigarette smoke-induced COPD. In the general population, there is a positive association between dietary intake of antioxidant vitamins and lung function. Epidemiological studies have demonstrated negative associations of dietary antioxidant intake with pulmonary function and with obstructive airway disease (75). Britton et al.(76), in a population of 2633 subjects, showed a positive association between dietary intake of the antioxidant vitamin E and lung function, supporting the hypothesis that this antioxidant may have a role in protecting against the development of COPD. Another study has suggested that antioxidant levels in the diet could be a possible explanation for differences in COPD mortality in different populations (77). Dietary polyunsaturated fatty acids may protect cigarette smokers against the development of COPD (78). These studies support the concept that dietary antioxidant supplementation including polyphenols may be a possible therapy to prevent or inhibit the oxidative stress and inflammatory response which are the key features in the development of COPD. Such interventional studies have been difficult to carry out but there is at least some evidence to suggest that antioxidant vitamin supplementation reduces oxidant stress in smokers, measured as a decrease in pentane levels in breath as an indication of lipid peroxides in the airways (38).

XV. OXIDATIVE STRESS AND SUSCEPTIBILITY TO COPD

Only 15–20% of cigarette smokers appear to be susceptible to the effects of cigarette smoke. These subjects show a rapid decline in FEV_1 and develop COPD. There has been considerable interest in identifying those who are susceptible and the mechanisms of that susceptibility (79), since this would provide an important insight into the pathogenesis of COPD as did the recognition of the association between α_1-antitrypsin and COPD.

Polymorphisms of various genes, in particular oxidant and antioxidant responsive genes, have been shown to be more prevalent in smokers who develop COPD. Microsomal epoxide hydrolase (an enzyme involved in the metabolism of highly reactive epoxide intermediates which are present in cigarette smoke) has been shown to be polymorphic in emphysema (80). The proportion of individuals with slow microsomal epoxide hydrolase activity (homozygotes) was significantly higher in patients with COPD and a subgroup of patients shown pathologically to have emphysema (COPD 22%; emphysema 19%), compared with control subjects (6%) (80,81). Similarly, the polymorphism of another antioxidant gene-glutathione-*S*-transferase is associated with decline in lung function in smokers (81). Variations in the levels of glutathione and the genetic polymorphism of its synthesizing gene γ-GCS, and HO-1 represent new oxidative stress susceptibility factor in the pathogenesis in COPD (44,82). It may be that a panel of "susceptibility gene" polymorphisms, of functional significance in enzymes involved in xenobiotic metabolism or antioxidant enzyme genes may allow individuals to be identified as being susceptible to the effects of cigarette smoke.

XVI. OXIDATIVE STRESS, NF-κB ACTIVATION, AND GENE EXPRESSION

A. NF-κB Activation

It is well known that various inflammatory genes, such as those for the cytokines, IL-8, TNF-α, and nitric oxide (iNOS), are regulated by NF-κB. NF-κB exists as a heterodimeric complex usually of p50 and p65/RelA subunits. In unstimulated cells, NF-κB is found in the cytoplasm as an inactive non-DNA binding form, associated with an inhibitor protein called inhibitory κB (IκB) which masks the nuclear translocation signal and so prevents NF-κB from entering the nucleus. Many stimuli, including cytokines and oxidants, activate NF-κB, resulting in ubiquitination, cleaving of IκB from NF-κB and the destruction of IκB in the proteosome (Fig. 12). The released NF-κB dimer can then be translocated into the nucleus and activate target genes by binding with high affinity to κB elements in their promoters. These critical events in the inflammatory response are redox sensitive. Recently, Di Stefano et al. (83) have demonstrated increased expression of p65 protein of NF-κB in bronchial epithelium of smokers and patients with COPD. The increased expression of p65 in epithelial cells was correlated with the degree of airflow limitation in patients with COPD. Similarly, Caramori et al. (84) have shown the p65 subunit of NF-κB was increased in sputum macrophages but not in sputum neutrophils during exacerbations of COPD suggesting that inflammatory response can be seen in diverse cell population. The activation of NF-κB in monocytes/macrophages can then trigger the release of pro-inflammatory mediators in lung epithelial fluid which

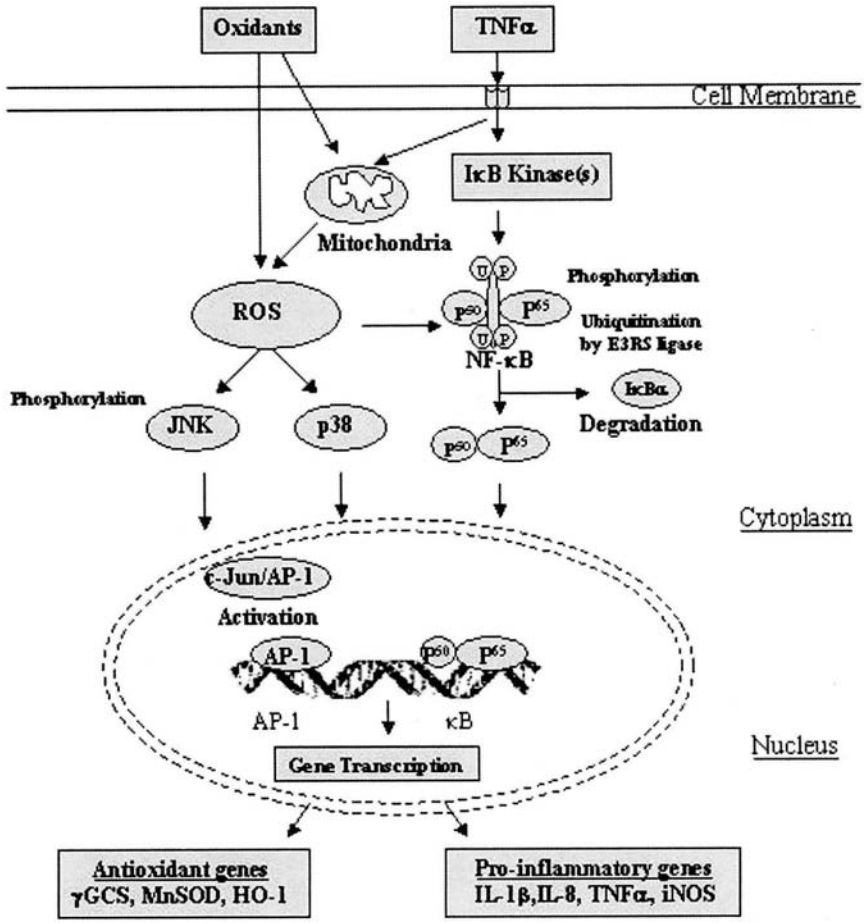

Figure 12 Model for the mechanism of NF-κB and AP-1 activation leading to the gene transcription in lung epithelial cells. TNF-α/oxidants act on mitochondria to generate ROS which are involved in the activation of NF-κB and AP-1. Activation of NF-κB involves the phosphorylation, ubiquitination, and subsequent proteolytic degradation of the inhibitory protein IκB. Free NF-κB then translocates into the nucleus and binds with its consensus sites. Similarly, AP-1 either c-Jun/c-Jun (homodimer) or c-Fos/c-Jun (heterodimer) is activated by the phosphorylation of JNK pathway leading to the activation AP-1 and binds with its TRE consensus regions. Activation of NF-κB/AP-1 leads to the co-ordinate expression of antioxidant protective and pro-inflammatory genes.

would then amplify the inflammatory cascade by activation of epithelial cells as well as recruitment of neutrophils in the airways.

Mochida-Nishimura et al. (85) have shown that cells obtained from BALF from smokers exhibited a 10-fold higher activation of NF-κB in

response to lipopolysaccharide (LPS) compared to that of non-smokers. This may be due to the elevated release of inflammatory mediators that may activate NF-κB. However, activation of the MAP kinase pathways [ERK, stress activated protein kinase (SAPK), and p38] was differentially regulated. Activation of p38 was more rapid in BAL cells from smokers compared to the activity of ERK and SAPK. They also suggested that the differences in activation of NF-κB and MAP kinases in BAL cells from smokers and non-smokers may be related to the differences in their microenvironment, which is affected by chronic exposure to cigarette smoke. The activation of p38, therefore, may be responsible for the elevated levels of TNF-α and IL-8 seen in BALF and sputum of patients with COPD (86).

B. Pro-Inflammatory Genes

Evidence from a large number of studies indicates that COPD is associated with airway and airspace inflammation and by the presence of markers of inflammation, including IL-8 and TNFα which are elevated in the sputum of patients with COPD (86). Studies in vitro show that treatment of macrophages, alveolar and bronchial epithelial cells with oxidants stimulate the release of inflammatory mediators such as IL-8, IL-1, and nitric oxide. This is associated with increased expression of the mRNA for the genes for these inflammatory mediators, and increased nuclear binding and activation of NF-κB (87,88). Similarly, several investigators have shown that cigarette smoke induces IL-8 release from human bronchial and endothelial cells which may contribute to airway inflammation in smokers (85,89). The increased IL-8 was significantly correlated with neutrophil counts in bronchial samples of BALF (85).

Cigarette smoke has been shown in vivo to be a cause of increased adherence of leukocytes to vascular endothelium (23). Shen et al. (90) have shown that cigarette smoke condensate induces the expression of a subset of cell adhesion molecules, such as intercellular adhesion molecule (ICAM-1), endothelial leukocyte adhesion molecule 1 (ELAM-1), and vascular cell adhesion molecule (VCAM-1) in human umbilical vascular endothelial cells associated with an increase in the binding activity of NF-κB suggesting the increased transendothelial migration of monocytes by cigarette smoking. The release of pro-inflammatory mediators, such as IL-lβ and sICAM-1, was increased by cigarette smoke exposure in bronchial epithelial cells cultured from biopsy materials obtained from patients with COPD compared to smokers (91). Similarly, primary cultured human bronchial epithelial cells obtained from patients with COPD showed higher levels of TNF-α-induced release of IL-6 and IL-8 compared to non-stimulated COPD cells (92). Surprisingly, the response was not different in cells obtained from current/ex-smokers. These studies suggest that patients with COPD have a greater susceptibility to the effects of cigarette smoke.

C. Antioxidant and Stress Response Genes

An important effect of oxidative stress in the lungs is the upregulation of protective antioxidant and stress response genes. Human studies have shown elevated levels of glutathione in epithelial lining fluid in chronic cigarette smokers compared with non-smokers (14,45). This increased level of glutathione is due to transcriptional upregulation of the gene for GSH synthesis (γ-GCS) by components within cigarette smoke in epithelial cells (43,44). Thus oxidative stress, including that produced by cigarette smoking, causes upregulation of the gene involved in the synthesis of glutathione as a protective mechanism against oxidative stress. However, the injurious effects of cigarette smoke may occur repeatedly during and immediately after cigarette smoking when the lung is depleted of antioxidants, including glutathione.

The cytokine tumor necrosis factor (TNF), which is present as part of the airway inflammation in COPD, has been shown to increase γGCS mRNA expression in alveolar epithelial cells (93). Corticosteroids have been used as anti-inflammatory agents in COPD, but there is still doubt over their effectiveness in reducing airway inflammation in COPD. Interestingly dexamethasone also causes a decrease in intracellular glutathione in airspace epithelial cells, by downregulating the GSH biosynthesis (93). Moreover, the induction of γGCS produced by TNF-α in epithelial cells is prevented by co-treatment with dexamethasone (93). These effects may have relevance for the treatment of COPD patients with corticosteroids. Transforming growth factor (TGF-β_1) has been shown to decrease antioxidant glutathione synthesis and is associated with increased ROS production in human alveolar epithelial cells and pulmonary artery endothelial cells in vitro (94). Increased TGF-β_1 expression was associated with fibrosis in the basement membrane in the lungs and depletion of GSH, suggesting that cigarette smoking interferes in normal repair (Fig. 13).

The activities of SOD and glutathione peroxidase (GP_X), manganese superoxide dismutase (MnSOD), and metallothionein (MT) have been shown to be higher in the lungs of rats exposed to cigarette smoke (95). Similarly, antioxidant enzyme activities were enhanced in alveolar macrophages in hamsters following cigarette smoke exposure, which resulted in reduced mortality when the animals were subsequently exposed to $>95\%$ oxygen (1). It is likely that alveolar macrophages undergo an adaptive response to chronic oxidant exposure that may ameliorate potential damage to lung cells from further oxidant stress by induction of antioxidant enzymes.

Extracellular glutathione peroxidase (eGPx) is an important antioxidant in the lungs and may be secreted by epithelial cells and macrophages, particularly in response to cigarette smoke or oxidative stress (96). Ishii et al. (97) have shown that glutathione-*S*-transferase PI (GSTP1) acts as a protective enzyme against cigarette smoke in the airway cells. Similarly,

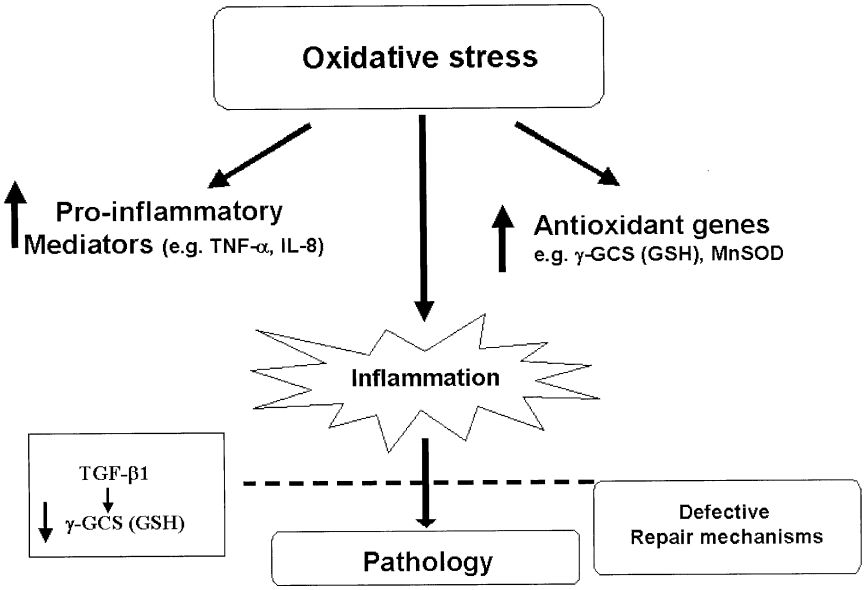

Figure 13 Oxidative stress/cigarette smoke can cause increased gene expression of both pro-inflammatory genes and also activation of protective genes, such as γ-glutamylcysteine synthetase. During sustained/chronic inflammation, the balance between genes for inflammatory mediators and antioxidant/phase II enzymes may be tipped in favor of pro-inflammatory mediators. It is possible that oxidative stress is enhanced during repair process by decreasing the GSH levels leading to pathology.

Maestrelli et al. (98) have shown that heme oxygenase-1 (HO-1) is induced in alveolar spaces of smokers suggesting that oxidative stress due to cigarette smoke may increase the gene expression of HO-1 leading to increased levels of exhaled CO (98). Cigarette smoke also induces heat shock protein 70 (HS 70) in human monocytes and HO-1, which have been implicated in the regulation of cell injury and cell death and, in particular, modulation of apoptosis in human endothelial cells and monocytes (99).

Thus oxidative stress, including that produced by cigarette smoke, causes increased gene expression of both pro-inflammatory genes and also activation of protective antioxidant genes. A balance may therefore exist between pro- and anti-inflammatory gene expression in response to cigarette smoke, which may be critical to whether cell injury is induced by cigarette smoking. Such an imbalance of an array of redox-regulated antioxidant vs. pro-inflammatory genes might, therefore, be associated with the susceptibility or tolerance to disease. It is possible that induction of antioxidant enzymes may provide initial adaptive or protective responses against oxidative stress and inflammatory mediators. However, during sustained/chronic

inflammation, the balance between genes for inflammatory mediators and antioxidant/phase II enzymes may be tipped in favor of pro-inflammatory mediators (Fig. 13).

XVII. CHROMATIN REMODELING (HISTONE ACETYLATION AND DEACETYLATION) AND GLUCOCORTICOID INEFFICACY IN RESPONSE TO SMOKING

Tightly bound DNA around a nucleosome core (histone proteins) suppresses gene transcription by decreasing the accessibility of transcription factors, such as NF-κB and AP-1 to the transcriptional complex. Acetylation of lysine residues in the N-terminal tails of the core histone proteins results in uncoiling of the DNA, allowing increased accessibility for transcription factor binding. Histone acetylation is reversible and is regulated by a group of acetyltransferases (HATs) which promote acetylation, and deacetylases (HDACs) which promote deacetylation.

Recently, Ito et al. (100) have shown a role for histone acetylation and deacetylation in IL-lβ-induced TNF- α release in alveolar macrophages derived from cigarette smokers. They have also suggested that oxidants may play an important role in the modulation of HDAC and inflammatory cytokine gene transcription. Furthermore, we have shown that both cigarette smoke/H_2O_2 and TNF-α caused an increase in histone acetylation (HAT activity) leading to IL-8 expression in monocytes and alveolar epithelial cells in vitro and in vivo in rat lungs (101–103).

It has been suggested that oxidative stress may have a role in the poor efficacy of corticosteroids in COPD. It has been shown that glucocorticoid suppression of inflammatory genes requires recruitment of HDAC-2 to the transcription activation complex by the glucocorticoid receptor (100,103). This results in deacetylation of histones and a decrease in inflammatory gene transcription. A reduced level of HDAC-2 was associated with increased pro-inflammatory response and reduced responsiveness to glucocorticoids in alveolar macrophages obtained from smokers and in vivo in rat lungs exposed to cigarette smoke (101–103). Culpitt et al. (104) have shown that cigarette smoke solution stimulated release of IL-8 and GM-CSF, which was not inhibited by dexamethasone, in alveolar macrophages obtained from patients with COPD compared to that of smokers. They suggested that the lack of efficacy of corticosteroids in COPD might be due to steroid insensitivity of macrophages in the respiratory tract. Thus, the cigarette smoke/oxidant-mediated reduction in HDAC-2 levels in alveolar epithelial cells and macrophages will not only increase inflammatory gene expression but will also cause a decrease in glucocorticoid function in patients with COPD (105). This may be one of the potential reasons for the failure of glucocorticoids to function effectively in reducing inflammation in COPD (Fig. 14).

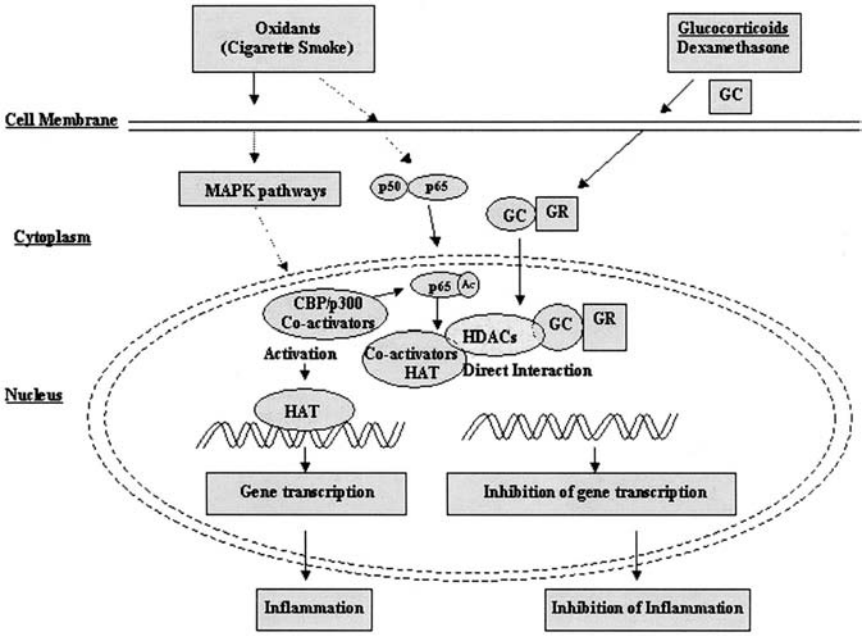

Figure 14 Model showing the possible mechanism of histone acetylation by oxidative stress and its repression by cortico steroids (GCs), leading to inhibition of gene transcription. MAP kinase signaling pathways may be activated by oxidative stress leading to histone acetylation. Direct interaction between co-activators (HAT), histone deacetylase, and the glucocorticoid receptor (GR) may result in repression of the expression of pro-inflammatory genes. HDAC forms a bridge with HAT to inhibit gene transcription. However, when the HDAC is inhibited by oxidants or the NF-κB subunit p65 is acetylated, steroids may not be able to recruit HDACs into the transcriptional complex to inhibit pro-inflammatory gene expression.

XVIII. ANTIOXIDANT THERAPEUTIC INTERVENTIONS

It is now evident that oxidant/antioxidant balance is altered in favor of oxidants in smokers, which plays an important role in the pathogenesis of COPD. Therefore, it would be logical to propose the rationale of antioxidant therapy in ameliorating the increased oxidative stress and consequently the inflammatory response in COPD. There are various options to enhance the lung antioxidant screen (Table 4).

A. Phosphodiesterase 4 Inhibitor

One approach is to target the inflammatory response by reducing the sequestration or migration of leukocytes from the pulmonary circulation into the airspaces. Possible therapeutic options for this are drugs that alter cell

Table 4 Antioxidant and Anti-inflammatory Therapeutic Interventions in COPD

Antioxidant compounds
Thiol compounds-*N*-acetyl-l-cysteine, *N*-acystelyn (NAL)
Inducers of glutathione biosynthesis
Spin traps
SOD and glutathione peroxidase mimetics
Anti-inflammatory/signal transduction inhibitors
p38 kinase inhibitor
NF-κB inhibitors
HDAC inducers
Better glucocorticoids

deformability, so preventing neutrophil sequestration or the migration of neutrophils, either by interfering with the adhesion molecules necessary for migration, or preventing the release of inflammatory mediators, such as IL-8 or Ieukotriene B_4, which result in neutrophil migration. It should also be possible to use agents to prevent the release of oxygen radicals from activated leukocytes or to quench those oxidants once they are formed, by enhancing the antioxidant screen in the lungs. Preliminary studies of a phosphodiesterase 4 inhibitor (PDE4) have shown some therapeutic benefit in patients with COPD (106). The mechanism by which such drugs act is by decreasing the neutrophil activation. In particular, the release of ROS by neutrophils may be decreased, since increasing cAMP blocks the assembly of NADPH oxidase.

B. Spin Traps and Antioxidant Enzyme Mimetics

The other approach would be to use specific spin traps such as α-phenyl-*N*-tert-butyl nitrone to react directly with reactive oxygen and reactive nitrogen species at the site of inflammation (107). The therapeutic purposes of this drug are currently in clinical development. Inhibitors that have a double action, such as the inhibition of lipid peroxidation and quenching radicals, could be developed. In a recent study by Smith et al. (108) have showed that intratracheal instillation of a catalytic antioxidant, manganese (III) meso-tetrakis (*N*,*N*′-diethyl-l,3-imidazolium-2-yl) porphyrin (AEOL 10150 and AEOL 10113) inhibited the cigarette smoke-induced inflammatory response (decreased number of neutrophils and macrophages) in rats after 2 days or 8 weeks (6 hr/day, 3 day/week) exposure (108). These compounds also mimic ECSOD and catalase scavenging both lipid peroxides and peroxynitrite, and have been shown to be effective in a number of animal models of lung disease. It has been shown that SOD mimetic M40419 blocked the development of emphysema and significantly reduced lung markers of oxidative stress in an animal model (108). Animal studies have shown that

recombinant SOD treatment can prevent the neutrophil influx to the airspaces and IL-8 release induced by cigarette smoking through a mechanism involving downregulation of NF-κB (109). This holds great promise if compounds can be developed with antioxidant enzyme properties which may be able to act as novel anti-inflammatory drugs by regulating the molecular events in lung inflammation.

C. Other Auxiliary Antioxidants

Another approach would simply be to supplement diet with small molecular weight antioxidants. This has been attempted in cigarette smokers using various antioxidants such as vitamin C and vitamin E (110). The results have been rather disappointing, although as described in Sec. IX, the antioxidant vitamin E has been shown to reduce oxidative stress in patients with COPD (111). Dietrich et al. (112) have recently shown that vitamin C or an antioxidant mixture containing vitamin C, α-lipoic acid, and vitamin E decreases plasma F_2-isoprostane levels in smokers with high body mass index suggesting that smoking-mediated oxidative stress is involved in lipid peroxidation. This suggests a variety of multiple antioxidants to protect against cigarette smoke mediated oxidative stress.

D. Thiol Compounds

1. *N*-acetyl-L-cysteine (NAC)

NAC, a cysteine-donating reducing compound, acts as a cellular precursor of GSH and becomes de-acetylated in the gut to cysteine following oral administration. NAC may also reduce cystine to cysteine, which is an important mechanism for intracellular GSH elevation in vivo in lungs. It reduces disulfide bonds (a property of a good reducing agent), but also has the potential to interact directly with oxidants. NAC is also used as a mucolytic agent (to reduce mucus viscosity and to improve mucociliary clearance).

Pharmacological approaches, particularly with thiol antioxidants, such as NAC have been used in an attempt to enhance lung GSH in patients with COPD with varying success (113,114). There have also been studies of patients with COPD where the administration of NAC has lead to a conflicting result, the number of exacerbations of COPD having been modified (114,115). This probably arose as a result of differing dosage regimens and disease severity in these studies (116). A multicenter study using NAC by metered dose inhalers in patients with chronic cough failed to show a positive effect on well being, sensation of dyspnoea, cough, or lung function (116). Van Schooten et al. (117) have reported that supplementation of oral dose of 600 mg twice daily for a period of 6 months in a randomized, double-blind, placebo-controlled, Phase II chemoprevention trial reduced various plasma and BAL fluid oxidative biomarkers in smokers.

In addition, the efficacy of oral NAC in COPD based on a quantitative systematic review and meta-analysis of published double-blind placebo-controlled clinical trials has been discussed (119,120). All of these studies have reported that treatment with NAC mucolytics when taken long term is associated with a reduction in risk of acute exacerbations, improves symptoms and days of illness in COPD. However, the results of phase III trial on the multicenter Bronchitis Randomized On NAC Cost-Utility Study (BRONCHUS) showed no effect on decline in FEV, but a reduction in over inflation in patients with severe COPD and exacerbation rate in patients who are not treated with inhaled glucocorticoids (118).

2. *N*-acystelyn (NAL)

NAL, a lysine salt of *N*-acetyl-L-cysteine, is a mucolytic and antioxidant (reducing) thiol compound. The advantage of NAL over NAC is that it has a neutral pH solution, whereas NAC is acidic. NAL can be aerosolized into the lung without causing significant side effects (121). Gillissen et al. (121) compared the effect of NAL and NAC and found that both drugs enhance intracellular glutathione in alveolar epithelial cells and inhibited hydrogen peroxide and $O_2^{\bullet-}$ released from human blood-derived PMN from smokers with COPD. NAL also inhibited ROS generation induced by serum-opsonized zymosan by human polymorphonuclear neutrophils. This inhibitory response was comparable to the effects of NAC. Recently, Antonicelli et al. (87) have shown that NAL inhibited oxidant-mediated IL-8 release in alveolar epithelial A549 cells suggesting the anti-inflammatory effect of NAL. Therefore, NAL may represent an interesting alternative approach to augment the antioxidant screen and thereby inhibiting the inflammatory responses in the lungs (Fig. 15).

3. *N*-isobutyrylcysteine (NIC)

Because NAC becomes hydrolyzed in biological systems, the measured bioavailability of the drug is low. Thus, it was speculated that a drug might be synthesized that possessed greater bioavailability than NAC, and could be used as a more effective treatment for chronic bronchitis. *N*-isobutyrylcysteine (NIC) is an NAC-Iike compound that does not undergo effective first-pass hydrolysis, and therefore has a higher oral bioavailability than NAC. This oral bioavailability can be as high as 80%, dependent on food intake. However, when evaluated as a therapy for exacerbations of chronic bronchitis, NIC performed no better than placebo drugs, and not as well as NAC. Recently a study of *N*-isobutyrylcysteine, a derivative of *N*-acetylcysteine, also failed to reduce exacerbation rates in patients with COPD (122).

4. Glutathione (GSH)

Direct increase of lung cellular levels of GSH/antioxidant screen would be a logical approach in COPD. In fact, extracellular augmentation of GSH has

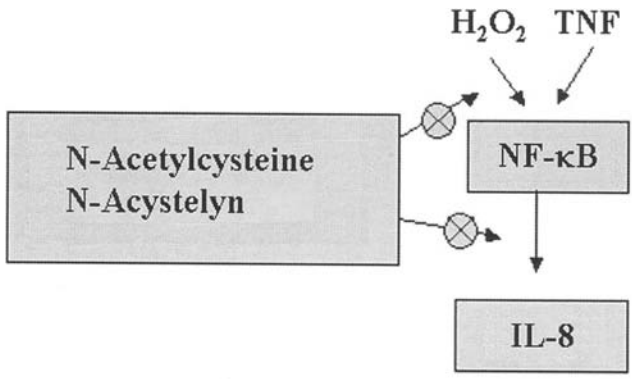

Figure 15 Schematic diagram showing oxidant- and TNF-α-mediated activation of NF-κB and IL-8 release. Both NF-κB and IL-8 release are inhibited by thiol antioxidants, such as NAC and NAL.

been tried through intravenous administration of GSH, oral ingestion of GSH, and aerosol/inhalation of nebulized GSH in an attempt to reduce inflammation in various lung diseases (2,44). However, all these lead to undesirable effects suggesting that GSH aerosol therapy may not be an appropriate way of increasing GSH levels in lung ELF and cells in COPD. In all of these studies, the question was raised about the bioavailability of GSH, pH, and osmalility at the site of microenvironment and the resultant formation of toxic products (GSSG and GSH-adducts). It seems rational to suggest that neutralizing the pH, providing GSH in salt form, liposome-entrapped GSH delivery and the maintenance of isotonicity would be useful in designing any GSH inhalation therapy in inflammatory lung diseases. Increasing the activity of γ-GCS by gene transfer techniques may increase cellular GSH levels. The induction of γ-GCS by molecular means to increase cellular GSH levels or γGCS gene therapy also holds great promise in protection against chronic inflammation and oxidant-mediated injury in COPD.

E. Glutathione Peroxidase Mimic

This is based on the approach that glutathione peroxidase be manipulated by small molecules with activity similar to this enzyme. Ebselen is seleno-organic compound, as it contains selenium, an important element in the glutathione peroxidase catalysis of the reaction between GSH and ROS. This increases the efficiency of GSH as an antioxidant, and can thus be used as a therapy against oxidative stress and inflammation. Recent studies have shown that ebselen inhibits airway inflammation (neutrophil recruitment and chemokine expression) in response to lipopolysaccharide in various

animal models (123,124). It would be interesting to see whether similar results can be obtained by ebselen in response to smoking in vivo.

F. Redox Sensor Molecules

There are other small redox molecules such as β-strand mimetic template MOL-294 and PNRI-299 which have been shown to inhibit NF-κB and AP-1-mediated transcription and blocks allergic airway inflammation in a mouse asthma model (125). The mechanism of inhibition is based on the reversible inhibition of redox sensor proteins (similar to redox effector factor-1). These redox compounds are novel and have been shown to reduce airway eosinophil infiltration, muscus hypersecretion, edema, and cytokine release in mouse asthma model. However, the use of these compounds against cigarette smoke-induced oxidative stress and the release of pro-inflammatory mediators have not been tested in vitro or in vivo.

G. Polyphenols

Curcumin (diferuloylmethane) is a naturally occurring flavonoid (polyphenol) present in the spice, turmeric, which has a long traditional use as a chemotherapeutic agent for many diseases. Curcumin is an active principle of the perennial herb *Curcuma longa* (commonly known turmeric). Turmeric has a long traditional use in the Orient for many ailments, particularly as an anti-inflammatory agent. Recent studies have reported that curcumin inhibits NF-κB expression/activation, IL-8 release cycloxygenase (COX-2), HO-1, and neutrophil recruitment in the lungs (126,127). Curcumin has multiple properties to protect against cigarette smoke-mediated oxidative stress (126). It acts as oxygen radical and hydroxyl radical scavenger which are formed by cigarette smoke, increases antioxidant glutathione levels by induction of γ-GCS and as an anti-inflammatory agent through inhibition of NF-κB and IL-8 release in lung cells. Resveratrol, a flavanoid found in red wine, is an effective inhibitor of inflammatory cytokine release from macrophages in COPD patients (128). This anti-inflammatory property of resveratrol may be due to its ability to induce sirtuins, and the HDAC activity (129). The molecular mechanisms of anti-inflammatory properties of dietary polyphenols against cigarette smoke/oxidative stress have not yet been studied.

IX. CONCLUSIONS

There is now considerable evidence for the increased generation of ROS in COPD, which may be important in the pathogenesis of this condition. ROS may be critical in amplifying the normal inflammatory response to cigarette smoke/environmental oxidants (noxious agents), through the upregulation of redox-sensitive transcription factors and hence pro-inflammatory gene expression, but are also involved in the protective mechanisms against the

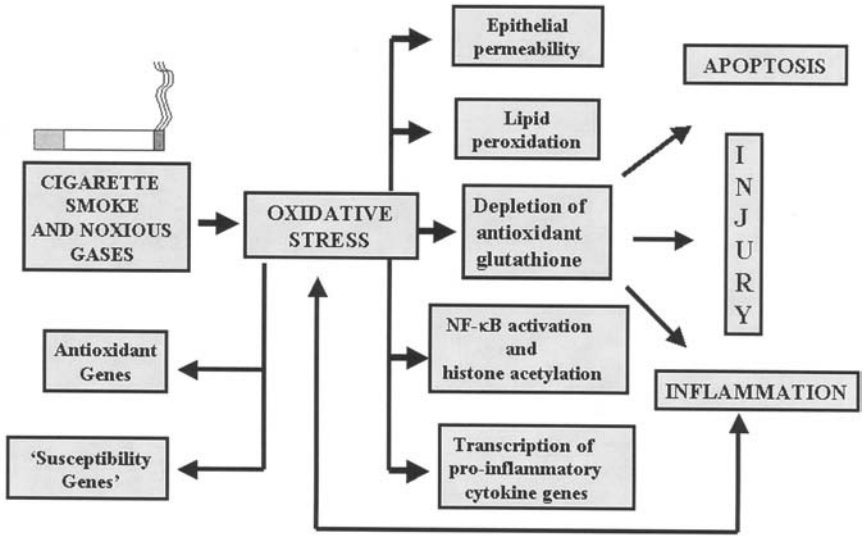

Figure 16 Summary diagram of cigarette smoke/oxidative stress-mediated lung injury and inflammation in smokers.

effects of cigarette smoke by the induction of antioxidant genes. The presence of an oxidative stress has important consequences on several events of lung physiology and for the pathogenesis of COPD (Fig. 16). Further understanding of the effects of ROS in basic cellular functions and molecular mechanisms such as amplification of pro-inflammatory and immunological responses, defective repair mechanism, signaling pathways, and apoptotic mechanisms will provide important information regarding basic pathological processes contributing to COPD. Since a variety of oxidants, free radicals and reactive aldehydes are implicated in the pathogenesis of chronic lung diseases it is likely that a combination of antioxidants may be effective of the intervention of COPD. The effective wide spectrum antioxidant therapy that has good bioavailability and potency is urgently needed to control the localized oxidative and inflammatory processes that occur in the pathogenesis of COPD. In addition, development of such novel antioxidant compounds would be therapeutically useful in monitoring the oxidative and inflammatory biomarkers in the progression/severity of COPD.

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Proteinases in COPD

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I. INTRODUCTION

The modern era of COPD pathogenesis arose in the early 1960s following two seminal observations, one experimental and the other clinical. In 1963, Laurell and Eriksson reported an association of chronic airflow obstruction and emphysema with deficiency of serum α_1-antitrypsin (α_1-AT) (1), and in 1964 Gross et al. (2) described the first reproducible model of emphysema in experimental animals by injecting the lungs with the plant cysteine protease papain. Together, these two observations formed the basis for the proteinase–antiproteinase hypothesis of emphysema that has been the prevailing concept of the pathogenesis of emphysema ever since.

The pathogenesis of garden-variety emphysema associated with cigarette smoking can be dissected into four inter-related events (Fig. 1): chronic exposure to cigarette smoke may lead to: (1) inflammatory cell recruitment within the terminal airspaces of the lung, (2) these inflammatory cells release elastolytic proteinases in excess of inhibitors in local microenvironments causing damage to the extracellular matrix (ECM) of the lung, (3) structural cells of the lung are killed either in response to lost matrix or as a primary event, and (4) ineffective repair of alveoli and elastic fibers and perhaps other extracellular matrix components result in airspace enlargement that defines pulmonary emphysema. This report will focus on the roles of proteinases in COPD.

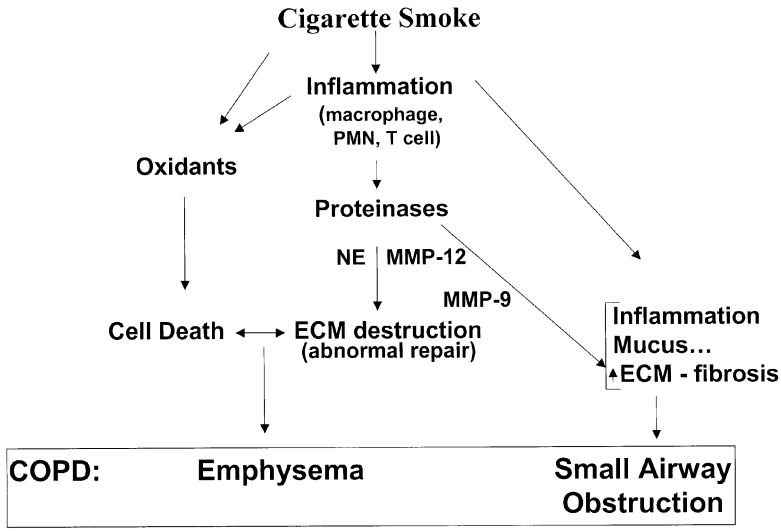

Figure 1 Pathogenesis of COPD. Chronic exposure to cigarette smoke leads to inflammation with subsequent release of proteinases including neutrophil elastase (NE) and MMPs such as macrophage elastase (MMP-12) and gelatinase B (MMP-9). Proteolytic destruction of extracellular matrix (ECM) coupled with inadequate repair results in airspace enlargement or emphysema. Loss of ECM support can result in cell death, alternatively, cigarette smoke and cellular production of oxidants also can initiate cell death. Ultimately, one must lose both ECM and alveolar cells to result in loss of alveolar units with coalescence into larger dysfunctional airspaces. In addition, cigarette smoke has a myriad of effects on the airways including inflammation, mucus production, and subepithelial fibrosis resulting in small airway obstruction. Of note, MMP-9 may activate TGF-β resulting in this fibrotic component.

II. PROTEINASE CLASSES

There are four classes of proteinases, serine, cystene, aspartic, and metal loproteinases that are distinguished by their mechanism of catalysis and endogenous inhibitors. Serine proteinases, particularly NE, have been studied most extensively in COPD. Recently, MMP activity has been appreciated as potentially important in COPD. Cysteine proteinases are largely intracellular, but they are potent and may be released from cells and thus contribute to extracellular proteolysis in COPD. Aspartic proteinases are intracellular enzymes that are generally not believed to contribute to COPD.

A. Serine Proteinases

The major serine proteinases implicated in COPD are neutrophil elastase (NE), and perhaps two other enzymes that are also found in primary or azuriphil granules of neutrophils, and to a small extent in monocyte (but not macrophage) granules. These serine proteinases are characterized by

conserved His, Asp, and Ser residues that form a charge relay system that functions by transfer of electrons from the carboxyl group of Asp to the oxygen of Ser which then becomes a powerful nucleophile able to attack the carbonyl carbon atom of the peptide bond of the substrate. The enzymes are synthesized as pre-proenzymes in the endoplasmic reticulum and processed by cleavage of the signal peptide (pre-) and removal of a dipeptide (pro-) by dipeptidylpeptidase I, and stored in granules as active packaged proteins. These serine proteinases are expressed in a lineage-restricted and developmentally specific manner. For example, neutrophil elastase, proteinase 3, and cathepsin G and their granules are formed during a very specific stage during the development of myeloid cells. Mature neutrophils contain serine proteinases, but they do not have the capacity to transcribe new proteinases in response to environmental stimuli. Upon neutrophil activation, a portion of NE and other azuriphil constituents translocate to the cell surface, but only a small fraction is released from the cell. Cell surface expression serves to focus proteolysis. Interestingly, surface-bound NE is unable to be completely inhibited by α_1AT.

1. Neutrophil Elastase

Neutrophil elastase (NE) has activity against a broad range of extracellular matrix proteins including elastin. Following the discovery of α_1-AT deficiency and the capacity of HNE to cause emphysema in experimental animals, NE has been considered to be of primary importance in the pathogenesis of pulmonary emphysema (see evidence below). The main roles of NE in COPD are thought to be to destroy lung elastin and act as a secretagogue. In addition, NE is involved in monoctye transvascular migration, and in some instances mediates neutrophil migration. However, it has been difficult to prove that NE, or any other proteinase for that matter, promotes neutrophil migration by proteolytically creating paths of degraded matrix. Despite long-term interest in NE and development of NE inhibitors, these inhibitors remain largely untested in COPD.

2. Proteinase 3

Proteinase 3 (PR3) is roughly 40% as potent as HNE against elastin. PR3 has been shown to cause emphysema in experimental animals (3). This molecule has been identified as the autoantigen target of cytoplasmic-staining anti-PMN autoantibody in Wegener's granulomatosis.

3. CathepsinG

Cathepsin G (CG) is stored in neutrophil primary granules and to a lesser degree in mast cells and a subset of peripheral blood monocytes. CG is a chymotryptic serine proteinase with capacity to degrade ECM components. CG has 20% of the elastolytic capacity of HNE.

4. Serine Protease Inhibitors

Serine proteinases are inhibited by α_1-AT as alluded to above. *Alpha-1-macroglobulin* is a large molecular weight inhibitor of multiple classes of prteinases that is restricted to the bloodstream. Two smaller molecular weight inhibitors include *secretory leukoprotease inhibitor* (SLPI), and *elafin.* SLPI is a 12-kDa protein produced by mucus-secreting and epithelial cells in the airway as well as type 2 pneumocytes. SLPI inhibits NE and CG, but not PR3. *Elafin*, also produced by airway secretory and epithelial cells, is released as a 12-kDa precursor which is processed to a 6-kDa form that specifically inhibits NE and PR3. These inhibitors are able to inhibit NE bound to substrate giving them an added dimension that API lacks.

α_1-Antitrypsin (α1-AT) is a glycoprotein of 52 kDa synthesized primarily by the liver, consisting of a single polypeptide chain of 394 amino acids (4). Fully processed α_1-AT has three carbohydrate side chains that account for 12% of the molecular mass. α_1-AT is an acute phase reactant. Plasma levels of α_1-AT rise with trauma, estrogen therapy, birth control pills, and pregnancy. Proteolytic inhibition of neutrophil elastase and other serine proteinases by α_1-AT involves cleavage of the "strained" reactive open center of α_1-AT between methionine^{358} and serine^{359}, resulting in an altered, "relaxed" α_1-AT conformation in complex with the proteinase. Formation of the complex renders the proteinase inactive and, because the complex is quite stable, inactivation is essentially permanent. α_1-AT inhibits many serine proteinases and does so on a 1:1 molar basis; however, α_1-AT associates with neutrophil elastase much faster than with trypsin or other serine proteinases suggesting that inhibition of neutrophil elastase is the primary function of α_1-AT. When α_1-AT is complexed with a proteinase the complex binds to receptors [called serpin:enzyme complex (SEC) receptors] on hepatocytes and monocytes. Cigarette smoke can oxidize a methionine residue in the reactive center of α_1-AT, inactivating its capacity as a proteinase inhibitor. The potential consequences of this reaction were demonstrated in a dog model in which animals treated with chloramine-T, an agent that profoundly depresses α_1-AT functional activity, developed pulmonary emphysema.

The nomenclature for the α1-AT polymorphism uses letters to specify the allelic variants. The original letters were chosen to reflect electrophoretic mobility: F = fast; M = medium; S = slow; and Z = ultraslow. Several α1-AT phenotypes are associated with very low serum concentrations of α1-AT. Of these, the Pi Z phenotype is by far the most common, accounting for $> 95\%$ of such individuals. The Pi Z phenotype is due to a point mutation involving a single nucleotide at codon 342 that results in coding for lysine instead of glutamic acid. This amino acid substitution alters the charge attraction and promotes dimerization of α1-AT molecules that lead to the aggregation of α1-AT in the endoplasmic reticulum that impedes secretion of the protein

from the cell and results in the low levels of α1-AT in plasma and other body fluids. Pi Z individuals have about 15% of the normal serum concentration of α1-AT. The prevalence of the Pi Z phenotype in the United States is about one in 3000 people. The Z allele is rare in orientals and African-Americans. The small number of other individuals with marked deficiency of α1-AT has Pi SZ, Pi null-null, or Pi null-Z phenotypes.

Many Pi Z individuals eventually become symptomatic with COPD, due to emphysema, but there is considerable variation and some indivi duals reach advanced age with minimal symptoms. Smoking has a marked effect on the age at which shortness of breath appears. On the average, Pi Z smokers have symptoms by age 40, about 15 years earlier than Pi Z non-smokers.

B. Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) comprise a family of 23 human matrix degrading enzymes believed to be essential for normal development and physiologic tissue remodeling and repair. Abnormal expression of MMPs has been implicated in many destructive processes, including tumor cell invasion and angiogenesis, arthritis, atherosclerosis, arterial aneurysms, and pulmonary emphy. MMPs are secreted as inactive pro-enzymes which are activated at the cell membrane surface or within the extracellular space by proteolytic cleavage of the N-terminal domain. Catalytic activity is dependent on coordination of a zinc ion at the active site and is specifically inhibited by members of another gene family, called TIMPs for tissue inhibitors of matrix metalloproteinases. Four TIMPs have been described. Optimal activity of MMPs is around pH 7.4. MMP family members share about 40–50% identity at the amino acid level, and they possess common structural domains. Domains include a pro-enyzme domain that maintains the enzyme in its latent form, an active domain that coordinates binding of the catalytic zinc molecule, and (except for matrilysins) a C-terminal domain involved in substrate, cell, and TIMP binding. The gelatinases A and B (MMP-2 and MMP-9, respectively) have an additional fibronectin-like domain that mediates their high binding affinity to gelatins and elastin. MMP-9 has one more domain with homology to type V collagen. Membrane-type MMPs (MT-MMPs 1–6 or MMP-14–19) have an additional membrane-spanning domain.

1. Tissue Inhibitors of Metalloproteinases

Tissue inhibitors of metalloproteinases (TIMPs) comprise a family of proteins, four to date, with molecular masses ranging between 21 (TIMP-2, non-glycosylated) and 27.5 (TIMP-1, glycosylated). Each TIMP inhibits MMPs via tight, non-covalent binding with 1:1 stoichiometry. TIMP-1 binds to the C-terminal domain of MMPs, but how this leads to inhibition of catalysis is unknown. Those MMPs that lack the C-terminal domain,

including MMP-7 and fully processed form of MMP-12, are still susceptible to TIMP inhibition although with a lower Ki. TIMP-2 is secreted complexed to MMP-2 in fibroblasts. A significant body of work has uncovered complex mechanisms, whereby TIMP-2, not only inhibits MMP-2, but also is involved in docking pro-MMP-2 to the cell surface where the enzyme is activated by MT1-MMP. TIMP-3 is expressed predominantly by epithelial cells and binds to extracellular matrix.

TIMPs are secreted from many cell types and are abundant in tissues. Alveolar macrophages secrete both a variety of MMPs as well as TIMP-1 and TIMP-2. Endotoxin induces synthesis of macrophage MMPs and TIMP-1, but inhibit TIMP-2 production (5). Other cytokines, such as interferon-γ, inhibit MMP-1 and MMP-3 expression in macrophages with little effect on TEVIP-1 (6). Thus, depending on the inflammatory stimulus, MMPs and TIMPs may be coordinately regulated, perhaps to limit tissue injury during normal remodeling associated with inflammation, or regulation may be discoordinate, potentially leading to tissue injury.

2. MMPs in COPD

Of the many MMPs, this discussion will focus on those that have elastolytic or collagenolytic capacity, and are expressed by inflammatory cells or other cells in the lung and hence might contribute to COPD. Discussed in the next section are specific MMPs that have been implicated in COPD directly.

Substrates: Elastin is a highly inert substance and there are few proteinases that are elastases; these include the gelatinases (MMP-2, MMP-9), matrilysin (MMP-7), and macrophage elastase (MMP-12). There are three major collagenases; interstitial collagenase (MMP-1, a human enzyme not found in rodents), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13). In addition, MMP-2 has been shown to cleave collagen as having membrane type MMP-1 (MT1-MMP). The collagenases, particularly MMP-1, have a limited substrate specificity, while MMP-7 and MMP-12, along with the stromelysins (MMP-3, MMP-10), have the capacity to catalyze the degradation of many extracellular matrix substrates excluding collagens.

The capacity of MMPs to degrade non-matrix proteins is also becoming appreciated. For example, most MMPs like the related ADAMs (a disintigrin and metalloproteinase domain) can cleave and activate latent TNF-α, thereby regulating inflammation. MMPs (7), particularly MMP-12 (8), degrade and inactivate α_1-AT, thus indirectly enhancing the activity of NE. Thus, MMPs play both direct and indirect roles in matrix-destruction associated with emphysema, and may indirectly influence cytokine release and angiogenesis that could influence the development and progression of COPD.

Cellular Expression: Macrophages and neutrophils are the major cell types associated with COPD, although many other hematopoietic and structural cells might be involved and produce MMPs.

Alveolar macrophages produce several MMPs including significant amounts of MMP-1, MMP-9, MMP-12, and smaller amounts of MMP-3 and MMP-7. MT1-MMP (MMP-14) also appears to be a macrophage product. Expression of these MMPs is highly regulated, and under quiescent conditions, such as in normal mature lung tissue, MMPs are essentially not expressed. They are induced and their production and activity are carefully controlled during normal repair and remodeling processes. With chronic inflammation, regulation of MMPs can go awry, and MMPs can be produced in excess and at inappropriate sites.

Neutrophils contain three MMPs. MMP-8 and MMP-9 are stored in specific granules, and MT6-MMP is on the cell surface. These enzymes are preformed but unlike serine proteinases in primary granules, they are readily released upon neutrophil activation. Both MMP-8 and MMP-9 also translocate to the cell surface upon activation. Similar to NE, it allows both focused proteolysis as well as resistance to inhibition, in this case by TIMPs (9).

Alveolar macrophages cultured from patients with COPD express MMP-1 and MMP-9, while macrophages from subjects without COPD did not express these enzymes (10). In this study, MMP-12 appeared to be induced in cigarette smokers with and without COPD. As discussed below, overexpression of MMP-1 in the lungs of transgenic mice led to enlarged airspaces characteristic of emphysema.

MMP expression by other lung cells. Many cells in the lung have the capacity to produce MMPs. Eosinophils produce significant amounts of the MMP-9. T-lymphocytes produce MMP-2, MMP-3, and MMP-9. Using gene-targeted mice, it was shown that contact hypersensitivity is dependent upon T cell MMP activity (11). Stromelysin-1 (MMP-3) was required for sensitization, whereas gelatinase B (MMP-9) was required for timely resolution of the reaction to antigenic challenge.

Various resident lung cells can produce MMPs, including fibroblasts, which are a potential prominent source of MMPs-1,–2, -3, and the MT-MMPs. Type II alveolar epithelial cells produce MMP-7 in addition to other MMPs. Endothelial cells also produce a variety of MMPs such as MMP-1, and -9. Considering the variety of lung cells capable of producing MMPs, it seems plausible that MMPs could participate in the lung destruction resulting in emphysema.

3. Cysteine (Thiol) Proteinases

Cysteine (thiol) proteinases (12) represent a large, diverse group of plant and animal enzymes with amino acid homology at the active site only. Human alveolar macrophages produce the lysosomal thiol proteinases, cathepsins B, H, L, and S. These enzymes share similar sizes of 24–32 kDa and high mannose side chains (typical of proteins targeted for lysosomal accumulation). *Cathepsins B and H* have little endopeptidase activity and may

function to activate other proteins similar to a distant relative, interleukin-converting enzyme. *Cathepsin C* or dipeptidyl peptidase I has limited extracellular matrix degrading activity but is required for activation of nearly all matrix degrading serine proteinase pro-enzymes to their active form. *Cathepsins L and S* have large active pockets with relatively indiscriminate substrate specificities that include elastin and other matrix components. These enzyme have an acidic pH optima but cathepsin S retains ~25% of its elastolytic capacity at neutral pH (making it approximately equal to NE). *Cathepsin K* is a potent elastase predominantly expressed in osteoclasts but also by macrophages in the vasculature and perhaps other tissues. Thus, these enzymes clearly have the capacity to cause lung destruction when targeted to the cell surface or extracellular space particularly in acidic microenvironments.

Cystatins: Cystatins represent families of cysteine proteinase inhibitors, some of which are strictly intracellular, while others, such as cystatin C, possess a signal peptide and are secreted by a variety of cells into the extracellular fluid. Cystatin C, comprised of a single non-glycosylated 120 amino acid peptide chain (13 kDa), forms reversible 1:1 complexes with enzymes in competition with substrates. Cystatin C is the most ubiquitous cystatin found in all human tissues and body fluids tested, providing general protection against tissue destruction by intracellular cathepsin enzymes leaking from dying cells. It is also a product of alveolar macrophages.

III. EVIDENCE FOR ROLE OF PROTEINASES IN EMPHYSEMA

A. Human Data

The older literature contains studies that either support, e.g. Refs. 13, 14, or refute, e.g. Ref. 15, an association of NE with COPD. Evidence supporting a role for NE include: (1) the presence of HNE and neutrophils in the lung tissue and BAL of patients with emphysema in some (but not all) studies, (2) smoking leads to an acute increase in a specific peptide released by HNE action on fibrinogen, and (3) cigarette smoke can oxidize a methionine residue in the reactive center of α_1-AT, inactivating α_1-AT, and thus altering the HNE:α_1-AT balance. Whether this inactivation occurs in vivo is uncertain. The strongest evidence for the role of NE in COPD comes from the fact that patients with α_1-AT deficiency are at increased risk of emphysema.

With respect to MMPs, correlative studies in human emphysematous lung tissue have demonstrated the presence of MMPs-1, -2, MT-1MMP (16). As mentioned, cultured macrophages found a correlation between MMP-1 and MMP-9 in smokers with emphysema as opposed to smokers without emphysema (10). This study suggests that expression of certain MMPs might predict those smokers susceptible to emphysema.

B. Animal Models

1. Elastase and Chemical-Induced Emphysema

Since Gross' initial experiments, investigators have instilled a variety of proteinases into the lungs of many small and large animals. A common feature is that administration of elastolytic enzymes including pancreatic elastase, neutrophil elastase, and proteinase 3 results in airspace enlargement (3,17–19). Pancreatic elastase produces consistent and impressive airspace enlargement. Instillation of non-elastolytic enzymes such as bacterial collagenase do not cause emphysema. Overexpression of proteinases, either by simple intratracheal instillation or more modern transgenic methods, can determine whether an enzyme has the capacity to cause emphysema (when applied to mature, fully developed lungs). However, these models cannot identify which proteinases are involved in the pathogenesis of emphysema associated with cigarette smoking, nor can they be used to decipher events upstream of proteinase release. Moreover, cigarette smoke exposure may cause a variety of other abnormalities not observed with simple overexpression of a proteinase. Nevertheless, the elastase model continues to have utility due to its relative simplicity and the fact that it allows for first order approximation for study of downstream events particularly alveolar repair. For example, elastase instillation has recently been used to demonstrate that retinoic acid has the capacity to promote alveolarization and lung repair in adult male rats (20). In addition, although instillation of elastase causes immediate airspace enlargement, there is an ensuing inflammatory response and further lung destruction from endogenous proteinases. Whether chemotactic mechanisms and participating proteinases are similar to COPD is unclear but of interest.

2. Cigarette Smoke-Induced Emphysema

A variety of chemicals and irritants have been used in experimental animals to induce inflammation and emphysema including LPS, cadmium chloride, nitrogen dioxide, inorganic dusts, and ozone. Results from these models have been reviewed elsewhere (21). These models have contributed to our knowledge of lung injury, but none have replicated exposure to cigarette smoke as a model for authentic COPD.

Several animal species have been exposed to cigarette smoke over the years including dogs, rabbits, guinea pigs, and rodents (21). Recent focus has been on the mouse since it provides unique opportunities for genetic manipulation. Other advantages of the mouse include extensive knowledge of mouse biology, abundant mouse cDNA probes and antibodies, rapid breeding, large litter sizes, small size (advantage for dosing expensive pharmaceutical agents, a disadvantage for surgical models), and relatively cheap housing. Exposure of mice to long-term cigarette smoke, using smoking

chambers similar to those described in the past for other species, results in a variety of changes in lung structure. The mouse upper airway differs from humans in that submucosal glands are sparse and restricted to the trachea. Similar to humans, mice have ciliated epithelial cells that are altered in response to cigarette smoke. Epithelial cells lose their cilia and columnar shape, and undergo metaplasia. Mice have less airway branching before terminating in acinar units. Also, unlike humans, the mouse lower airspace lacks respiratory bronchioles and the alveolar to airspace dimensions are significantly less in the mouse than observed in other species including rats. Overall, the mouse alveolar space is very similar to humans, and following exposure to cigarette smoke, inflammatory cell recruitment and airspace enlargement closely mimic the human response (22).

The main strength of mouse models is the ability to develop genetic gain of function and loss of function models. Overexpression of proteinases in transgenic mice was used by D'Armiento et al. (23) who found that a human collagenase-l (MMP-1) transgene driven by the haptoglobin reporter unexpectedly resulted in lung-specific expression in several independent founder lines. These mice developed enlarged airspaces characteristic of emphysema. This was the first demonstration that an MMP could directly cause emphysema. Also, since MMP-1 is inactive against mature elastin, this result suggested that collagen degradation was sufficient to cause emphysema. This study raises the important concept that collagen turnover is involved in emphysema. Clearly, there is clearly loss of collagen from destroyed alveoli but there is also excess collagen accumulation in the small airways (24). Thus, collagen turnover in emphysema is likely important but complicated.

As discussed above, overexpression has limited ability to decipher disease pathogenesis in that it may tell us that a protein in excess can cause a pathologic picture similar to a disease process, but does not prove that in the context of a disease, this protein is responsible. Gene targeting or targeted mutagenesis by homologous recombination in embryonic stem cells has allowed investigators to generate strains of mice that lack individual proteins, providing specific loss of function models. Combination of gene targeting with the cigarette smoke exposure model provides an opportunity to perform highly controlled experiments that differ with respect to expression of a single protein in mammals. Strains of mice deficient in individual candidate proteinases can be compared to determine their contribution to the development of emphysema in response to cigarette smoke. However, if the protein is expressed during development, then one might confuse developmental effects from acquired. In the case of airspace enlargement, this is of particular concern.

Macrophage elastase (MMP-12), nearly undetectable in normal macrophages, is expressed in human alveolar macrophages of cigarette smokers and in patients with emphysema, but not normal lung tissue. Applica-

tion of wild-type (MMP-$12^{+/+}$) mice to long-term cigarette smoke exposure led to inflammatory cell recruitment followed by alveolar space enlargement similar to the pathologic defect in humans. However, mice deficient in macrophage elastase (MMP-$12^{-/-}$) were protected from development of emphysema despite long-term smoke-exposure (22). Surprisingly, MMP-$12^{-/-}$ mice also failed to recruit monocytes into their lungs in response to cigarette smoke. Because MMP-12 and most other MMPs are only expressed upon differentiation of monocytes to macrophages, it appeared unlikely that monocytes require MMP-12 for transvascular migration.

The working hypothesis is that cigarette smoke induces constitutive macrophages, which are present in lungs of MMP-$12^{-/-}$ mice, to produce MMP-12 that in turn cleaves elastin thereby generating fragments chemotactic for monocytes (Fig. 2). This positive feedback loop perpetuates macrophage accumulation and lung destruction. The concept that proteolytically generated elastin fragments mediate monocyte chemotaxis is not original. Independent studies by Senior et al. (25,26) as well as Hunninghake et al. (27) from the early 1980s demonstrated that elastase-generated elastin fragments were chemotactic for monocytes and fibroblasts. Gene targeting is merely reinforcing this as a major in vivo mechanism of macrophage accumulation in a chronic inflammatory condition. Whether human emphysema is also dependent on this single MMP is of course uncertain. At the very least, this study demonstrates a critical role of macrophages in the development of emphysema and unmasks a proteinase-dependent mechanism of inflammatory cell recruitment that may have broader biological implications.

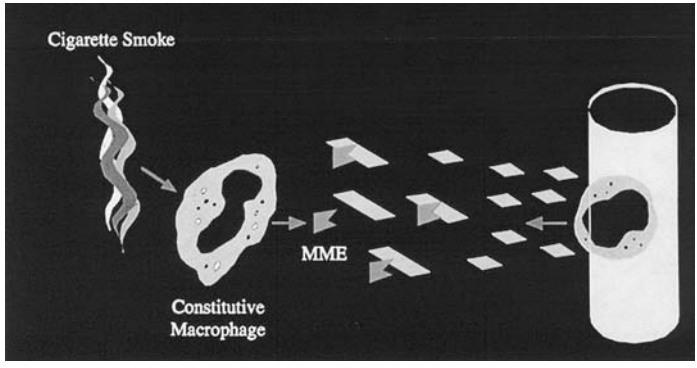

Figure 2 Potential role of elastin fragments in monocyte recruitment in COPD. Cigarette smoke induces constitutive macrophages to produce macrohage elastase (MME or MMP-12) that in turn cleaves elastin thereby generating fragments chemotactic for peripheral blood monocytes.

Subsequently, several other genetically engineered mice have confirmed the capacity of MMPs/MMP-12 to cause airspace enlargement. Inducible, lung-specific transgenic mice expressing either: (1) the Th2 cytokine IL-13 (28), or (2) the Thl cytokine IFN$_\gamma$ (see Ref. 29), induce expression of MMP-12, MMP-9, and cysteine proteinases with subsequent airspace enlargement, (3) SP-D$^{-/-}$ mice demonstrate macrophage activation MMP production and consequent emphysema (30), (4) $\alpha_v\beta6^{-/-}$ mice develop macrophage recruitment, MMP-12 activation airspace enlargement with age (31). Emphysema is abrogated upon crossing $\alpha_v\beta6^{-/-}$ with MMP-12$^{-/-}$ mice or crossing $\alpha_v\beta6^{-/-}$ with TGF-β. Since $\alpha_v\beta6$ activates TGF-β, and TGF-β is known to inhibit MMP-12, this study shows that in the absence of TGF-β, there is induction of MMP-12 and emphysema. However, too much TGF-β could cause fibrosis, hence a delicate balance of TGF-β appears to be critical (Fig. 3). In fact, in MMP-9, production in the IL-13 transgenic mice is responsible for TGF-β activation and small airway fibrosis in the face of emphysema (28) (Fig. 3).

Neutrophil elastase (NE) deficient mice have also been generated by gene targeting. These mice have demonstrated a role for NE in killing bacteria (32,33). Multiple mechanisms of NE-mediated bacterial killing have subsequently been identified including proteolytic degradation of Omp proteins on the outer wall of gram-negative bacteria (34), degradation of bacterial toxins (35), activation of cathelicidins (36). NE–/– mice have now also been applied to cigarette smoke exposure, and NE–/– smoke-exposed mice were significantly protected from the development of emphy-

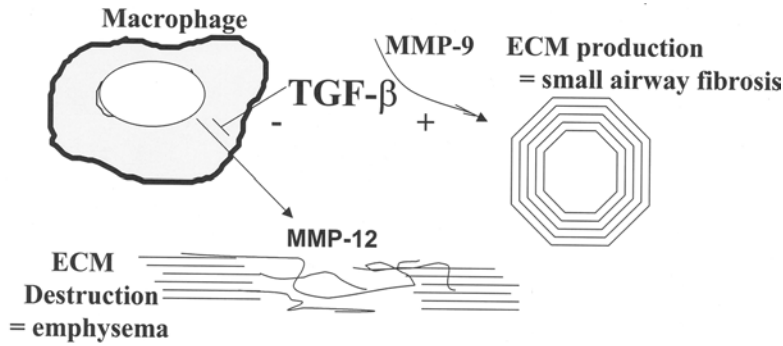

Figure 3 Potential role of TGF-β in COPD. In the absence of TGF-β activation, as is seen in β_6-deficient mice, there is loss of inhibition of MMP-12 production resulting in ECM destruction/depletion and emphysema. However, too much TGF-β results in ECM accumulation and airway fibrosis. Proteinases such as MMP-9 have the capacity to activate TGF-β. Thus, proteinases are associated with both ECM depletion in the alveolar space resulting in emphysema as well as ECM accumulation in the small airways. Both of these processes are characteristic of COPD.

sema (37). NE were protected by 2/3 from the development of emphysema in response to cigarette smoke (38). This was both a direct effect of NE as well as from its ability to inactivate tissue inhibitors of metalloproteinases (TIMPs) and mediate monocyte migration into the lung. In turn, MMP-12 degrades α1-AT, and MMP-12 may influence neutrophil recruitment via TNF-shedding (39). Thus, NE and MMP-12 interact to enhance the activity of the other in COPD.

IV. SUMMARY

In summary, proteinases have been strongly implicated in the pathogenesis of COPD for decades. Recently, we have realized that there are multiple proteinases involved including serine, cysteine, and matrix metalloproteinases. Moreover, they are expressed by a variety of inflammatory and immune cells, as well as structural cells of the lung. In addition to their matrix destructive role, we now appreciate additional functions such as their participation in inflammatory cell movement by generating and removing chemotactic gradients. Future studies should focus on the relationship between inflammation-structural cell apoptosis and the role of proteinases in these processes. Finally, we have yet to apply proteinase inhibitors long term for COPD. Determining the safety and efficacy of inhibition should be a priority.

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13

Antiproteases

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I. INTRODUCTION

The function of antiproteases is to inhibit the activity of cognate proteases thereby preventing potentially damaging degradation of host tissue. In the context of the respiratory tract, the primary antiproteases of interest are alpha-1-antitrypsin (AAT), secretory leukoprotease inhibitor (SLPI), elafin, tissue inhibitors of metalloproteases (TIMPs), and cystatins. The primary protease targets of AAT, SLPI, and elafin are the serine proteases, neutrophil elastase (NE), proteinase 3, and cathepsin G, which are released by activated neutrophils. Tissue inhibitors of metalloproteases inhibit the activity of matrix metalloproteases (MMPs) that are released from activated neutrophils and macrophages, whereas cystatins inhibit the activity of elastolytic cathepsins that are released from macrophages, fibroblasts, and epithelial cells. Despite the emphasis on protease inhibitory function, it has become apparent in recent years that antiproteases possess other biological functions including antibacterial, anti-inflammatory, and immunomodulatory properties. In the course of this chapter, we will outline the biological function of each of the antiproteases mentioned earlier and discuss how antiprotease activity may be compromised in the setting of lung disease.

II. ALPHA-1 ANTITRYPSIN

A. Structure

The alpha-antitrypsin (AAT) gene is located on chromosome 14 (q31–32.3), spanning 12.2 kb and consisting of seven exons and six introns (1). The coding exons (II–V) follow three exons (Ia, Ib, Ic), which code for the untranslated region of the AAT gene (2,3). The gene is translated into a 418 amino acid protein incorporating a 24 amino acid signal peptide. Glycosylation of AAT occurs at three ASN sites—ASN 46, 83, and 247—in the endoplasmic reticulum, and the protein is packaged in the Golgi apparatus prior to release. The final 52-kDa protein is produced primarily in hepatocytes but has also been shown to be expressed in epithelial cells, macrophages, and neutrophils (4–6). Owing to the large number of AAT mRNA transcripts present in the hepatocyte, it is believed that serum AAT is largely derived from the liver from where it diffuses throughout the body and into the lung.

B. Activity

Alpha-antitrypsin is a member of the serpin family of serine protease inhibitors (7). The tertiary structure of members of this family is similar as is the exposed reactive center loop containing the inhibitory active site sequence (8). Alpha-antitrypsin inhibits many proteases including trypsin, plasmin, thrombin, factor X, and cathepsin G (4). However, the main cognate protease of AAT is NE. Neutrophil elastase is a 29-kDa, 220 amino acid protein synthesized in the promyelocytic stage of neutrophil development (9). Neutrophil elastase is packaged into azurophilic granules from which it can be rapidly released. Studies from NE knockout (–/–) mice have revealed that the major function of NE is its microbicidal activity towards gram-negative bacteria (10). Neutrophil elastase possesses an active site containing a catalytic triad of residues—Ser173–His41–Asp88. Upon binding to a protein substrate, molecular changes occur resulting in the transfer of an electron to the Ser173 residue converting it to a reactive nucleophile that can cleave a peptide bond in the target protein (11). However, when NE binds to the active site of AAT, the Met358–Ser359 bond, cleavage of this Met–Ser bond results in the generation of a tight, noncovalent interaction between NE and AAT thereby inhibiting NE activity and release of the 36-amino acid C-terminal Ser359–Lys394 peptide (12).

The oxidation susceptibility of AAT renders it less active against NE. The association rate constant of AAT drops from 6×10^{-7} M^{-1} sec^{-1} to 3×10^{-4} M^{-1} sec^{-1}, a drop of almost 2000-fold (13). The molecular basis of this oxidation was initially shown to be oxidation of the active site Met358 residue (14). However, using recombinant-derived AAT, it has since been demonstrated that Met351, another active site residue, is also involved in the AAT–NE interaction (15). There are nine methionine residues present in the AAT sequence; however, only Met 351 and 358 are fully surface exposed

and thereby bind to NE. In the case of PPE, only Met358 appears to be important for binding to, and inhibiting, this protease (16). The differences in binding of oxidized AAT to NE and PPE can most likely be explained by the previous observation that oxidized AAT is a much less potent inhibitor of NE, although it will still bind and inhibit NE albeit at a much slower rate. However, oxidized AAT does not inhibit PPE indicating that the inhibitory mechanisms of AAT for PPE and NE are different (13,17).

c. AAT Deficiency

Alpha-antitrypsin deficiency is a lethal, hereditary disorder characterized by liver disease and early-onset emphysema. The most common phenotype associated with this condition is the Z variant of AAT (18). The Z mutation occurs as a result of a single amino acid substitution (Glu^{342}–Lys^{342}), which affects the secondary structure of AAT (2). This mutation destroys a salt bridge and affects the secondary structure of AAT, a perturbation involving a unique molecular interaction between the reactive center loop of one molecule and the A sheet of another (19). This leads to polymerization of AAT molecules resulting in accumulation of Z AAT polymers in the endoplasmic reticulum of hepatocytes. The liver disease, in AAT deficiency, is thought to result from the accumulation of Z AAT deposits in the hepatocyte (20). Such Z AAT deposits in the liver result in the disruption of cell function and integrity leading eventually to cell death (7). However, this results in decreased circulating Z AAT leading to an increased risk for the development of emphysema by the third or fourth decade of life (21). Early-onset emphysema is believed to occur as a result of decreased levels of AAT in the lung allowing greater and prolonged exposure to NE released from neutrophils. In addition, Z AAT is a less effective antiprotease than its wild-type counterpart (22). It has been demonstrated recently that a significant portion of the Z AAT present on the respiratory tract is polymerized, a state in which Z AAT is inactive as an antiprotease and may act as a neutrophil chemoattractant (23). Therefore, the fourfold problem of lower levels, decreased activity, polymerization of Z AAT, and neutrophil chemoattraction predisposes to emphysema in affected individuals. A further complication associated with early-onset emphysema in AAT deficiency is cigarette smoking. Owing to the large number of oxidants present in cigarette smoke, together with increased oxidant released from activated neutrophils, an even greater loss of Z AAT activity is thought to result from this increased oxidant burden, and individuals with AAT deficiency who smoke have demonstrably shortened life expectancy of >20 years (18).

d. Non-AAT Deficient Emphysema

The majority of patients with COPD have normal circulating concentrations, especially lung concentrations, of AAT. However, oxidation of the

AAT active site residues by oxidants in cigarette smoke may partially explain the development of protease-mediated lung damage in non-AAT deficient individuals. Other mechanisms may also be important. For example, oxidation of AAT can be reversed by the enzyme methionine sulfoxide reductase (Msr), although little is known about the activity of Msr in the lung (24). Proteolytic cleavage of AAT and the other significant antiprotease, SLPI, may also be important (25). Alterations in the levels of proteases (cathepsins, MMPs, serine proteases) or protease inhibitors (SLPI, elafin, TIMPs, cystatins) due to polymorphisms in the gene coding for these proteins may be responsible for alterations in protease:antiprotease levels in the lung which may predispose some, but not all smokers, to the development of COPD (26,27). However, research in the area of polymorphisms and lung disease is still in its infancy, and therefore, it is too early to tell whether this field will uncover new information to help explain the development of COPD in some smokers but not others.

e. Other Functions

Alpha-antitrypsin has a wider role to play in inflammation resolution than simply as an inhibitor of serine proteases (Fig. 1). In this regard, it appears that AAT may play an important part as an anti-inflammatory protein, regulating the proinflammatory effects of NE and other proteases (28). Neutrophil elastase has been shown to induce IL-8 release from epithelial cells (29). Inhibition of NE by AAT may therefore be important in preventing neutrophil recruitment and preventing an ongoing cycle of inflammation. Alpha-antitrypsin can regulate the NE cleavage of the phosphatidylserine receptor (PSR), a macrophage cell surface protein, which is crucial in clearance of apoptotic neutrophils (30). The process of apoptotic neutrophil clearance is important in the context of inflammation resolution. Alpha-antitrypsin has also been demonstrated to inhibit the cytotoxic effects of alpha-defensins by blocking defensin-induced IL-8 production by epithelial cells (31). Plasma-purified AAT can suppress *Pseudomonas* colonization in a model of chronic lung infection (32). One of the primary reasons for this may be related to improved opsonization and removal of *Pseudomonas* by phagocytosis, a process that is hindered by the action of free NE (33). Alpha-antitrypsin may also play an important role in inhibiting the proinflammatory effects of proteinase 3, which has a central role in the pathogenesis of Wegener's Granulomatosis (34). Therefore, given the multiple deleterious effects of a chronic burden of NE in the lung which includes connective tissue degradation, increased IL-8 production, decreased bacterial clearance and impaired apoptotic cell removal, the use of AAT in the treatment of chronic lung disease may be therapeutically relevant in the near future.

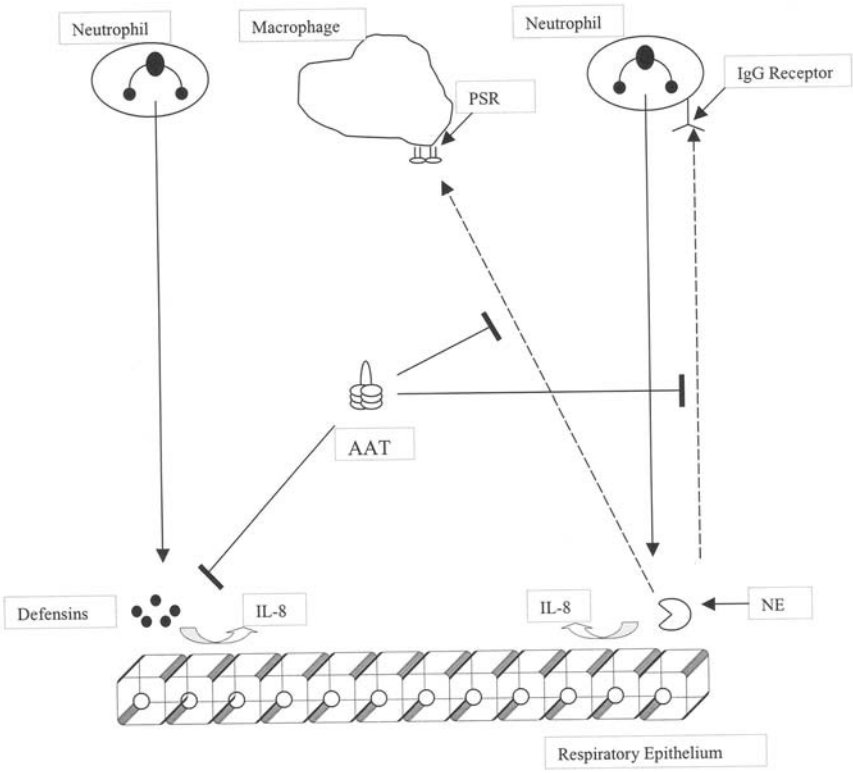

Figure 1 Inhibition of NE-mediated degradation and cleavage reactions by AAT. Neutrophil elastase is released from neutrophils and causes the activation of IL-8 by respiratory epithelium, as well as the cleavage of IgG receptor on neutrophils and the PSR on macrophages. These events are inhibited by AAT. In addition, AAT prevents defensin-induced IL-8 production by respiratory epithelium.

III. SECRETORY LEUKOPROTEASE INHIBITOR

Secretory leukoprotease inhibitor was initially identified as serine protease inhibitor present in a number of bodily secretions including nasal, bronchial, salivary, tear, and seminal secretions (35–38). The SLPI gene is 2.65 kb in length and is composed of four exons and three introns (39). The mature protein is 11.7 kDa in size, consisting of 107 amino acids and comprising two domains (40). Each domain is homologous to the whey acid protein (WAP) motif, a protein sequence composed of four disulfide bridges and ~50 amino acids in length (41). Secretory leukoprotease inhibitor and elafin (described later in this chapter) are the best

characterized of the WAP family of proteins, which currently stands at 15 members, and are situated in a tight cluster on chromosome 20ql2–13 (41). Expression sites for SLPI are found primarily in mucosal tissue, but can also be made by inflammatory cells including macrophages and neutrophils (42,43). Secretory leukoprotease inhibitor has been shown to inhibit NE, cathepsin G, chymotrypsin, and trypsin (40). Interestingly, SLPI does not inhibit the other neutrophil serine protease, proteinase 3, in contrast to AAT and elafin (44). Owing to its high association rate constant for NE, SLPI was considered to be the major inhibitor of NE in the upper respiratory tract. Recent research has shown that SLPI possesses antibacterial properties, which may be important in front-line defense of the upper airways. However, it is the work describing SLPI as an anti-inflammatory/immunomodulatory protein that has gathered most attention in the recent past (Fig. 2).

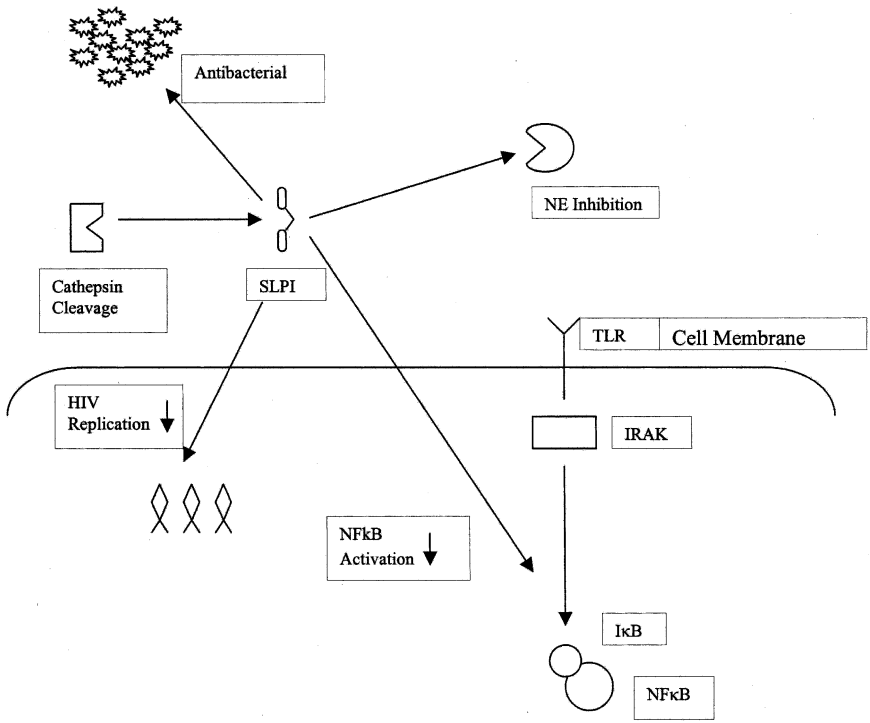

Figure 2 Schematic describing the multifunctional role of SLPI. Extracellularly, SLPI inhibits NE activity, inhibits bacterial growth, and is itself susceptible to cleavage by elastolytic cathepsins. Secretory leukoprotease inhibitor also prevents intracellular HIV replication and LPS-induced NF-κB activation in monocytes.

A. Antiprotease Activity

The crystallographic structure of SLPI reveals a boomerang-like shape with an N-terminal domain of residues 1–54 and a C-terminal domain composed of residues 55–107 (45). The antiprotease active site of SLPI has been located on a loop (residues 67–74) on the C-terminal domain containing the scissile bond Leu72–Met73. As with AAT, the association rate constant of SLPI for NE is very high (10^{-7} M^{-1} sec^{-1}) and the rate of dissociation is low and contains an active site methionine residue (Met73), which is susceptible to oxidative inactivation, rendering it a less effective antiprotease (46). It has also been shown that the Thr67–Tyr68 bond of the active site loop is susceptible to cleavage by members of the elastolytic cathepsin family, cathepsin B, L, and S (25). This is particularly relevant in emphysema where cleavage products of SLPI have been demonstrated in bronchoalveolar lavage (BAL) from these individuals. Recent studies strongly suggest that cathepsins play a putative role in the cleavage of SLPI in emphysema resulting in decreased SLPI levels and activity (25). In studies of individuals with community-acquired pneumonia, SLPI levels were found to be increased in the infected and uninvolved lobes, in comparison to control lobes. The overall anti-NE activity in infected BAL was low consistent with evidence of SLPI cleavage in infected BAL samples (47). These studies show that while SLPI levels can increase in certain disease states in response to infection, activity can be compromised by proteolytic cleavage.

B. Antibacterial Activity

Secretory leukoprotease inhibitor has been shown to have antibacterial activity towards *Escherichia coli* and *Staphylococcus aureus*. The activity appears to reside in the N-terminal domain of the protein (48). Secretory leukoprotease inhibitor is one of the most abundant antimicrobial proteins of the respiratory tract, and, together with lactoferrin and lysozyme, it has been shown to act synergistically, an effect that is lost upon increasing ionic strength (49). The SLPI production by uterine epithelial cells, from pre- and postmenopausal women, differ with greater amounts of SLPI produced by premenopausal women thus enhancing antibacterial activity (50). Some bacteria have evolved ways of inhibiting the antibacterial activity of SLPI and other respiratory tract antimicrobial proteins. Some virulent strains of *Streptococcus pyogenes* produce an extracellular protein called streptococcal inhibitor of complement (SIC) which is capable of binding to SLPI and inhibiting its antibacterial activity against an M1 strain of group A streptococci (51). Lysozyme is also inhibited by SIC but not lactoferrin. Interestingly, binding of SIC to SLPI did not affect the antiprotease activity of SLPI, suggesting that SIC may be binding to the N-terminal domain of SLPI but not the C-terminal domain containing the antiprotease active site.

C. Anti-inflammatory/Immunomodulatory Activities

Secretory leukoprotease inhibitor expression is induced in LPS-treated macrophages, but IFN-γ decreases SLPI expression (42). Secretory leukoprotease inhibitor also has the ability to inhibit LPS-induced NF-κB activity in macrophages with resulting decreases in TNF-α and NO production. In further studies, the ability of SLPI to inhibit LPS-induced NF-κB activation in U937 cells by preventing degradation of the key regulatory proteins,Interleukin-1 Receptor Associated Kinase (IRAK), IκB-α, and IκB-β, has been demonstrated. Interestingly, in the presence of SLPI, the phosphorylated version of IκB-α actually accumulates in the cell in addition to polyubiquitinated phosphorylated IκB-α. Oxidized SLPI treatment with LPS does not prevent degradation of IRAK, IκB-α, and IκB-β indicating that the LPS inhibitory effects of SLPI are dependent on its antiprotease activity (52).

In a model of acute lung injury, induced by intrapulmonary deposition of IgG immune complexes in rats, prior administration of SLPI attenuates pulmonary recruitment of neutrophils and decreases lung injury (53). Investigation of nuclear extracts from rat whole lungs revealed that deposition of IgG immune complexes resulted in a significant increase in nuclear translocation of NF-κB. However, administration of SLPI resulted in greatly reduced NF-κB activation in whole lung samples, although downregulation of NF-κB activation was not observed in alveolar macrophages (AMs) isolated by BAL from these animals. Other studies have demonstrated that SLPI increases LPS-induced expression of TGFβ and IL-10 from macrophages, as well as inducing HGF production by lung fibroblasts but not by skin fibroblasts (54,55). Two recent studies have also indicated new roles for SLPI in wound healing and apoptosis. The SLPI knockout (–/–) mice have been shown to exhibit impaired cutaneous wound healing with increased inflammation which is linked to increased elastase activity and release of active TGFβ, a major contributing factor to aberrant wound repair (56). Secretory leukoprotease inhibitor has also been shown to bind to proepithelin, an important anti-inflammatory protein, and prevent its conversion to epithelin, a proinflammatory agent, by elastase (57). Release of SLPI by macrophages during clearance of apoptotic cells also indicates a possible role in inflammation resolution (58).

Another significant biological property of SLPI is its antiviral activity. Secretory leukoprotease inhibitor has been shown to inhibit HIV infectivity of monocytes by blocking viral DNA synthesis by a mechanism that does not involve binding to HIV directly but is most likely due to interaction with the host cell (59). In this study, SLPI was shown to bind a 55-kDa protein on the surface of monocytes by which it may be mediating its antiviral properties. Subsequent investigations reveal that SLPI can bind the scramblase protein, which is involved in membrane phospholipid movement and apoptosis (60). However, whether SLPI can affect the activity of scramblase is currently unknown.

IV. ELAFIN

The mature form of elafin is a 6-kDa protein possessing a WAP domain, homologous to the WAP domains present in the SLPI protein and is found in bronchial secretions and skin (61,62). Elafin is expressed not only by many cell types present in the lung, but also by endothelium and AMs (63–65). The elafin gene is 2.3 kb in length, composed of three exons and two introns, and possesses transcription factor binding sites for AP-1 and NF-κB (65–67). Elafin is expressed as a 117-amino acid protein, which includes a 22-residue signal peptide (65). TNF-α and IL-1 have both been demonstrated to induce elafin expression in pulmonary epithelial cells, although LPS does not (67). The primary function of elafin is the inhibition of the neutrophil serine proteases NE and proteinase 3 but not cathepsin G (68,69). In contrast, SLPI inhibits cathepsin G but not proteinase 3 (44). As with SLPI, elafin is a potent inhibitor of NE with an association rate constant of $3.6 \times 10^{-6}\ M^{-1}\ sec^{-1}$. Other activities of elafin have been described. For example, overexpression of elafin improves acute lung injury induced by *Pseudomonas aeruginosa* in mice, although it is not clear if this effect is dependent on the antiprotease activity of elafin or an antibacterial/anti-inflammatory effect (70). Recently, it has been demonstrated that pre-elafin can protect against LPS-induced lung inflammation in mice by decreasing neutrophil influx into the lung, decreasing gelatinase activity, and reducing levels and expression of a number of proinflammatory mediators (71). However, the full extent of elafin's antibacterial/anti-inflammatory properties has not been fully elucidated.

V. TISSUE INHIBITORS OF METALLOPROTEASES

Four human TIMPs (TIMP-1, -2, -3, and -4) have been identified to date and their expression is regulated during development and tissue remodeling (72). Mature TIMP proteins have similar molecular weights of between 21 and 28 kDa (73). Each TIMP is composed of an N-terminal and C-terminal domain, and each domain contains three disulfide bridges that make the protein quite stable (74). Most of the biological functions of TIMPs reside within the N-terminal domain, although the C-terminal domain mediates interactions with some MMP zymogens. The primary function of TIMPs is to act as endogenous inhibitors of MMP activity and, as with other antiproteases, TIMPs play an important role in controlling extracellular protease activity in order to prevent undesired tissue damage by MMPs (Fig. 3). However, as with the other antiproteases described so far, TIMPs have a multiplicity of roles, which are not related to inhibition of MMP activity and include growth-promoting activity, apoptosis, and immunomodulation of growth factor-induced cell growth.

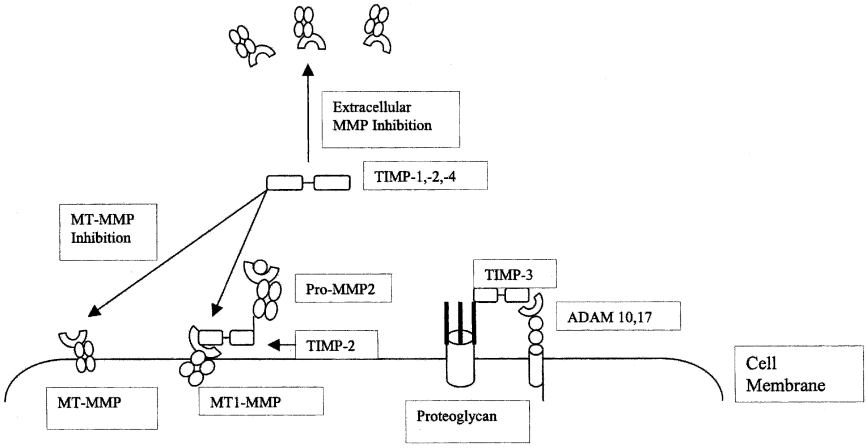

Figure 3 Inhibition of MMPs by TIMPs. The TIMP-1, -2, and -4 inhibit extracellular MMP activity, as well as MT-MMPs. The TIMP-2 acts in conjunction with MT1-MMP to act as a receptor for the proform of MMP-2. The TIMP-3 is bound to heparin sulphate proteoglycans and acts as an inhibitor of ADAMs.

A. MMP Inhibition

Tissue inhibitors of metalloproteases inhibit most MMPs examined with the exception of TIMP-1 that does not possess inhibitory activity towards membrane type (MT) 1-MMP (75). Tissue inhibitors of metalloproteases are secreted proteins, but may also be found at the cell surface in association with membrane-bound proteins (73). However, TIMP-3 is sequestered to the extracellular matrix where it binds to heparin-sulfate-containing proteoglycans (Fig. 3) (76). In addition, TIMP-3, and to a lesser extent TIMP-1, can inhibit members of the ADAMs family, e.g., TIMP-3 can inhibit ADAM-17 (TACE), whereas TIMP-1 will inhibit ADAM-10 (77,78). The first structure determinations of TIMP–MMP interactions came following X-ray crystallographic studies of the TIMP-1–MMP-3 and TIMP-2–MT1-MMP complexes (79,80). Crystallographic studies have shown that TIMP-1 is a wedge-shaped molecule with an exposed ridge structure that inserts into the catalytic site and substrate-binding groove of MMP-3. Mutagenesis studies have revealed that residues situated in and around Cysl and Cys70, which are disulfide-bonded in TIMP-1, are necessary for binding to MMPs (81). In particular, Cys1 to Val4 and Met66 to Val69 form most of the protein–protein interaction sites with MMP-3. Cys1 to Val4 bind to the active site of the MMP, and Ser68 and Val69 fit into the substrate binding sites. The α-amino and carbonyl groups of Cys1 bidentately co-ordinate the catalytic Zn^{2+} of the MMP, and the side chain of Thr2 extends into the large substrate specificity pocket of MMP-3. Binding of TIMP-1 to MMP-3

results in a conformational change, which results in the disruption of a salt bridge between Phe83 and Asp237. As a consequence of this, the N-terminal region of MMP-3 moves and an interaction is set up with Met66 of TIMP-1.

B. MMP:TIMP Ratios in Lung Disease

The balance between MMPs and TIMPs is essential for the prevention of tissue breakdown and inappropriate remodeling of tissue by excess MMP activity. Studies in mice that are null for specific MMP or TIMP genes have shed some light on changes that can occur in the lung due to alterations in MMP or TIMP levels. Mice deficient in MMP-12 (metalloelastase) do not develop emphysema following long-term exposure to cigarette smoke compared with wild-type controls (82). Likewise, TIMP-3 null mice exhibit enlarged airspaces at 2 weeks of age, increased collagen turnover, and live only half as long as wild-type mice, indicating that loss of TIMP activity disturbs the MMP/TIMP balance with resulting increased MMP activity and subsequent lung destruction (83). Increased release of MMP-9 and TIMP-1 by smokers compared with nonsmokers has been observed in AM supernatants (84). In a follow-up study, greater amounts of MMP-9 were released from the AMs from patients with COPD compared with healthy smokers and nonsmokers. In contrast, nonsmoker AMs released greater amounts of TIMP-1 compared with AMs from healthy smokers and patients with COPD (85). Type II pneumocytes exhibit increased MMP-1 expression in patients with emphysema, but is absent from the parenchyma of normal control subjects (86). Polymorphisms have been identified in the TIMP-2 gene, which occur with greater frequency in patients with COPD compared with controls, and it has been speculated that these polymorphisms may lead to decreased expression or mRNA stability of TIMP-2 and predispose to development of COPD (26). Increased MMP-9:TIMP-1 ratios have been observed in acute asthma, although TIMP-1 levels exceed MMP-9 in chronic stable asthma patients (87,88). Therefore, early membrane degeneration caused by MMP-9 in asthma may be followed at a later stage by impaired tissue repair and increased extracellular matrix deposition due to increased TIMP-1 levels.

C. Other Functions of TIMPs

Tissue inhibitors of metalloproteases possess activities that are independent of their MMP-inhibitory activities. The TIMP-1 and -2 have been found to have mitogenic effects on a number of cell lines, although overexpression of these inhibitors causes reduced tumor cell growth (89). The TIMP-1 has also been shown to increase MMP-1 production by fibroblasts (90). The TIMP-3 induces apoptosis of human colon carcinoma cells and melanoma cells possibly by stabilizing TNF-α receptors perhaps via reduced receptor shedding (91). The proapoptotic effect of TIMP-3 is in contrast to TIMP-1 and -2,

which have been shown to inhibit apoptosis of B cells (92). Therefore, similar to AAT, SLPI, and elafin, it seems clear that TIMPs possess biological properties that exceed their capacity to inhibit MMP activity.

VI. CYSTATINS

Cysteine protease inhibitors of the cystatin superfamily are present in a number of human tissues and body fluids and can be grouped into three protein families (93). Family 1 members consist of cystatins A and B and are approximately 11–12 kDa in size, do not possess disulfide bridges, and are not expressed with a signal peptide. Cystatin A is present on chromosome 3 and cystatin B is found on chromosome 21 (94,95). Family 2 members of the cystatin group are secreted proteins, are slightly bigger than Family 1 members (13–14 kDa), and possess two disulfide bridges. Members of this family include cystatin C, D, S, SN, and SA and are present on chromosome 20 (96,97). The Family 3 cystatins contain Family 2 cystatin-like domains, and members of this group are L-kininogen and H-kininogen, the latter of which is found on chromosome 3 (98). All members of this family are tight-binding enzyme inhibitors with specificity for cathepsin B, L, and S (99).

A. Cathepsin Inhibition

The crystal structure of chicken egg white cystatin reveals the protein is wedge shaped and composed of a five stranded antiparallel β-pleated sheet wrapped around a central long α-helix with a first and second β-hairpin loop forming a molecular edge (100). From this model, the mode of interaction of cystatins with their cognate enzymes was first suggested and later confirmed by the structure elucidation of the papain–stefin B (cystatin B) complex (101). Cystatin binds into the primed S1′ to S4′ subregion of papain via the β-hairpin loops and the N-terminal region interacts with the nonprimed S1 to S3 subsites in such a way as to prevent proteolytic cleavage by the active site of papain. It was also confirmed in this model that N-terminally truncated forms of the inhibitor could still bind to papain although with reduced affinity.

The interaction of cystatins with cathepsin B is somewhat different from that proposed with other cysteine proteases. Cathepsin B possesses an ''occluding loop'' comprising residues 104–126, which partially blocks the active site and impacts on the inhibitor-binding properties of the enzyme (102). Further studies have revealed that inhibition of cathepsin B by cystatin C occurs by a two-step mechanism in which an initial weak interaction is followed by a conformational change. This initial interaction most likely involves binding of the N-terminal region of the inhibitor to the S2 and S3 subsites. Evidence from these studies also suggests that conformational change that occurs is due to the inhibitor displacing the occluding loop of

cathepsin B, which normally obscures the active site. Subsequent mutagenesis experiments have revealed that mutation of His110 residue (which anchors the occluding loop of cathepsin B to the main body of the enzyme) alters the binding of cystatin C to cathepsin B from a two-step reaction to an apparent one-step reaction (103).

B. Cystatins and Disease

A large body of research has centered on the role of cathepsins and cystatins in tumor invasion, tumor growth, and angiogenesis (104,105). There is good evidence to show that increased levels of cathepsin B are secreted from tumor cells, which are not counterbalanced by increased cystatin expression thus resulting in greater degradation of the extracellular matrix (105). This degradation may also promote the formation of tumor-associated angiogenesis (106).

Cystatins are also involved in nonlung tumor disease. Bronchoalveolar lavage samples from patients with emphysema have demonstrated elevated levels of cathepsin L activity which may have implications not only for disruption of the extracellular matrix, but also degradation of soluble defense proteins of the respiratory tract including SLPI and AAT (25). Transgenic murine models, which overexpress IL-13 and IFNγ, develop emphysematous-like changes in the lungs (107,108). This overexpression of IL-13 and IFNγ is associated with increased expression of cathepsin B, L, and S. However, small synthetic cathepsin inhibitors can significantly decrease inflammation in this model, thus confirming that cathepsins do play a significant role in IL-13-induced emphysema and tissue inflammation. Elevated cathepsin activity has also recently been described in BAL samples from CF patients and this can result in degradation of β-defensins with concomitant loss of their antimicrobial activity (109). Therefore, increased cathepsin activity and overwhelming of the cystatin screen in CF and COPD may have consequences for lung tissue degradation, as well as predisposing these individuals to infection.

C. Other Functions of Cystatins

Recent literature has indicated that members of the cystatin family may play a role in modulating immune responses, particularly by macrophages. Cystatins have been demonstrated to increase nitric oxide production from IFNγ-stimulated macrophages by a process that involves upregulation of TNF-α and IL-10 production (110). Cystatins have also been shown to inhibit growth of the pathogen *P. gingivalis* by a mechanism that does not involve inhibition of *P. gingivalis* cysteine protease activity (111). In addition, peptides based on the protease-binding center of cystatin C can inhibit growth of several bacterial species including group A streptococci (112). Cystatins also exhibit antiviral properties by being able to inhibit replication

of the herpes simplex virus type 1 (113). Finally, cathepsin B has been implicated in the IL-8 mediated transactivation of the epidermal growth factor receptor, an effect that can be inhibited by small synthetic inhibitor of cathepsin B (114). Therefore, cystatins may have an additional role in inhibiting cell migration and chemotaxis in microvascular endothelial cells.

VII. THERAPEUTIC APPLICATIONS OF ANTIPROTEASES IN LUNG DISEASE

A large variety of lung diseases are characterized by the presence of multiple unopposed protease activities (serine protease, MMPs, and cathepsins). Lung tissue is susceptible to the direct effect of proteolysis in addition to its effect on other soluble host defense proteins present on the respiratory surface. As lung tissue is susceptible to the direct and indirect effects of these proteases, it would seem reasonable to hypothesize that antiprotease therapy directed against these activities would be of benefit. Small synthetic inhibitors based on the active sites of many of the naturally occurring antiproteases are currently being tested in animal models of emphysema (115–118). However, some of these inhibitors possess certain drawbacks—decreased half-life, increased toxicity, and the induction of an immune response—that may limit their use. In addition, many of these inhibitors have not yet been tested on humans. Much of the clinical work in this area has centered on the use of recombinant and plasma-purified versions of the naturally occurring antiproteases, particularly AAT and SLPI. Clinical trials have already been conducted using AAT and SLPI in CF and AAT deficiency.

Alpha-antitrypsin augmentation therapy with plasma-purified AAT has been available for the treatment of AAT deficiency since 1987. Intravenous administration of plasma-purified AAT augments serum and lung levels of AAT and increases antielastase capacity (119,120). There are new emerging data to show that long-term AAT augmentation in AAT deficient patient results in a less rapid decline in lung function, loss of lung tissue, and decreased mortality in these individuals (121,122). However, the number of individuals with AAT deficiency greatly exceeds the plasma available as a source of AAT posing a problem with the use of plasma-purified AAT (123). In addition, there are also fears concerning the use of plasma-purified product, in general, regarding the transmission of infectious agents. In this regard, recombinant AAT (rAAT) has been evaluated in augmentation trials. Initial trials showed that intravenous administration of rAAT to animals resulted in rapid renal clearance most likely due to the absence of carbohydrate side chains, which stabilize the AAT structure, obviating its use as an intravenous agent (124). As an alternative, aerosolized rAAT was evaluated in AAT deficient patients. This route of administration

resulted in increased ELF AAT levels and antielastase capacity (125). In addition, rAAT could be detected in the serum of these individuals, indicating that the lower respiratory tract epithelium is permeable to rAAT and that rAAT provides antiprotease protection for the interstitium of the lung, as well as the epithelial surface. However, the use of rAAT in augmentation trials has been limited and alternative sources of glycosylated AAT, which will be more stable than the nonglycosylated AAT, are now being produced in transgenic animals, and the results of these studies are expected soon.

Recombinant SLPI (rSLPI) has also been administered to individuals with CF resulting in decreased active NE and decreased IL-8 levels on the epithelial surface of the lung. The latter effect is partly due to the ability of SLPI (as well as AAT) to inhibit NE-induced upregulation of IL-8 by the respiratory epithelium (126–128). Pharmacokinetics of aerosolized rSLPI show that although SLPI levels and antielastase capacity are increased in the ELF of CF patients and healthy control postaerosolization, rSLPI did not accumulate on the respiratory epithelial surface (127).

Although the administration of SLPI and AAT may prove useful in neutralizing serine protease activity in neutrophil-dominated lung diseases, they may have little effect on the other nonserine proteases present on the epithelial surface. The old paradigm of emphysema, being due to unopposed activity of NE, is no longer the full explanation, and in COPD, a condition characterized by significantly increased numbers of alveolar macrophages, with resultant elevated cathepsin and MMP activity, and by a combined antiprotease therapeutic strategy using cystatins and TIMPs, as well as AAT or SLPI, may prove to be the most useful way to combat protease-mediated lung destruction.

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14

Corticosteroid Resistance in COPD

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I. INTRODUCTION

Corticosteroids are the most potent anti-inflammatory agents used to treat chronic inflammatory diseases such as asthma, rheumatoid arthritis, and inflammatory bowel disease. However, some populations of these patients are corticosteroid-insensitive (1–3), and almost of all patients with COPD show a poor response to corticosteroids (4). If the molecular mechanisms for corticosteroid insensitivity are better understood especially in COPD, this may provide insight into the mechanisms of corticosteroid action and allow a rational way to treat these patients whose disease tends to be severe. Elucidation of the cause for the relative lack of corticosteroid response in COPD may have important implications for other chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease.

II. THE MOLECULAR BASIS OF INFLAMMATION IN CHRONIC LUNG DISEASES INCLUDING COPD

Inflammation is a central feature of many chronic lung diseases, including COPD and asthma (5,6). The specific characteristics of the inflammatory response and the site of inflammation differ between these diseases, but all involve the recruitment and activation of inflammatory cells and changes in the structural cells of the lung. These diseases are characterized by an increased expression of many mediators involved in the inflammatory

cascade including cytokines, chemokines, growth factors, enzymes, receptors, and adhesion molecules (7–9). Especially, increased production of tumor necrosis factor-α (TNFα), interferon-γ (IFNγ), interleukin-8 (IL-8), macrophage inflammatory protein lα (MlP-lα), monocyte chemoattractant protein I (MCP-1), GROα, matrix metalloproteinase (MMP)-9 are found in COPD (7–9).

A. Transcription Factors

Increased inflammatory gene transcription is regulated by pro-inflammatory transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). There is evidence that these transcriptional factors are activated in COPD and also in cells exposed to oxidative stress (10–13). For example, NF-κB appears to be activated in sputum macrophages during exacerbations of COPD (11). Bronchial biopsies in smokers with normal lung function and COPD patients show increased expression of NF-κB, p65 protein, predominantly in the bronchial epithelium. Cigarette smoke and hydrogen peroxide (H_2O_2) induce marked NF-κB and AP-1 activation in epithelial cells (13–15) and these transcription factors regulate many of the inflammatory genes that are abnormally expressed in corticosteroid-resistant (CR) asthma and steroid-insensitive Crohn's disease (1,2). NF-κB is ubiquitously expressed and is able to not only control induction of inflammatory genes in its own right but also can enhance the activity of other cell and signal-specific transcription factors (16). In addition, it is a major target for corticosteroids (16). NF-κB is activated by all the stimuli relevant to chronic respiratory diseases including cytokines, such as TNFα and interleukin-lβ (IL-lβ), viruses, and oxidative stress including cigarette smoke (14,16,17). Activation of cell surface receptors leads to phosphorylation of receptor-associated kinases. These kinases in turn phosphorylate specific intracellular kinases (inhibitor of NF-κB kinase; IKK). Phosphorylation of IKKs results in phosphorylation of the NF-κB cytoplasmic inhibitor (I-κBα), which targets I-κBα for proteosomal degradation. This releases NF-κB from its inactive state enabling nuclear translocation and binding to specific DNA response elements within the regulatory regions of responsive genes (18).

AP-1 is a transcription factor complex that is formed by dimerization of members of the *Fos* (c-Fos, Fral, and Fra2) and *Jun* (c-Jun, Jun B, and Jun D) protooncogene families and is defined by binding to the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-response element (TRE) (19,20). In the resting cells, AP-1 is composed of dimers of the Jun family and has weak DNA binding and gene transactivating activities. When the cell is activated, the components of AP-1 change rapidly to Fos:-Jun heterodimers of which c-Fos:c-Jun is the most abundant and is much more active than the resting homodimer. Inducible AP-1 is formed after

activation of specific mitogen-activated protein kinases (MAPKs) of which JNK is a central component (20). Jun N-terminal kinase increases DNA binding and gene transactivating activity of AP-1 by increasing the production of c-Fos and by increasing the affinity of c-Jun for c-Fos. JNK also phosphorylates Elk-1, which enhances *c-Fos* transcription (20) by binding to the serum response element in its promoter. c-Jun transcriptional activation is mediated by a TRE that is bound by the transcriptional activator ATF-2, either as a homodimer or as heterodimer with c-Jun. In this way, c-Jun may autoregulate expression of its own gene. In addition, ATF-2 is phosphorylated by JNK (19,20) leading to an increase in c-Jun expression.

B. Chromatin Remodeling

Alterations in the structure of chromatin are critical to the regulation of gene expression (21). This chromatin structure is composed of nucleosomes, which are particles consisting of ~146 bp DNA associated with an octomer of two molecules each of core histone proteins (H2A, H2B, H3, and H4). In the resting cell, DNA is tightly compacted around these basic core histones, excluding the binding of the enzyme RNA polymerase II, which activates the formation of messenger RNA. This conformation of the chromatin structure is described as closed and is associated with suppression of gene expression. Acetylation of lysine residues on histones induces a relaxed Chromatin structure allowing gene transcription to occur. Transcriptional coactivators such as CREB binding protein (CBP) have intrinsic histone acetyltransferase (HAT) activity, which is further activated by the binding of transcription factors. Changes in the phosphorylation status of HATs also affect their activity. Increased gene transcription is therefore associated with an increase in histone acetylation, whereas hypoacetylation is correlated with reduced transcription or gene silencing, which is regulated by histone deacetylase (HDAC) (21,22).

III. HOW CORTICOSTEROIDS SWITCH OFF INFLAMMATION

We now have a much better understanding of the molecular mechanism by which corticosteroids switch off the expression of inflammatory genes in diseases such as asthma (23). Corticosteroids exert their effects by binding to a cytoplasmic glucocorticoid receptor (GR), which is a 777 amino acid receptor, a member of the nuclear hormone receptor superfamily. Glucocorticoid receptors are expressed in almost all cell types and are modular in structure (1,2). Thus, GR has several functional domains including a ligand-binding domain (LBD), a DNA-binding domain, and two domains that are involved in transactivation of genes, once binding to DNA has occurred via association with other proteins (activation function, AF-1 and AF-2). The second activation domain (AF-2) lies within the LBD. The inactive GR is bound to

a protein complex that includes two subunits of the heat shock protein hsp90, which thus act as molecular chaperones preventing the nuclear localization of unoccupied GR. As illustrated in Fig. l, once the ligand binds to GR, hsp90 dissociates allowing the nuclear localization of the activated GR–steroid complex and its binding as a dimmer to specific DNA sequences

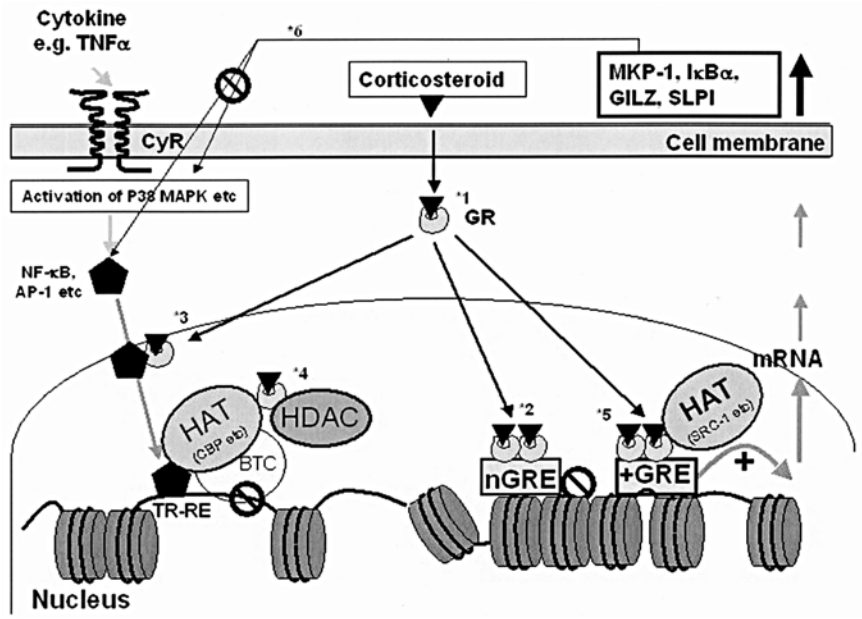

Figure 1 Molecular mechanism of steroid action. Inflammatory genes are activated by inflammatory stimuli, such as IL-lβ or TNFα, acting through cytokine receptors (CyR), resulting in activation of p38 MAPK and the transcription factors, such as NF-κB and AP-1. Upon activation, these are able to bind to specific recognition sites within the promoter regions of responsive genes (TF-RE) and HAT complex are recruited to transcription factors. Histone acetyltransferase acetylates histone and induces DNA unwinding. Transcription of inflammatory genes such as cytokines and other mediators is stimulated following recruitment of the BTC. Glucocorticoid receptors after activation by corticosteroids translocate to the nucleus (1) and bind to either an nGRE in the promoter of inflammatory genes inhibiting gene transcription (2) or, more commonly, interact with and block the ability of AP-1 and NF-κB from enhancing gene expression (3). Or, at lower doses of corticosteroids, activated GR binding to transcriptional factors–HAT complex inhibits HAT activity and recruits HDAC2 to the complex, which reverses histone acetylation and switches off the activated inflammatory genes (4). Homodimer of activated GR–HAT complex binds to +GRE binding site and induces anti-inflammatory proteins expression (5). Especially, production of MKP-1, inhibitory-κBα (IκBα) or GILZ inhibit inflammatory cytokine-induced p38 MAP kinase, NF-κB, or AP-1 activation (6) and inhibits transcription of inflammatory genes. SLPI: secretory leukoprotease inhibitor.

[GR response elements (GREs), GGTACAnnnTGTTCT] and interaction with coactivator complexes (such as steroid receptor coactivator 1 (SRC-1), p160 coactivator, and CBP via the AF-2 domain of GR (24,25). The coactivators have HAT activity and play an intriguing role in gene transcription that involves modulation of chromatin structure. Glucocorticoid receptors binding to this partial palindromic promotor sequence-GRE induce the transcriptional induction of anti-inflammatory genes such as secretary leukocyte protease inhibitor (SLPI), lipocortin, mitogen-activated protein kinase phosphatase-1 (MKP-1), IL-1 type II receptor, NF-κB inhibitor I-kBα, AP-1 inhibitor-glucocorticoid-induced leucine zipper (GILZ), and β-adrenergic receptor (2,24,26). Recent studies have demonstrated that corticosteroids may also play a role in repressing the action of MAPKs such as the extracellular regulated kinase (ERK) and JNK (27–29). Corticosteroids inhibit the phosphorylation and activation of JNK, resulting in a failure to phosphorylate c-Jun and Elk-1, reduced *c-fos* transcription and a marked reduction in AP-1 activity (28). More recently, it has been shown that dexamethasone can rapidly induce the dual specificity MAPK inhibitor MKP-1 and thereby attenuate p38 MAPK activation (30–33). Rogatsky and colleagues (34) have in turn shown reciprocal inhibition of rat GR reporter gene activity by JNKs by a direct phosphorylation of serine 246, whereas ERK can inhibit GR action by an indirect effect possibly through phosphorylation of a cofactor. This GR–GRE binding also induced tyrosine aminotransferase (TAT) and phosphoenolpyruvate carbocykinase (PEPCK), which are involved in corticosteroid-induced side effects (35,36).

As well as transactivation by GR, the homodimer of activated GR may also bind to a negative GRE (nGRE) in a manner similar to that described for positive gene regulation, and induces transrepressive effect on genes, such as IL-6 and prolactin (24).

In chronic inflammation, there is a coordinated expression of multiple inflammatory genes, including cytokines, chemokines, MMP, adhesion molecules, and inflammatory enzymes that have been activated by proinflammatory transcription factors, such as NF-κB and AP-1. This increase in gene expression is also brought about by acetylation of core histones around which DNA is wound and these transcription factors bind. Corticosteroid-bound GR directly interacts with transcriptional factors, such as NF-κB and AP-1, impairing their ability to induce gene expression by decreasing the availability of NF-κB or AP-1 to interact with DNA (25). As the dimeraization defective mutant (GRdim) is capable of inhibiting NF-κB function, monomer of activated GR can bind to transcriptional factors (37). More importantly, corticosteroids at lower dose reverse the hyperacetylation of histone at promoter of inflammatory gene by reversing histone acetylation through the recruitment of histone deacetylase-2 (HDAC2) to the activated coactivator complex (38). This then results in rewinding and compacting of DNA, the exclusion of basal transcription complex (BTC) including RNA

polymerase and the suppression of inflammatory gene transcription. This mechanism can account for the anti-inflammatory effect of corticosteroids in asthma and other inflammatory diseases. Thus, in bronchial biopsies of untreated asthmatic and COPD patients, there is an increase in HAT activity, which is reversed in patients controlled on inhaled corticosteroids (39). This new understanding of the anti-inflammatory mechanisms of corticosteroids may now help us to understand how these become impaired in COPD (Fig. 1).

IV. CORTICOSTEROID INSENSITIVITY IN COPD

Although inhaled corticosteroids (ICS) are highly effective in asthma, they provide much less clinical benefit in COPD, despite the fact that airway and lung inflammation is present (4,40). Recently, four large 3-year studies of ICS in COPD have been published (41–44). The results showed that ICS do not affect the rate of decline in FEV_1, which was the primary endpoint in all of the studies. This means that long-term treatment with high doses of ICS fails to reduce the accelerated progression of airway obstruction in COPD, which is thought to be a consequence of a chronic inflammatory process (45). Analysis of the secondary endpoints (such as reducing in symptoms and in exacerbations) in some of the studies suggests ICS at high dose may benefit for some patients with COPD. Thus, in airway inflammation in COPD, high doses of inhaled or even oral corticosteroids fail to reduce inflammatory cells, cytokines, or proteases (46–48) though in asthma these are suppressed by low doses of inhaled corticosteroids in most patients. Alveolar macrophages play a critical role in orchestrating the chronic inflammation in COPD through the release of proteases such as MMP-9, inflammatory cytokines such as TNFα, and chemokines such as IL-8 that attract neutrophils into the airways. Corticosteroids effectively suppress the release of these inflammatory mediators in alveolar macrophages from normal nonsmokers, but are less effective in cells from smokers with normal lung function and virtually ineffective in macrophages from patients with COPD (Fig. 2) (49,50). We also found that H_2O_2, an oxidative stress, mimicked this in U937 monocytic cell line, and enhanced lipopolysaccharide (LPS)-induced IL-8 production and decreased the inhibitory effect of steroids (Fig. 2). This suggests that alveolar macrophages have developed resistance to corticosteroids in patients with COPD.

V. MOLECULAR MECHANISMS UNDERLYING CORTICOSTEROID INSENSITIVITY

At a molecular level, resistance to the anti-inflammatory effects of corticosteroids is reported to be induced by several mechanisms in asthma and other diseases. The reduction in corticosteroid responsiveness observed in cells

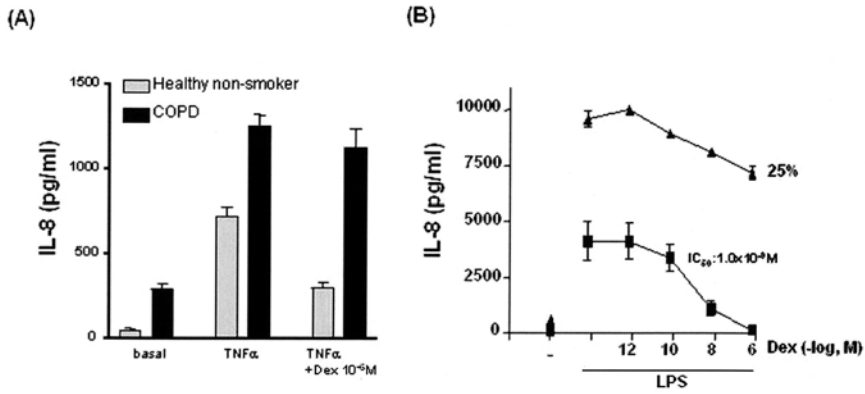

Figure 2 Steroid insensitivity in COPD alveolar macrophages and H_2O_2-treated U937 cells. (A) Interleukin-8 production in alveolar macrophages from healthy non-smokers and COPD patients. Alveolar macrophages were stimulated with TNFα (10 ng/mL) with or without 30-min pretreatment of dexamethasone (Dex: $10^{-}6$ M). In alveolar macrophages from COPD, dexamethasone was not effective on TNFα-induced IL-8 production. (B) Dose-dependent inhibition of LPS-induced IL-8 production by Dex in U937 cells. The cells were stimulated with LPS (500 ng/mL) with (triangle) or without (square) 4 hr pretreatment of H_2O_2 (l00 μM). Various concentrations of Dex were pretreated for 30 min before LPS stimulation. H_2O_2 treatment enhanced IL-8 production and decreased corticosteroid action. (From Ref. 94.)

from these subjects has been ascribed to a reduced number of GR, altered affinity of the ligand for GR, reduced ability of the GR to bind to DNA, or increased expression of inflammatory transcription factors, such as AP-1, that compete for DNA binding or reduction of HDAC (2,51).

A. Defects in GR Sequence and Pharmacokinetics

Unlike familial corticosteroid resistance, where there is a mutation in the LBD of GR and a subsequent resetting of the basal cortisol level, CR asthma patients have normal cortisol levels and are not Addisonian (52). Plasma morning serum cortisol or 24 hr urinary cortisol excretions reported to be normal or increased in COPD (53,54). Using standard steroid suppression tests, CR asthmatics and COPD patients do not have an altered secretory rate of endogenous cortisol or an altered sensitivity of the HPA axis (55,56). By chemical mutational analysis, no mutations in the GR of CR patients were observed in CR asthmatics (57). This was confirmed in a later study, which used RT-PCR and direct sequencing of the GR (58). It is therefore unlikely that the defect in CR asthmatics and COPD lies in the structure of the GR.

B. Defects in Ligand Binding

Using whole cell binding assays no significant changes in the dissociation constant (K_d) of monocyte and T-cell and receptor density (Bmax) of GR in patients with CR asthma (59). GRα mRNA levels are reported to be slightly lower in patients with COPD than normal (60), although this has not been confirmed in other epithelial cells from smokers (healthy smokers and COPD) (61). However, a statistically significant difference was found between K_d values in human bronchial epithelial cells from smokers (healthy smokers and COPD) compared to nonsmokers. The reduction of GR expression and altered affinity of GR may reflect either an intrinsic defect in the GR in these patients or may relate to changes in the receptor induced by inflammation or oxidative stress in more severe COPD or asthma. Pretreatment with TNFα, which is a dominant proinflammatory cytokine in COPD, diminishes the ability of dexamethasone to suppress IL-6 secretion in whole-blood cell cultures and to enhance IL-1 receptor antagonist secretion by U937 cells. Tumor necrosis factor-α resulted in a 60% decrease of the GR number without any change in the binding affinity after 48 hr (62). Nitrosylation of GR by oxidative stress is reported to decrease its affinity for corticosteroids (63). The reversal of the reduced binding affinity is mimicked by incubation of cells with high concentrations of IL-2/IL-4 or IL-13 treatment in peripheral blood mononuclear cells (PBMCs) (2,64). Two explanations for the effect of IL-2/IL-4 or TNFα on ligand binding characteristics have been proposed. One theory is that steroid resistance is due to increased expression of the dominant negative isoform of GR, GRβ (2). As the result of alternative splicing of the GR pre-mRNA, there are two homologous mRNAs and protein isoforms, termed GRα and GRβ. Both mRNAs contain the same first eight exons of the GR gene. The remainder is derived by alternative splicing of the last exon of the GR gene, resulting in either inclusion or exclusion of exon 9α. The two protein isoforms have the same first 727 NH_2-terminal amino acids. GRβ differs from GRα only in its COOH terminus with replacement of the last 50 amino acids of GRα with a unique 15 amino acids sequence lacking a steroid-binding domain. These differences render GRβ unable to bind glucocorticoids, reduce its binding affinity for DNA recognition sites, abolish its ability to transactivate glucocorticoid-sensitive genes, and make it function as a dominant inhibitor of GRα, possibly through formation of antagonistic GRα/GRβ heterodimers. In COPD, GRβ mRNA levels are reported to be increased in patients with COPD compared with normal subjects (60), and also increased numbers of cells expressing GRβ have been reported in skin biopsies from CR patients (65). In contrast, others have been unable to detect enhanced GRβ expression in PBMCs from CR asthmatics patients (64,66).

The other theory relates to phosphorylation of GR. The effects of IL-2/IL-4 on GR ligand binding and dexamethasone regulation of IL-10 release are reversed by the p38 MAPK inhibitor SB203580 (64). Tumor necrosis factor-α as well as IL-2/IL-4 activates p38 MAPK (67) and this results in serine phosphorylation of GR and reduced dexamethasone repression of LPS-stimulated granulocyte-macrophage colony-stimulating factor (GM-CSF) release (64). These data show that p38 MAPK inhibitors may have the potential to reverse glucocorticoid insensitivity and reestablish the beneficial effects of glucocorticoids in patients with severe asthma and COPD. It is unclear whether this is a direct or indirect effect of p38 MAPK or whether GR phosphorylation alters ligand-binding affinity directly. This may result from either a change in GR conformation due to association of distinct cofactors, or partial blocking of the ligand-binding domain, due to association of GR with nuclear transcriptional modulating proteins. Similar results have been seen following NO treatment of GR, whereby nitrosylation of GR at an hsp90 interaction site modifies ligand binding (63). Serine 226 and the sequences immediately surrounding it are highly conserved suggesting that its phosphorylation may alter or disrupt the protein–protein interactions regulating GR action.

C. Cross-Talk with Transcription Factors

The major anti-inflammatory effects of corticosteroids are thought to be due to repression of inflammatory and immune genes. The inhibitory effect of corticosteroids is due largely to protein–protein-complex interactions between activated GR and transcription factors, such as NF-κB and AP-1, which mediate the expression of these inflammatory genes (Fig. l). The interplay between proinflammatory transcription factors and GR may reflect differing effects on histone acetylation/deacetylation (38).

There is an increase in the basal levels of AP-1 DNA binding in the nuclei from CR patients, although no differences in the sequences of *c-fos* and *c-jun* mRNA are detectable (58). These results suggested that AP-1 was altered in CR patients and that increased levels of AP-1 might inhibit GR function. In a subsequent study using nuclear run-on, RT-PCR and Western blotting, a 2–4-fold greater increase in the *c-fos* transcription and mRNA and protein expression in PBMCs isolated from CR compared with corticosteroid-sensitive (CS) asthmatics and normal subjects were demonstrated (68). When cells were stimulated with PMA, the time- and concentration-dependent induction of c-Fos was greater in the CR group. Overexpression of c-Fos induced by stimulation of PBMCs derived from CS subjects PMA for 6 hr, attenuated the ability of these cells to induce GR–GRE binding after l hr dexamethasone treatment. In these experiments, GR–GRE binding was reduced to levels similar to that seen in CR subjects. Incubation of PBMCs derived from CR subjects with

dexamethasone and with antisense oligonucleotides directed against *c-fos* increased GR–GRE binding to similar levels seen in CS individuals. These findings suggested that increased c-Fos under basal conditions is the predominant inhibitory activity on GR–DNA binding in CR asthma. In addition, excessive and constitutive epithelial activation of NF-κB and AP-1 in steroid-resistant Crohn's disease has also been demonstrated (69). Oxidative stress, such as cigarette smoke and H_2O_2, enhances AP-1 and NF-κB activation (70–75). In COPD patients or smokers, NF-κB and AP-1 are activated excessively and constitutively, probably by TNFα and other proinflammatory cytokines (11,12).

A marked increase in the expression of activated phosphorylated c-Jun, enhanced expression of JNK, and greater upregulation of c-Fos expression is found in the CR compared with the CS asthmatics (76). Corticosteroid resistance is strongly associated with perinuclear expression of active JNK and p38 activation in nucleus of epithelial cells in CR Crohn's disease (69).

The data to date suggest that increased levels of c-Fos and increased activation of c-Jun in patients with CR asthma accounts for the increased AP-1 activity seen in vitro and probably relates to increased activation of JNK in these subjects. Jun N-terminal kinase regulates the expression and activation of both major components of AP-1. Elevated JNK activity could be critical to the mechanisms of steroid-resistant patients and failure to inhibit JNK phosphorylation by glucocorticoids may be a major cause for the lack of response to glucocorticoids in CR asthma. In addition, excessive and constitutive epithelial activation of JNK and p38 may in turn suppress GR function resulting in a feed-forward loop of increasing inflammation and reduced corticosteroid responsiveness in these patients (69). As oxidative stresses, such as hydrogen peroxide, induce JNK activation and phosphorylation, elevated JNK activity may be involved in the mechanism of steroid insensitivity in COPD (77,78).

Immunoprecipitation experiments have shown that phosphorylated STAT5 and the GR form immune complexes. This association might lead to retardation of GR nuclear translocation because IL-2 is not able to induce steroid insensitivity in splenocytes from STATS knockout mice. This study demonstrates a novel role for STATS in IL-2-induced steroid sensitivity (2). Oxidative stress (H_2O_2) also stimulates STAT5 activation and constitutive and excessive expression of STATS by oxidative stress (79) might also be involved in steroid resistance in COPD.

D. Neutrophilic Inflammation

Neutrophils have been implicated in the pathogenesis of many diseases, including COPD, severe asthma, psoriasis, and a variety of collagen-vascular diseases (6,80,81). These cells are less sensitive to glucocorticoids

than other white blood cell types (82). The corticosteroids beclomethasone, budesonide, dexamethasone, fluticasone propionate, hydrocortisone, and prednisolone inhibit apoptosis in a concentration-dependent manner as assessed by flow cytometric analysis, annexin-V binding, and morphological analysis (83). The maximal inhibition of apoptosis was 50–60%. This is in contrast to T cells and eosinophils, where steroids induce apoptosis (84,85). Moreover, glucocorticoids slightly enhance the inhibitory effect of GM-CSF on neutrophil apoptosis. This indicates that corticosteroids prolong human neutrophil survival by inhibiting apoptosis at clinically relevant drug concentrations via an effect on GR.

The mechanisms by which human T cell, eosinophil, and neutrophil apoptotic responses to glucocorticoids differ are unknown. The ratio of GRβ to GRα has been reported to be 73-fold greater in neutrophils than in PBMCs (86). This reflects differences observed in relative abundance of the mRNAs. GRα/GRβ heterodimers have only 15–20% of the transactivating activity of GRα homodimers. In these studies, the prosurvival activity of dexamethasone is associated with elevated GRβ in freshly isolated neutrophils, perhaps through formation of GRα/GRβ heterodimers.

Formation of 52 and 30 kD GR fragments due to proteolysis by neutrophil elastase (a 28 kD serine protease) is found in cytosol of leukemia cells (87). Receptor fragmentation in the cytosol is inhibited by methoxysuccinyl-alanyl-alanyl-prolyl-valyl-chloromethylketone, a highly specific inhibitor of neutrophil elastase. The addition of as few as 5% neutrophils to a lymphoid cell suspension provides sufficient elastase to produce receptor fragmentation.

E. Latent Viral Infection

Epidemiologic studies have implicated childhood respiratory infection as an independent risk factor for the subsequent development of persistent asthma and COPD (88). Excess amounts of adenoviral E1A DNA have been described in patients with COPD (89) with the ElA protein expressed in the epithelial cells lining the bronchi, the bronchial glands, gland ducts, the cells lining the bronchioles, and the type 2 cells lining the alveolar surface (90). ElA-transfected airway epithelial cells after LPS exposure produce an excess amount of IL-8 and intercellular adhesion molecule (ICAM)-l. Latent adenovirus infection reduces the inhibitory effects of glucocorticoids on airway inflammation in ovalbumin-sensitized guinea pigs (91). The mechanism is not yet fully understood though excessive AP-1 induced by adenovirus infection may be involved in steroid resistance (92). Thus, adenoviruses appear to persist as latent infection in the airways of patients with COPD and adenoviral E1A proteins capable of activating host transcription factors and amplifying host gene expression, including those involved in cigarette smoke-induced lung inflammation and COPD may contribute to steroid insensitivity in COPD.

More recently, it has been proposed that rhinoviral infection can prevent GR nuclear translocation via an NF-κB-mediated process (93).

F. Reduced Histone Deacetylase Activity in COPD

We have demonstrated that alveolar macrophages of cigarette smokers have reduced HDAC activity and reduced expression of HDAC2 and its activity compared to cells from normal subjects whereas there is no reduction of HDAC 1 expression (Fig. 3). This is correlated with increased in basal and IL-1β-stimulated release of TNFα and IL-8 in alveolar macrophages (94). The level of HDAC activity is inversely correlated with maximal TNFα and IL-8 production in these cells. Repression of HDAC activity can lead to enhanced histone acetylation and increased gene expression. We have

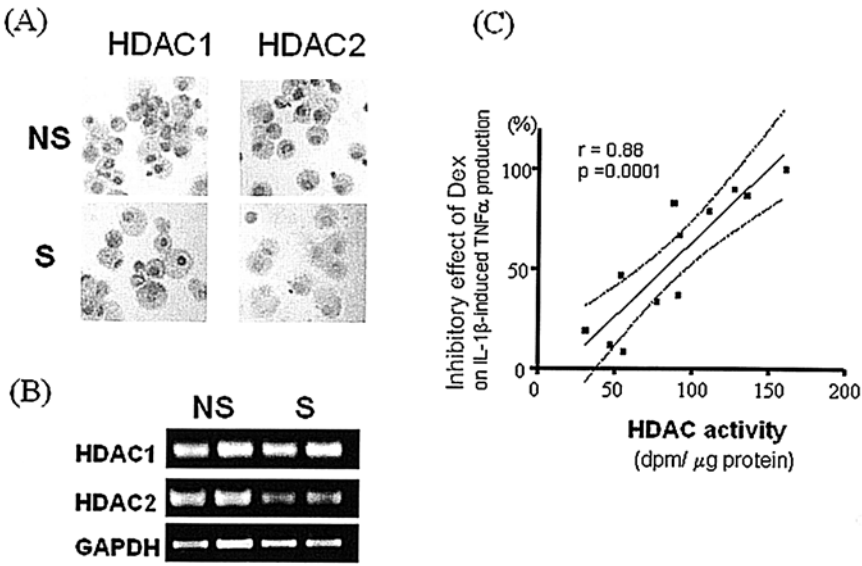

Figure 3 Histone deacetylase expression and correlation between HDAC activity and corticosteroid inhibitiory action in IL-1β-induced GM-CSF production. (A) The localization of the expression of the HDAC1 and HDAC2 within BAL macrophages of nonsmokers (NS) and smokers (S) was detected by immunocytochemistry. Histone deacetylase-2 expression selectively reduced in smokers. (B) The relative expression of HDAC1 and HDAC2 mRNA in BAL macrophages isolated from S and NS. GAPDH mRNA expression is used as a control for mRNA. (C) Correlation between HDAC activity and steroid inhibitory action in IL-1β-induced TNFα production in BAL macrophages obtained from NS and S groups. Total HDAC activity was measured by ^{3}H-acetic acid release from labelled histones. Inhibitory effect of dexamethasone (Dex) in IL-1β-induced TNFα production was measured with ELISA. (From Ref. 94.)

previously shown that glucocorticoid suppression of inflammatory genes requires recruitment of HDAC2 to the activation complex by the GR (38). This implies that reduced HDAC2 activity will not only increase inflammatory gene expression but will also cause a reduction in the inhibitory effect of a corticosteroid, dexamethasone, on the expression of these cytokines. Indeed, we have shown that this is the case in bronchoalveolar lavage (BAL) macrophages from smokers and in A549 and U937 cells following oxidative stress (H_2O_2)(Fig. 3) (94). Furthermore, in peripheral lung tissue and alveolar macrophage, there is a reduction in HDAC activity and the expression of HDAC2 in normal smokers and a striking reduction in patients with COPD (95). We propose that this reduction in HDAC2 prevents corticosteroids from exerting their anti-inflammatory effects in COPD (Fig. 4) (96). Although the mechanism of HDAC reduction is not yet

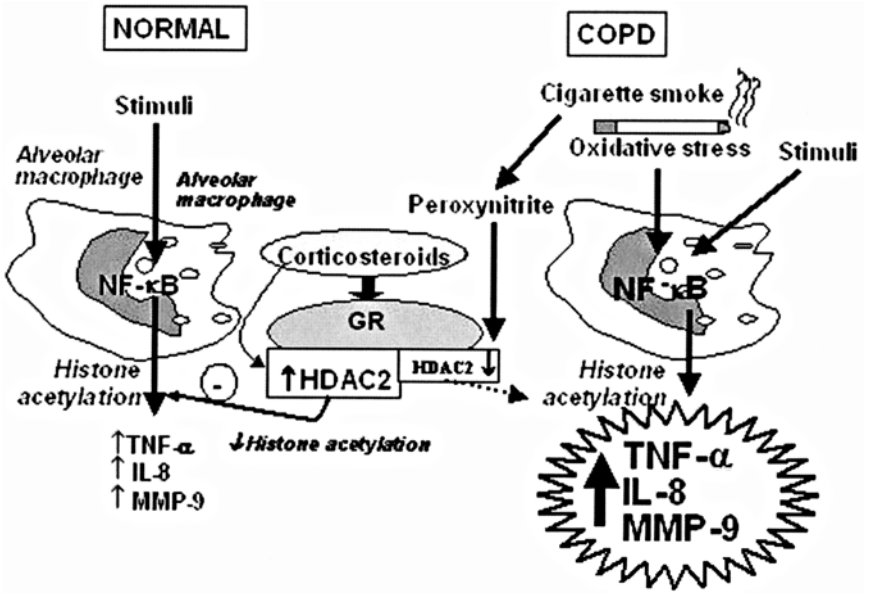

Figure 4 Reduction of HDAC2 and corticosteroid resistance in COPD. Stimulation of normal alveolar macrophages activates NF-κB and other transcription factors to switch activate histone acetyltransferase (HAT), histone acetylation and thus to genes encoding inflammatory proteins, such as TNFα, IL-8, and MMP-9. Corticosteroids reverse this by binding to glucocorticoid receptors and recruiting HDAC2, which reverses histone acetylation and switches off the activated inflammatory genes. In COPD patients, cigarette smoke activates macrophages, as in normal subjects, but oxidative stress perhaps acting through the formation of peroxynitrite, impairs the activity of HDAC2. This amplifies the inflammatory response to NF-κB activation, but also reduces the anti-inflammatory effect of corticosteroids as HDAC2 is now unable to reverse histone acetylation. (From Ref. 96.)

certain, we propose that it may be mediated via oxidative stress. Exhaled markers of oxidative stress, such as 8-isoprostane and ethane, are increased in normal smokers with a much greater increase in patients with COPD, even when they have stopped smoking (97,98). Oxidative stress and cigarette smoking increase histone acetylation and switch on inflammatory gene transcription (15,99). We propose that oxidative stress impairs the function of HDAC2, resulting in resistance to corticosteroids and this has been demonstrated in an epithelial cell line, monocytic cell line, and primary epithelial cells from healthy volunteer in vitro (94). Furthermore, oxidative stress also reduces the inhibitory effect of corticosteroids on cytokine release in vitro, an effect that is mimicked by a nonspecific HDAC inhibitor trichostatin A (94). Thus, by impairing the function of HDAC2, oxidative stress may both amplify inflammation and impair the anti-inflammatory action of corticosteroids (Figs. 3 and 4) (96).

However, the mechanisms whereby oxidative stress leads to this impairment in activity of HDAC remain to be determined by future studies. One possibility is that oxidative stress in the presence of increased nitric oxide (NO) formation may generate peroxynitrite, which then nitrates tyrosine residues on HDAC or associated proteins. In COPD, there is certainly evidence for nitrotyrosine formation in alveolar macrophages (100). This could be directly investigated by measuring nitration of tyrosine residues on HDAC in vitro and in COPD cells and tissues. Another possibility is phosphorylation of specific HDAC, thus changing their function by some kinases, which are activated by oxidative stress, such as Akt/PKB, phosphoinositol 3 kinase, and p38 MAPK (101–103).

VI. THERAPEUTIC IMPLICATIONS

The proposed mechanism of steroid resistance in COPD involving a reduction in HDAC2 activity and expression as a consequence of oxidative stress has important therapeutic implications. We predict that there are several strategies that might overcome steroid resistance in COPD so that corticosteroids become able to switch off the multiple inflammatory cytokines, chemokines, and proteases that mediate the disease. We predict that effective antioxidants that are able to neutralize oxidative stress would increase the response to corticosteroids. However, currently available antioxidants are unlikely to reduce the high level of oxidative stress in severe COPD and more potent drugs, or perhaps an inhaled delivery, are needed in the future. If peroxynitrite formation mediates the effects of oxidative stress, then inhibiting inducible NO synthase, which is upregulated in COPD macrophages (100) or peroxynitrite scavenger (104,105) might be a more efficient approach. Potent, selective, and long-lasting inducible NO synthase inhibitors are now in clinical development (106).

Another approach would be administration of low doses of theophylline, which have recently been shown to increase the activity of HDAC through a novel mechanism independent of phosphodiesterase inhibition and adenosine antagonism (107) (Fig. 5). In vitro theophylline at lower than therapeutic concentrations is able to restore the reduced HDAC activity induced by oxidative stress and restore steroid responsiveness. Alternatively, it may be possible to use MAP kinase inhibitors as steroid-sparing agents reducing the dose of corticosteroid needed to obtain effective therapy. Recent preliminary data suggest that the combination of long-acting β adrenergic receptor agonist (LABA) and inhaled steroid may be more effective than monotherapy of inhaled steroid in COPD (40), although the magnitude of the improvement in lung function are smaller than those reported in the earlier asthma studies. Ligand-independent activation of GR by LABAs has been demonstrated in a human cell line in vitro (108). Long-acting β adrenergic receptor agonist also may affect some kinases involved in the regulation of GR function (109,110), or even indirectly modify HAT/HDAC activity.

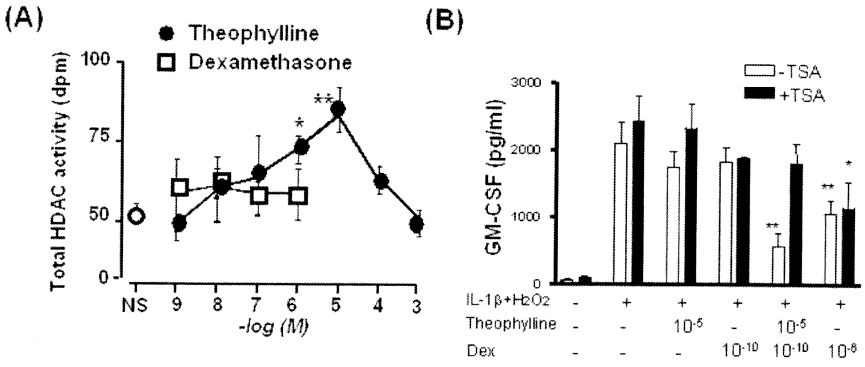

Figure 5 Effect of theophylline on HDAC activity and H_2O_2-induced steroid insensitivity in A549 cells. (A) Theophylline and dexamethasone were added to nuclear extracts containing HDAC proteins from A549 cells. Total HDAC activity was measured by ^{3}H-acetic acid release from labelled histones. Theophylline dose-dependently increased HDAC activity up to 10^{-5} M, whereas dexamethasone showed no effect. **:$p < 0.01$, *:$p < 0.05$. (B) A549 cells were stimulated with IL-1β (1 ng/mL) after 4-hr pretreatment of H_2O_2 (100 μM). Theophylline was treated 20 min before and dexamethasone was treated 30 min before IL-1β stimulation. Granulocyte-macrophage colony-stimulating factor production was measured with ELISA. Dexamethasone (10^{-10} M) or theophylline (10^{-5} M) alone was less effective in IL-1β stimulation under H_2O_2 treatment, but low dose theophylline enhanced the suppressive effect of dexamethasone. This effect was attenuated by trichostatin A (TSA), an HDAC inhibitor, indicating that theophylline can attenuate oxidative stress-induced steroid resistance via an increase in HDAC activity. (From Ref. 107.)

In the future, it might also be possible to identify new classes of drug that may activate HDACs.

VII. CONCLUSIONS

Several mechanisms have been proposed to account for a failure to respond to corticosteroids including a reduced number of GR, altered affinity of the ligand for GR, reduced ability of the GR to bind to DNA, reduction of HDAC or increased activation of transcription factors, such as AP-1, which compete for DNA binding. However, information about the molecular mechanism of steroid resistance in COPD is still limited. One potential reason for the failure of corticosteroids to function effectively in reducing inflammation in COPD is that oxidative stress may reduce HDAC activity and expression (Fig. 4). Patients with severe asthma also have increased oxidative stress and this could account for the need for high doses of inhaled or oral corticosteroids in these patients. Asthmatic patients who smoke also have a marked reduction in the anti-inflammatory response to corticosteroids (111,112), and this may be explained by the effect of cigarette smoking on HDAC activity in asthmatic airways as well as COPD. Patients with other severe inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel diseases, are also likely to have local oxidative stress, which may reduce their responsiveness to corticosteroids. ''Resensitization'' of steroid responsiveness using the therapeutic strategies described above may considerably reduce the requirement for corticosteroids and therefore the risk of systemic side effects that currently limit the use of oral corticosteroids in these common diseases. Thus, add-on treatment of the compounds enhancing HDAC2 activity specifically may give some resolutions of corticosteroid resistance.

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Molecular Genetics

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I. INTRODUCTION

Cigarette smoking is clearly the major environmental determinant of COPD. However, interestingly, only a minority of chronic cigarette smokers develop COPD. This observation demonstrates that other factors are involved and there are epidemiological data to suggest that some of those factors are genetic. In this chapter, we will summarize the family and twin studies that indicate that there is a genetic basis to the development of airflow limitation. We then review the genes that have been investigated as potential susceptibility factors for COPD. In common with other complex genetic diseases, it is likely that several genes will be implicated in the pathogenesis of this COPD and that there will be an interaction with environmental factors such as cigarette smoke and air pollution.

II. GENETIC EPIDEMIOLOGY

A. Family Studies

A condition that is least partially genetically determined would be expected to cluster in families and this has been shown for COPD (1–3). However, familial clustering may occur due to common environment conditions rather than a common genetic susceptibility. Evidence to indicate that genetic factors are responsible for familial clustering includes the results of studies where it was shown that the relative risk for COPD was increased in the

relatives of cases compared with the relatives of controls (i.e., individuals without respiratory disease) (4–8). This increased prevalence could not be explained by factors such as age, sex, and smoking history. In addition, the prevalence of COPD and the correlation of lung function have been shown to decrease with increased genetic distance (e.g., in second degree relatives vs. first degree relatives) (9,10). In the general population unselected for airflow limitation, there was a higher correlation of lung function between parents and their children or between two siblings than between spouses (11,12).

B. Twin Studies

Twin studies provide a means to estimate the relative contributions of genes and environment to a trait, by comparing the correlation of the trait in monozygotic (MZ) twins with the correlation in dizygotic (DZ) twins.

Webster et al. (13) studied 45 pairs of apparently healthy MZ twins, comparing maximum expiratory flow at 60% of total lung capacity in smokers and nonsmokers, and found that this test could discriminate smokers from nonsmokers, among pairs of twins in which one member smoked and the other did not. The intra-pair difference of this test in pairs where both members smoked was the same as in pairs in which both members did not smoke, supporting the view that genetic factors are important in determining the vulnerability of the airways to cigarette smoke.

Studies of twins raised together may reflect the similar childhood environments as much as the genetic make-up of the twins. In order to overcome this problem, Hankins et al. (14) studied 15 pairs of MZ twins and one set of MZ triplets, who were separated soon after birth and raised apart. Six twin pairs were concordant for nonsmoking and six were discordant. Three pairs and the triplets were concordant for smoking. The results of this study, along with those of the previous studies of twins raised together, support the conclusion that genetic factors are important in determining susceptibility to COPD due to chronic cigarette smoke exposure.

The proportion of the variability of a trait due to genetic factors is known as the heritability of that trait, and for forced expiratory volume in one second (FEV_1), this proportion ranges from 50–80% (15,16). Variation in heritability estimates in these studies could be due to differences in exposure to environmental factors and/or differences in genetic make-up of the respective populations.

C. Segregation Analyses

Although twin studies provide evidence for a genetic basis to a disease, they are unable to identify the nature or number of inherited factors involved. In order to achieve this, a technique known as segregation analysis can be

employed. In this approach, the level of pulmonary function is investigated in families, and statistical models are fitted to the data. In this way, information regarding the mode of inheritance of a trait (dominant or recessive inheritance, the number of genes involved, etc.) can be gained. The results of such studies have confirmed a significant genetic component to pulmonary function (17,18). Generally, the results suggested that the best model for this genetic component was that there were several genes, each with a small effect, rather than a single major gene. Most recently, Kurzius-Spencer et al. (19) reported the results of a study of 746 white, non-Mexican families from Tucson. Significant correlations of FEV1 levels were observed between parents and offspring and between siblings, but not between spouses. The correlations between the siblings were higher than between parents and offspring, which may reflect shared childhood environmental factors. All of the correlations were similar whether or not smoking was adjusted for, suggesting that exposure to similar levels of cigarette smoke did not account for the familial aggregation of FEV1. As in previous studies, segregation analysis of FEV1 showed significant familial effects with no evidence for a major gene. In summary, results of these epidemiological studies demonstrate that COPD is a complex genetic disease, i.e., there is a genetic component to COPD, but it is unlikely that there is a major susceptibility gene in the majority of families.

III. IDENTIFICATION OF GENES THAT INFLUENCE SUSCEPTIBILITY TO DISEASE

Two major strategies have been used to identify genes containing mutations or polymorphisms (common sequence variants), which contribute to the development of COPD. The first strategy is positional cloning that involves searching the entire human genome for disease-causing genes. The genes are identified solely on the basis of their position in the genome. The second strategy is the candidate gene approach in which individual genes are directly tested for their involvement in a disease process. The genes selected for this approach are those that, because of their function, are plausible candidates for being the disease gene. Traditionally, positional cloning has been performed using family data using a technique known as linkage analysis, whereas candidate genes have been tested by association studies of unrelated subjects.

A. Genome Screens for COPD

To perform linkage analysis, phenotypic data and DNA are collected from affected families of at least two generations. Each family member is typed for genetic markers that are scattered throughout the genome thereby

resulting in a complete genome screen. Linkage analysis determines whether any of the markers are inherited with the disease more often than predicted by chance. If so, that disease is said to be "linked" to that marker on a certain chromosome. An advantage of linkage analysis is that completely novel genes can be identified and implicated in the pathogenesis of a disease. This is because linkage of a disease to a genetic marker depends only on the close proximity of that marker with the disease–causing gene, and no data concerning the function of the gene is required.

COPD is defined as irreversible airflow obstruction, which can be assessed by the reduction in the level of FEV_1 and by the ratio of FEV_1 to forced vital capacity (FVC). Evaluating linkage to these quantitative spirometric indices (FEV_1, FVC, FEV_1/FVC ratio) may be a more powerful approach for finding disease susceptibility genes and may be less subject to genetic heterogeneity than linkage to pulmonary disease (20). To date, several genome scan studies have been performed and a number of candidate regions were suggested to be in linkage to lung function levels measured by spirometry (Table 1) (21–23).

A genome scan exploring genetic linkage to lung function was performed in the Framingham Heart cohort, a longitudinal cohort started in 1948 (22). This cohort contained numerous extended pedigrees and provided a unique population for the analysis of genetics of pulmonary function (17). Joost et al. estimated the heritability of FEV1, FVC, and FEV1/FVC and reported a suggestive linkage of FEV1 to a locus on chromosome 6q (LOD 2.4). In a follow-up study, fine mapping for the linkage on chromosome 6q and assessment of the association between lung function and specific markers supported the idea that chromosome 6q harbored a gene that was important for lung function (23).

In addition to the study performed in Framingham Heart cohort, another genome scan was performed in the National Heart, Lung, and Blood Institute Family Heart Study (NHLBIFHS) cohort (24). These authors reported different loci that may influence the variability in pulmonary function (24). The FEV_1/FVC ratio was linked to chromosome 4 (LOD score 3.5), and FEV1 was linked to chromosome 18 (LOD score 2.4). The authors suggested that the discrepancy between this study and the Framingham study might be due to differences in subject ascertainment and spirometric data collection (24).

An additional cohort used for the linkage analysis of lung function was composed of severe early-onset COPD probands without severe AAT deficiency and control probands matched for age and gender (7). Owing to the stringent enrolment criteria, the size of this cohort was not large (585 subjects, 72 pedigrees). Both qualitative phenotypes, including airflow obstruction and chronic bronchitis, and quantitative phenotypes, including pulmonary function and bronchodilator responsiveness (BDR), were evaluated, and several linked genomic regions were identified (25–27).

Table 1 Summary of Genome-Wide Linkage Analysis in COPD

Cohort	Population	Size of studying cohort (No. of families)	Trait	Location	LOD score	Candidate genes	Reference
Early-onset COPD patients	Caucasian	585 (72)	Moderate airflow obstruction	12p (36 cM)	1.7	MGST1, MGP	25
			Mild airflow obstruction	8 (76 cM)	1.4	—	
				19(42 cM)	1.1		
			Chronic bronchitis	19 (42 cM)	1.2		
				22 (36 cM)	1.4		
			FEV_1/FVC	2q (222 cM)	4.1	IL8RA	26
			FEV_1	12p(37 cM)	2.4	MGST1, MGP	
			FVC	1 (13 cM)	2.1	—	
			BDR	4p, 4q, 3	1.3–1.6	—	27
			Airflow obstruction	2q, 8p	3.3, 4.4		
Framingham Study	Caucasian	1,578 (330)	FEV_1	6q terminus	2.4	—	22
			FVC	21p terminus	2.6		
			FEV_1/FVC ratio	6q terminus	1.4		
		1,115 (182)	FEV_1	6q (185 cM)	5.0	—	23
NHLBI Family Heart Study	Caucasian	2178 (391)	FEV_1	18 (31 cM)	2.4	—	24
			FVC	18(79 cM)	2.9		
			FEV_1/FVC ratio	4 (28 cM)	3.5	Superoxide dismutase 3	

Note: FEV1, forced expiratory volume at one second; FVC, forced vital capacity; BDR, bronchodilator responsiveness; NHLBI Family Heart Study, National Heart, Lung, and Blood Institute Family Heart Study; IL8RA, interleukin-8 receptor alpha gene; MGST1, microsomal glutathione S-transferase 1; MGP, matrix Gla protein.

B. Candidate genes for COPD

COPD is characterized by a slowly progressive irreversible airflow limitation that is primarily due to two pathophysiological changes in the lung: peripheral airway inflammation and a loss of lung elastic recoil resulting from parenchymal destruction (Fig. 1). Many inflammatory cells, mediators, and enzymes have been implicated, and these offer potential targets for genetic investigations. It seems certain that there will be a complex interaction between several different genetic and environmental factors. To date, the genes that have been implicated in the pathogenesis of COPD are involved in antiproteolysis, metabolism of toxic substances in cigarette smoke, antioxidation, the inflammatory response to cigarette smoke, and mucociliary clearance. The genes involved or potentially involved in the pathogenesis of COPD are summarized in Table 2.

1. Proteolysis–Antiproteolysis

Severe α_1 -Antitrypsin deficiency: α_1-Antitrypsin (α_1-AT) is an acute phase protein synthesized by the hepatocytes and to a lesser extent by

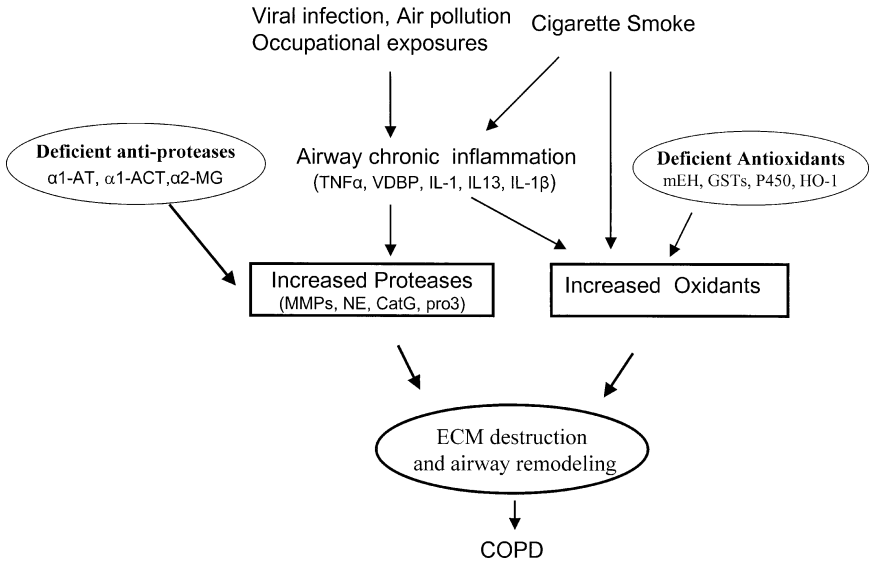

Figure 1 Summary of pathways and possible candidate genes involved in the pathogenesis of COPD. TNFα, tumor necrosis factor α; VDBP, vitamin D-binding protein; IL-Iβ, interleukin 1β; α_1-AT, α_1-antitrypsin; α_1-ACT, α_1-antichymotrypsin; α_2-MG, α_2-macrglobulin; MMPs, matrix metalloproteinases; NE, neutrophil elastase; CatG, cathepsin G; Pr3, proteinase 3; mEH, microsomal epoxide hydrolase; p450, cytochrome P-450; GST, glutathione S-transferase; HO-1, heme oxygenase-1; ECH, extracellular matrix.

Table 2 Summary of Candidate Genes and Polymorphisms Implicated in the Pathogenesis of COPD

Candidate gene	Candidate risk allele	Effect of risk allele	Association studies
α_1-AT	Z(Lys^{342})	ZZ homozygotes reduce plasma α_1-AT level to 10–15% of normal	ZZ individuals have a marked increased in risk for COPD (29,37,38,41–43,64)
		MZ heterozygotes reduce plasma α_1-AT level to 60% of normal	MZ individuals have a modest increase in risk (53–58,63,65)
	S (Val^{264})	SZ heterozygotes reduce plasma α_1-AT level to 60% of normal	Inconsistent results regarding the increased risk for COPD (51,52,64)
	3' UTR (1237 A)	Attenuated upregulation of gene expression in vitro	Conflicting data regarding risk for developing COPD (58,66–69)
α_1-ACT	Ala^{229}	α_1-ACT deficiency	Associated with COPD in some populations (75,76)
	Pro^{55}	Defective α_1-ACT protein	Associated with COPD (75)
MMP-1	-1607GG	Higher levels of gene expression.	Paradoxically associated with lower rate of decline of lung function (84)
MMP-9	-1562T	Higher level of gene expression	Associated with emphysema (85,86)
MMP-12	Asn^{357}	None known	Haplotype consisting of MMP1-1607G and MMP12 Asn^{357} was associated with a rapid decline of lung function (84)
mEH	His^{113}	40–50% Decrease in mEH activity	Associated with emphysema, COPD, COPD severity, and the rate of decline of lung function (58,92,93,95)
	His^{139}	25% Increase of enzyme activity	Associated with COPD and rate of decline of lung function in combination with His^{113}(58)
GSTM1	Null	Lack of protein production	Associated with emphysema, severe chronic

(*Continued*)

Table 2 Summary of Candidate Genes and Polymorphisms Implicated in the Pathogenesis of COPD (*Continued*)

Candidate gene	Candidate risk allele	Effect of risk allele	Association studies
			bronchitis, and rapid decline in lung function in combination with GSTTl null genotype and GSTPl Ile allele (97,98,104)
GSTTl	Null	Lack of protein production	Associated with rapid decline in lung function in combination with GSTMl null genotype and GSTPl Ile allele (104)
GSTPl	Ile^{105}	Reduced catalytic activity for some xenobiotic substrates	Associated with COPD and rapid decline in lung function in combination with GSTTl null genotype and GSTMl Ile allele (102,104)
HO-1	-(GT)*n*	The L allele may suppress gene transcription and affect enzyme activity	L allele was associated with emphysema, and more susceptible to oxidant-induced apoptosis (109,111), but not with the rate of decline in lung function (104)
CYP1A1	Val^{462}	Increase in catalytic activity	Associated with COPD but only in patients who also had lung cancer (106)
VDBP	IF	Not known	Associated with increased risk of COPD (116,118); but not with lung function level and decline rate of lung function (58,119)
	2	Not known	Associated with a decreased risk of COPD (116,117)

TNF	-308A	Increased TNF production	Inconsistent result about the association with COPD (58,126–131)
IL-lβ	-511T	Increased levels of IL1 and IL1RN	Haplotype consisting of IL-lβ -51 IT and IL1RN allele 2 was associated with attenuated rate of decline of lung function (142)
IL-IRN	86 lbp tandem repeat	Allele 2 in combination with IL-lβ T allele was associated with higher levels of IL-IRN	Associated with normal rate of decline in lung function (142)
		Allele 1 in combination with IL-lβ T allele was associated with lower levels of IL-IRN	Associated with rapid decline in lung function (142)
IL-13	-1055T	Increased production of IL-13	Associated with COPD (145)
IL-4RA	Arg^{551}	Enhanced responsiveness to IL-4	Associated with rapid decline in lung function (146)
CFTR	IVS8-5T	Reduced CFTR expression	Conflicting data regarding the role of this polymorphism in C0PD (161,162)
	Met^{470}	Possible influence on CFTR chloride activity when combined with other Polymorphisms	Association with COPD (163)

Note: α_1-AT, α_1-antitrypsin; α_1-ACT, α_1-antichymotrypsin; α_2-MG, α_2-macroglobulin; MMP, matrix metalloproteinase; mEH, microsomal epoxide hydrolase; GST, glutathione S-transferase; HMOX1 heme oxygenase-1; CYP1A1, Cytochrome P4501A1; VDBP, vitamin D binding protein; TNFα, tumor necrosis factor-α; IL-1, interleukin-1; IL-1RN, interleukin-1 receptor antagonist; IL-I3, interleukin-13; IL-4RA, Interleukin 4 receptor α chain; CFTR, cystic fibrosis transmembrane conductance regulator.

alveolar macrophages. This antiprotease provides the major defense against proteolytic digestion of the lung by neutrophil serine proteases, such as neutrophil elastase, cathepsin G, and proteinase 3 (28). It has been known since the early 1960s that individuals who have extremely low levels of α_1-AT have an increased prevalence of emphysema (29). A genetic basis for α_1-AT deficiency was demonstrated by the observation that the deficiency followed a simple Mendelian pattern of inheritance and was usually associated with the Z isoform of α_1-AT (30–32). The two most common deficiency variants of α_1-AT, S and Z, result from point mutations in the α_1-AT gene (33–35) and are named on the basis of their altered electrophoretic mobility on isoelectric focusing gels compared with the normal M allele (36). Homozygosity of the Z variant (which contains lysine rather than glutamic acid at amino acid position 342) results in a severe deficiency that is characterized by plasma α1-AT levels $\sim$10% of the normal M allele. Individuals with the ZZ phenotype have a clearly accelerated rate of decline in lung function (37), sometimes even in the absence of smoking (38). However, the homozygous state is rare in the population (39) and thus can explain only a small percentage of the genetic susceptibility to cigarette smoke.

Despite the strong association of the ZZ genotype with early-onset COPD, the clinical course of the disease is highly variable (40), as is common with other genetic disorders. Exposure to cigarette smoke plays an important role in determining this variability (41). Several large series of AAT deficient individuals have clearly shown that PI Z subjects who smoke cigarettes tend to develop more severe pulmonary impairment at an earlier age than nonsmoking PI Z individuals (38,42,43). In addition, a follow-up study of participants in the Swedish AAT Deficiency Register has shown that the rate of decline in FEV_1 is significantly higher in PI Z current smokers than in never smokers or exsmokers (41). Few studies have considered whether factors other than smoking influence the development of lung disease. Black and Kueppers (44) studied 54 PI Z individuals and found significant variability in pulmonary function and clinical symptoms, especially among nonsmoking PI Z individuals. Piitulainen et al. (45) studied more than 200 nonsmoking PI Z subjects from the Swedish AAT Deficiency Register; so, they were able to examine the impact of risk factors for reduced pulmonary function without confounding by smoking. Not surprisingly, increasing age was associated with reduced pulmonary function in these nonsmoking PI Z subjects. Male sex, wheezing symptoms, and occupational exposures to gas, fumes, or dust were also associated with reduced pulmonary function. Subsequent analyses in this population suggested that agricultural occupation and use of a kerosene heater were associated with reduced FEV_1 levels; however, the generalizability of these findings may be limited as only a small number of PI Z subjects had these exposures (46). Mayer et al. (47) recently assessed occupational exposures as a potential contributor to variable expression of lung disease in 128 PI Z subjects. They found that

high mineral dust exposure was associated with reduced FEV1 and chronic cough, independent of smoking history. Although genotype–environment interactions have not been formally demonstrated, occupational and other environmental exposures may also be important determinants of the development of lung disease in PI Z subjects.

However, the rate of decline of lung function in ZZ subjects who are lifelong nonsmokers is also highly variable (44). In studies in which index and nonindex cases have been compared, many nonindex ZZ subjects show normal lung function (48) and a survival similar to the normal population (49) if they are nonsmokers. Therefore, the effect of the ZZ genotype in increasing the risk of clinically significant airflow limitation is likely to have been overestimated in some studies due to selection bias. It is possible that other genetic factors influence the clinical course in ZZ homozygotes. Polymorphisms in the endothelial nitric oxide synthase (NOS3) gene were shown to contribute to the development of COPD in ZZ individuals (50), although this association has not been confirmed in an independent study.

Intermediate α_1-Antitrypsin Deficiency: With the clear association of severe α_1-AT deficiency with COPD, it was a natural question to ask whether individuals with intermediate deficiency were also at risk for airflow limitation. Individuals who have one copy of either S or Z alleles are present in Caucasian populations at ~10% or 3%, respectively. These MS and MZ heterozygotes have reductions in α_1-AT levels to ~80% and 60% of normal, respectively. The SZ compound heterozygotes are rare but have levels even lower at ~40% of normal. In one study, SZ heterozygotes were shown to have an increased risk for COPD if they smoked (51). However, in a study from Spain, no association between SZ phenotype and COPD was found (52). To date, there have been no studies to convincingly demonstrate that MS heterozygotes are at increased risk of airflow limitation.

The results of many case–control studies have shown an increased prevalence of MZ heterozygotes in COPD patients compared with controls (53–57). In such studies, the odds ratio (OR) for MZ typically ranges from 1.5 to 5.0. However, in some of these studies, the controls were not selected from the same population as the cases, and this may lead to spurious associations of the MZ genotype with COPD due to differences in MZ frequency between populations. In addition, some of these case–control study. results were not adjusted for confounding variables such as smoking history and age. Recently, the MZ genotype was investigated as a risk factor for increased rate of decline of lung function in smokers (58). The study group consisted of 283 smokers with rapid decline of lung function (mean $\Delta FEV_1 = -154\,mL/year$) and 308 smokers with no decline (mean $\Delta FEV_1 = +15\,mL/year$). Rapid decline of FEV1 was associated with the MZ (OR = 2.8) and the association was stronger for a combination of a family history of COPD with MZ (OR = 9.7). These data suggest that the MZ genotype results in an

increased rate of decline in lung function and interacts with other familial factors. Some of these familial factors could be genetic and others could be environmental.

Investigators have also assessed the risk of the MZ genotype by studying lung function in the general population (57,59–62). In these studies, a population sample is phenotyped for α1-AT variants and the prevalence of COPD in those with the MZ phenotype is compared with those with the MM phenotype. A weakness of many of these studies was that they were based on small numbers of individuals and therefore had insufficient power to detect an effect of the MZ or MS phenotype. In a large cohort study from Denmark, Seersholm et al. (63) compared the prevalence of obstructive pulmonary disease in 1551 MZ individuals vs. 14,484 controls from the general population of unknown α1-AT genotype. Obstructive pulmonary disease was defined as a hospital discharge diagnosis of asthma or chronic bronchitis or emphysema. The risk for obstructive pulmonary disease was significantly increased in the MZ individuals compared with the controls (relative risk = 2.2). However, only first-degree relatives of ZZ COPD patients had a significantly increased risk, suggesting that other genetic or environmental factors were contributing to the increased risk in these patients. Dahl et al. (64) performed a large cross-sectional study of 9187 individuals from the general population of Copenhagen in Denmark. The study subjects underwent pulmonary function testing (FEV_1 and FVC) and were genotyped for the S and Z α1-AT variants. Only the SZ and ZZ individuals in this population showed an increased prevalence of COPD ($FEV_1 < 80\%$ predicted). There was no association of either MS or MZ genotype with lower level of lung function in individuals without clinically established COPD.

However, among the COPD patients, FEV_1 was < 655 mL in MZ individuals compared with MM individuals ($p < 0.05$) after adjustment for confounding variables. The observation that the MZ genotype was associated with airflow limitation only in those with COPD suggests that other predisposing factors exist, consistent with the results of Seersholm et al. (63). In addition to this cross-sectional study, Dahl et al. (65) performed a longitudinal study to test whether MZ genotype affects lung function decrease and other clinical outcomes of COPD on the same study cohort. Three lung function measurements obtained from 1976 to 1978, 1981 to 1983, and 1991 to 1994 were used to calculate annual change in FEV1. The results showed that the MZ genotype was more prevalent in subjects with airway obstruction and COPD and was associated with more rapid decline rate in FEV1 than the MM genotype. The author also concluded that the MZ genotype accounts for almost the same proportion of COPD cases as the ZZ genotype (65).

α_1-Antitrypsin Polymorphisms Not Associated with Deficiency: There are several polymorphisms of the α1-AT gene that are not associated with α_1-AT deficiency. The most extensively studied example is a polymorphism

in the 3′ untranslated region of the α_1-AT gene that has been associated with COPD in some populations (66,67), but not others (58,68,69). In vitro, this polymorphism was associated with decreased binding of a transcription factor and decreased gene expression (70). The most likely transcription factor is nuclear factor of interleukin (IL)-6, which is activated by IL-6 and subsequently increases the expression of α_1-AT (71). Thus, the 3′ mutation could affect the acute phase response leading to reduced α_1-AT synthesis in response to inflammation. However, in contrast to the in vitro data, the 3′ polymorphism was not associated with a reduced α_1-AT acute phase response in patients undergoing open-heart surgery (72) or in patients who had cystic fibrosis (CF) (73).

Therefore, the role of the 3′ polymorphism in the pathogenesis of COPD remains to be determined.

Another polymorphism in the 3′ region of the α_1-AT gene has been associated with COPD (74). The polymorphism was associated with normal α_1-AT levels and was found in eight out of 70 COPD patients but in none of 52 controls. There have been no follow-up studies to confirm this association.

Other Antiprotease Genes: The association of airflow limitation with genetic defects in the α_1-AT gene also led to a search for genetic variants of other antiproteases that may be involved in protection against lung destruction, α_1-Antichymotrypsin (α_1-ACT) is another serine protease inhibitor synthesized in the liver and alveolar macrophages and airway epithelia. Several functional single nucleotide polymorphisms have been identified. The $Leu^{55}\rightarrow Pro$ mutation leads to a defective α_1-ACT protein and was associated with COPD (75). Another polymorphism causes the $Pro^{229}\rightarrow Ala$ substitution and α_1-ACT serum deficiency and may predispose subjects to COPD (75,76). Other investigators have found no association of these polymorphisms with COPD or related phenotypes (69,77). α_2-Macroglobulin is a broad-spectrum protease inhibitor that is also synthesized in hepatocytes and in alveolar macrophages. Several polymorphisms of the α2-miacroglobulin gene have been described (78). However, all of these polymorphisms in other antiproteases are rare and the evidence that they contribute to susceptibility to COPD is weak.

Matrix Metalloproteinase Genes: Matrix metalloproteinases (MMPs) are a structurally and functionally related family of proteolytic enzymes that play an essential role in tissue remodeling and repair associated with development and inflammation (79). Several studies in animals and humans have provided evidence that MMPl (interstitial collagenase), MMP12 (human macrophage elastase), and MMP9 (gelatinase B) are important in airway inflammation and the development of emphysema. In 1992, D'Armiento et al. (80) demonstrated that transgenic mice, over-expressing human MMPl in their lungs, developed morphologic changes strikingly similar to human

pulmonary emphysema (80). The MMPl2 knockout mice did not develop emphysema following exposure to cigarette smoke compared to wild-type mice (81), suggesting that the presence of MMP12 is critical in smoke-induced lung injury. Smokers with airway obstruction show increased expression of MMPl and MMP9 compared with smokers without COPD and nonsmokers (82,83).

A promoter polymorphism in the MMPl gene (G-1607GG) was associated with rate of decline of lung function in smokers (84). In the same study, polymorphisms of MMP9 and MMPl 2 were not individually associated with rate of decline of lung function (84). However, combination of alleles (i.e., haplotypes) from the MMPl G-1607GG and MMP12 Asn357Ser polymorphisms revealed an association with rate of decline of lung function ($p = 0.0007$). These data suggest that the polymorphisms in the MMPl and MMP12 genes investigated by Joos et al. are either causative factors in smoking related lung injury or are associated with causative polymorphisms.

Minematsu et al. (85) examined the association between a MMP9 promoter polymorphism (C-l 562T) and the development of emphysema in Japanese smokers. They demonstrated that the T allele frequency was higher in subjects with distinct emphysema on chest CT-scans than in those without it ($p = 0.02$). In addition, the diffusing capacity of the lung for carbon monoxide per liter of alveolar volume was lower ($p = 0.02$) and emphysematous changes were more conspicuous ($p = 0.03$) in subjects with C/T or T/T than those with the C/C genotype (85). These data are consistent with the higher level of gene expression associated with the T allele in an in vitro assay (86).

2. Xenobiotic Metabolizing Enzymes

Xenobiotic metabolizing enzymes are a class of molecules that play an important role in detoxifying potentially damaging organic compounds found in cigarette smoke (87,88). There is considerable interindividual variation in the catalytic efficiencies of these enzymes in many, if not all, human populations. Therefore, these molecules have been studied to determine whether genetically determined deficiencies in xenobiotic metabolism may predispose an individual to the development of airflow limitation in response to cigarette smoke.

Microsomal Epoxide Hydrolase: Microsomal epoxide hydrolase (mEH) is an enzyme that plays a critical role in the lung's ability to metabolize highly reactive epoxide intermediates which may be found in cigarette smoke. Microsomal epoxide hydrolase is expressed in a variety of different cell types including hepatocytes and bronchial epithelial cells. In the coding region of the mEH gene, two relatively common genetic polymorphisms have been identified (89,90). The polymorphism in exon 3 resulted in the $Tyr^{113} \rightarrow$His substitution and a 40–50% decrease in mEH activity, and was named the "slow allele." Conversely, another polymorphism in exon 4

was referred as "fast allele" because it caused the $His^{139} \rightarrow Arg$ substitution and 25% increase of enzyme activity (89).

However, similar correlation between the polymorphisms and the mEH activity were not shown in liver tissue samples (91). The slow allele of mEH was found in a higher proportion of patients with emphysema (22%) and COPD (19%) than in control subjects (6%), yielding an OR of ~5 (92). In a smaller Japanese study, the slow allele of mEH was associated with more severe COPD (93). These results were not confirmed in a Korean population (94). However, the slow allele was found associated with rapid rate of decline of lung function in smokers (58) and COPD in Caucasians (95). Therefore, despite the one inconsistent study, overall these data suggest that genetic variation in the mEH gene does modify an individual's risk of COPD.

Glutathione S-Transferases: Glutathione S-transferases (GSTs) are members of a family of enzymes critically involved in detoxification of various toxic substances found in cigarette smoke, including polycyclic aromatic hydrocarbons that have been implicated in the pathogenesis of COPD. Most GSTs exist as soluble enzymes and can be divided into four main classes: alpha(A), mu (M), pi (P), and theta (T) (96). The GST genes are polymorphic. Homozygous deletion of the GSTM1 gene occurs in ~50% of Caucasians and therefore results in complete absence of this enzyme in these individuals. Homozygous deficiency for GSTM1 was associated with emphysema in Caucasian patients who had lung cancer (OR = 2.1) (97) and severe chronic bronchitis in heavy smokers (OR = 2.8) (98). However, in a Korean study, there was no association of the GSTM1 deletion with COPD (94). These discrepant results may be due to differences in the ethnicity of the study subjects. Certain genetic variants may be associated with disease only in certain ethnic groups due to interactions with population-specific environmental factors or other genetic factors.

A null polymorphism has also been identified in the GSTT1 gene (99), as well as two single nucleotide polymorphisms in the GSTP1 gene. The polymorphism in exon 5 of GSTP1 at nucleotide 313 ($Ile^{105} \rightarrow Val$) was shown to result in altered enzyme activity (100,101). Homozygotes for the isoleucine allele were significantly increased in Japanese patients with COPD compared with the controls (OR = 3.5) (102), However, this result was not replicated in a larger study of Korean COPD patients and controls (103). In a recent study, He et al. (104) investigated the association between the polymorphisms of the GSTM1, GSTT1, and GSTP1 gene and accelerated decline rate in FEV_1 in a Caucasian population. They found that none of the polymorphisms individually had a significant effect on the decline of lung function. However, a significant association was observed for concurrent deletion of the GSTM1 and GSTT1 genes and presence of homozygous GSTP1 Ile allele (OR = 2.83, P = 0.03). These data suggest that individuals

who had a defective genotype for more than one of these genes were at greater risk for smoking-induced decline in lung function than those who had only one defective genotype.

Cytochrome P4501A1: Cytochrome P4501A1 (CYP1A1) also metabolizes xenobiotic compounds to enabling them to be excreted. Cytochrome P4501A1 is expressed throughout the lung and may play a role in the activation of procarcinogens. A polymorphism in exon 7 of CYP1A1 causes an amino acid substitution ($Ile^{462} \rightarrow Val$) that results in increased CYP1A1 activity in vivo (105). The high-activity isoform (Val^{462}) was associated with susceptibility to centriacinar emphysema in patients who had lung cancer (OR = 2.5) (106).

3. Antioxidants

Heme Oxygenase-1: Heme oxygenase (HO) degrades heme to biliverdin and has been demonstrated to provide cellular protection against heme and nonheme-mediated oxidant injury (107,108). A polymorphism consisting of variable numbers of guanine–thymine (GT) nucleotides within the HO gene (HO-1) promoter was identified. The distribution of the number of (GT) *n* repeats was trimodal: short alleles (S: < 27 GT), middle alleles (M: 27–32 GT), and long alleles (L: ≥ 33 GT) (109). An association study in a Japanese population found the L allele was associated with pulmonary emphysema in smokers, yielding an OR of 2.4 (109). The authors hypothesized that the reason for the association was the effect of GT repeats in promoting the formation of a conformation of DNA known as Z DNA. The Z DNA has been shown to decrease gene expression (110), and therefore a high number of GT repeats in the HO-1 promoter may suppress expression of the gene and leave the individual susceptible to oxidant-induced lung injury. In support of this hypothesis, the authors showed that a high number of GT repeats resulted in decreased in vitro gene expression in response to hydrogen peroxide. In a recent study, Hirai et al. (111) reported different HO-1 mRNA expression level and HO-1 activity between the S/S genotype and the L/L genotype, and cell lines with S/S genotype were more resistant to oxidant-induced apoptosis than those with L/L genotype, which provided another possible mechanism for association with susceptibility to oxidative stress-mediated diseases. However, there was no association found between HO (GT) *n* alleles and the rate of decline in lung function in smokers in a subsequent study of Caucasian individuals (104).

4. Inflammatory Mediators

Vitamin D Binding Protein: Vitamin D binding protein (VDBP) is a protein secreted by the liver, which is able to bind vitamin D, extracellular actin, and endotoxin. Vitamin D Binding Protein enhances the chemotactic activity of two complement factors (C5a and C5a des-Arg) for neutrophils by one to two orders of magnitude (112). Chemoattractants, such as these

complement factors, are believed to play an important role in the accumulation of neutrophils in lung that is seen in COPD (113). In addition, VDBP can be converted to a potent macrophage-activating factor (114). Thus, besides its vitamin D binding function, VDBP could have important influences on the intensity of the inflammatory reaction in the lung in response to cigarette smoke.

There are three major isoforms of this protein termed 1S, 1F, and 2, and these isoforms are due to two common substitutions in exon 11 of the VDBP gene (115). Individuals who had one or two copies of allele 2 were shown to be protected against COPD (116,117). In addition, Horne et al. (116) demonstrated that 1F homozygous individuals had a significantly increased risk of developing COPD. This association was confirmed in a Japanese population (118). In contrast, no association of this genotype with lung function level and accelerated decline of lung function was found (58,119).

Schellenberg et al. (117) examined whether the associations of VDBP isoforms with COPD could be due to the effect of VDBP on neutrophil chemotaxis. However, there were no significant differences between the three VDBP isoforms in their ability to enhance chemotaxis of neutrophils to C5a. It remains possible that the mechanism for the association with COPD is related to the activation of macrophages at sites of inflammation. However, no investigators have examined the influence of these genetic variants on the ability of the protein to act as a macrophage-activating factor.

Tumor Necrosis Factor α: Tumor necrosis factor α (TNFα) and tumar necrosis factor β (TNFβ) (lymphotoxin) are proinflammatory cytokines that have many effects of relevance to the pathogenesis of COPD, e.g., neutrophil release from the bone marrow and neutrophil activation. A recent in vitro study suggested that metalloproteases mediate cigarette smoke-induced inflammation via the release of TNFα from macrophages (120). Elevated TNFα was found in BAL fluid, bronchial biopsy specimens, and induced sputum of patients with COPD (121).

The TNFα and TNFβ genes contain several polymorphisms including a G$\rightarrow$A transition in the TNFα gene promoter (TNFα G-308A) and an A$\rightarrow$G transition in the first intron of the TNFβ gene (TNFβ A252G). These polymorphisms have been shown to be associated with the level of TNFα and TNFβ production in vitro (122). In addition, the TNFα -308A allele has been associated with several diseases including cerebral malaria (123) and asthma (124,125). An association of the TNFα -308A allele with COPD was found in a Taiwanese population (126). The patients were selected on the basis of the presence of chronic bronchitis and airflow limitation ($FEV_1 < 80\%$ predicted and $FEV_1/FVC < 69\%$). The prevalence of the TNFα -308A allele was considerably increased in the patients compared with the controls, yielding an OR of 11.1 for chronic bronchitis.

Recently, this association was confirmed by Sakao et al. (127) in a Japanese population. In this study, 106 patients were selected on the basis of an $FEV_1 < 80\%$ and an $FEV_1/FVC < 80\%$, and these individuals were compared with 110 asymptomatic smokers or exsmokers and 129 adult blood donors. The presence of the TNFα -308A allele (homozygotes and heterozygotes combined) was significantly increased in cases (27%) compared with both control groups (12%) yielding an OR of 2.6. Evidence for the role of the TNFα-308A allele in the pathogenesis of COPD was further strengthened by the observation that this allele was associated with more severe emphysema, as judged by high resolution CT (128).

In contrast, no association of the -308A allele with COPD was found in a study of 53 physician diagnosed COPD patients and 65 controls from the Japanese population (129). However, the sample size in this study was small and there were considerably fewer subjects (2%) with the -308A allele than in the study by Sakao et al. (127). Studies of Caucasian populations have found no association of TNFα –308A with COPD (130) or rate of decline of lung function (58). In a recent study in Caucasians from Italy, the authors used "moderate-to-severe COPD associated with emphysema" as narrower criterion for the COPD phenotype and investigated its association with the polymorphisms in TNF family genes (131). The results suggested that none of these genes was a major genetic risk factor for COPD. Interestingly, a study of a Caucasian population from the Netherlands also reported no association of TNFα -308A with COPD (132). However, these authors did find an association of COPD with the presence of the A allele of another TNFα polymorphism (TNFα G489A). This association was only found in patients who had no evidence of emphysema based on high resolution CT scans, consistent with the hypothesis that TNFα polymorphisms would affect airway inflammation rather than proteolytic destruction of the lung. In summary, the role of TNFα polymorphisms in COPD has yet to be established, but this may be an another example of ethnic group specific genetic risk factors.

IL-1 Complex: The IL-1 family consists of two proinflammatory cytokines, IL-1α and IL-1β, and a naturally occurring anti-inflammatory agent, the IL-1 receptor antagonist (IL1RN). The two forms of IL-1 are the products of different genes, but they are structurally related and bind to the same receptor. IL-1α and IL-1β are synthesized by a variety of cell types, but mainly monocytes and macrophages. IL1RN is a protein that binds to the IL-1 receptor with the same affinity as IL-1, but does not possess agonist activity and therefore acts as a competitive inhibitor of IL-1 (133). The genes of the IL-1 complex are found in close proximity on the long arm of human chromosome 2 (134), and each of the genes is polymorphic. The IL-1β gene (IL1B) has a single nucleotide polymorphism in the promoter region (C-511T) (135), and the IL1RN gene has a polymorphic

site in intron 2 containing two to six repeats of an 86 base pair sequence (136). There is evidence that allele 2 of the IL1RN gene (IL1RN*2) is associated with increased susceptibility or more severe outcome in chronic inflammatory diseases such as ulcerative colitis, systemic lupus erythematosus, and alopecia areata (137–139). The *IL1B* C-511T has been associated with inflammatory bowel disease (140), as well as with plasma levels of IL1B and IL1RN (141).

In a recent study, individual IL-1 genotypes were not associated with rate of decline of lung function in smokers. However, there was a significant influence of combinations of IL1RN/IL1B alleles in these individuals (142). The association of these haplotypes with the decline of lung function may represent an interaction between the genes. A smaller study in a Japanese population found no association with individual IL1B and IL1RN genotypes and COPD (129).

IL-13 and IL-13 Receptor: Targeted expression of IL-13 in the adult murine lung has been shown to cause emphysema, elevated mucus production, and inflammation reminiscent of human COPD (143). Interleukin-13 operates through the IL-13 receptor (IL13R), which is composed of one IL-4 receptor α (IL4RA) subunit and either a low-affinity IL13RA1 or a high-affinity IL13RA2 subunit (144). IL13, IL13RA1, and IL4RA are polymorphic. A study found an association of a promoter polymorphism (C-1055T) of IL-13 with COPD in a Caucasian population (145). Another recent study suggested the IL4RA 551RR genotype was associated with rapid decline of lung function (OR = 2.24) (146).

Interleukin-10: Interleukin-10 is an important immunoregulatory cytokine produced by many cell types. Its main biological function is to inhibit the production of a variety of proinflammatory mediators (147). In addition, IL-10 may regulate the balance between proteases and antiproteases produced by airway macrophages (148,149). Therefore, IL-10 may be involved in chronic airway inflammation observed in asthma and COPD. Studies have found that airway IL-10 level was significantly lower in asthma and COPD patients and in healthy smokers compared with that in healthy nonsmokers (150). Polymorphisms in the IL-10 promoter could affect the level of IL-10 expression (151,152). A recent study found that homozygosity for the 3368G allele was associated with rapid decline of lung function in woman smokers (153).

5. Mucocilliary Clearance

The rate at which particulate matter is cleared from the lungs is highly variable between individuals (154). The tracheobronchial clearance rate of 6–7μm particles was studied in nine pairs of MZ and nine pairs of DZ twins (155). The intrapair correlation in clearance rates was significantly higher in the MZ twins vs. the DZ twins, suggesting that genetic factors may affect an

individual's mucocilliary clearance rate. This may have important implications for an individual's cumulative exposure to the compounds found in cigarette smoke.

Cystic Fibrosis Transmembrane Regulator: The cystic fibrosis transmembrane conductance regulator (CFTR) forms a chloride channel at the apical surface of airway epithelial cells and is involved in the control of airway secretions. In 1989, mutations in the CFTR gene were identified as the cause of CF. The CF carriers may also be predisposed to respiratory disease. The CF heterozygotes had increased bronchial reactivity to methacholine (156) and increased incidence of wheeze accompanied by decreased FEV_1 and FEF_{25-75} (157).

The most frequent CF-causing variant is ΔF508 and heterozygosity for this mutation was increased in patients who had disseminated bronchiectasis (158,159) and in patients who had "bronchial hypersecretion" (160). The prevalence of ΔF508 was not increased in patients who had chronic bronchitis (159). Other CFTR mutations were increased in patients who had disseminated bronchiectasis and normal sweat chloride levels (161,162). One of these mutations is a variable length thymine repeat in intron 8 of the CFTR gene (IVS8). The IVS8-5T allele results in reduced CFTR gene expression. Studies of IVS8-5T as a risk factor for COPD have yielded conflicting results (161,162). In another study, patients with obstructive lung diseases were screened for variants in the whole CFTR coding region (163). The study compared 12 COPD patients with 52 controls, both groups from the Greek population. There was no significant increase in CF-causing mutations in the patients vs. the controls. However, the frequency of the methionine allele of the Met470Val polymorphism was increased in the patients (71%) compared with the controls (36%). However, the Met470 variant is associated with increased CFTR chloride channel activity compared with the Val variant (164), and therefore the reason for the association with COPD is unclear. A recent study performed in a Korean population found a different CFTR polymorphism pattern from that in Caucasian populations (165). These authors also found several polymorphisms and their corresponding haplotypes were associated with bronchiectasis and chronic pancreatitis. In a functional study, the E2I7G and Q1352H polymorphisms were shown to significantly affect CFTR chloride activity in the presence of Met470Val polymorphism (165). These data suggest that interactions between multiple genetic variants affect the final phenotype of the CFTR gene.

In summary, CFTR variants have been consistently associated with disseminated bronchiectasis. This may be due to the effect of these variants on the rate of mucociliary clearance. However, it is not clear whether the patients who have disseminated bronchiectasis represent a clinically distinct group or have mild, undiagnosed CF with an unknown CFTR mutation on

their other chromosome (166). In addition, all the studies described earlier were based on small numbers of subjects and only three (161–163) compared cases to controls. The other studies compared frequencies in the cases with published allele frequencies, and therefore the results of these studies are far from definitive.

IV. CONCLUSIONS

Although there is clear evidence of a genetic contribution to the pathogenesis of COPD, few specific genes have been implicated to date. Most studies have been case–control candidate gene studies and were often too small in size to be powerful enough to detect genes of modest effect. In addition, the reported studies have been mostly limited to known biologically plausible candidates. In earlier onset diseases, such as asthma, large-scale family studies have provided clues as to the location of susceptibility genes using the technique of genome-wide screening. This technique can identify susceptibility genes irrespective of whether the biological function is known.

In the future, more information about the role of genetic risk factors in the development of COPD may be provided by large-scale family studies, genome-wide association studies, and investigation of an increased number of possible candidate genes identified by the Human Genome Project.

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16

Mechanisms of Systemic Effects

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I. INTRODUCTION

Current definitions of chronic obstructive pulmonary disease (COPD) focus exclusively in the lungs (1–4). Over the past 10 years, however, different studies have clearly shown that COPD is often associated with significant extrapulmonary abnormalities, the so-called ''systemic effects'' of COPD (5). Table 1 shows the main systemic effects described to date.

The systemic effects of COPD are clinically relevant because they influence significantly several aspects of the disease process with great clinical impact, such as exercise tolerance, quality of life, morbidity, and mortality (5). This chapter discusses the potential mechanisms and implications of the main systemic effects of COPD described to date and propose some new ones that may require future investigation.

Table 1 Systemic Effects of COPD

Systemic inflammation
Oxidant stress
Increased plasma levels of cytokines and acute phase proteins
Activated inflammatory cells (neutrophils/lymphocytes)
Weight loss and skeletal muscle dysfunction
Loss of muscle mass
Abnormal structure/function
Exercise limitation
Cardiovascular effects
Endothelium dysfunction
Abnormal left ventricular function
Nervous system effects
Abnormal brain metabolism
Depression
Abnormal ANS function
Osteoporosis
Anemia

II. SYSTEMIC INFLAMMATION

An excessive/inadequate inflammatory response to a variety of noxious inhaled gases or particles (mostly cigarette smoking) is considered a key pathogenic mechanism of COPD (4). This inflammatory response is characterized by the presence in the lung parenchyma of increased concentrations of inflammatory cells (including neutrophils, macrophages, and T-lymphocytes with a CD8+ predominance) and proinflammatory cytokines (such as leukotriene B4 (LTB_4), interleukin-8 (IL-8), and tumor necrosis factor alpha (TNFα)), as well as by the presence of oxidative stress (6). A similar pattern can also be detected in the systemic circulation of these patients.

A. Systemic Oxidative Stress

Rahman et al. (7) were the first to show evidence of systemic oxidative stress in patients with COPD. These authors showed that both the Trolox-equivalent antioxidant capacity and the levels of products of lipid peroxidation were significantly increased in the plasma of patients with COPD, particularly during the episodes of exacerbation of the disease (7). Subsequently, Praticó et al. (8) have reported increased urinary levels of isoprostane F2α-III in COPD. Isoprostane F2α-III is a stable prostaglandin isomer formed by reactive oxygen species (ROS)-dependent peroxidation of arachidonic acid (9). Again, this was particularly pronounced during exacerbations of the disease (8).

B. Circulating Inflammatory Cells

Patients with COPD characteristically show increased number of circulating neutrophils. Besides, several studies have now clearly identified potentially relevant functional abnormalities of these cells in COPD. These include: (a) enhanced chemotaxis and extracellular proteolysis (10); (b) a higher capacity to produce ROS, respiratory burst (11); (c) increased surface expression of several adhesion molecules, particularly Mac-1 (CD11b) (12). Interestingly, this persisted during the process of apoptosis in vitro (13), an observation that can be relevant for neutrophil clearance by macrophages from inflamed tissues; and (d) down-regulation of one type of G-protein subunit (Gas) involved in the intracellular signal transduction pathway of a variety of cellular processes including CD11b expression (12) and the control of the intracellular vesicular trafficking (14). The latter is required for the activation of NADPH oxidase, the enzyme which is eventually responsible of the respiratory burst in neutrophils (11).

Although circulating lymphocytes have been less studied than circulating neutrophils in COPD, there are also some hints of abnormal lymphocyte function in these patients. For instance, Sauleda et al. (15) showed that the activity of cytochrome oxidase, the terminal enzyme in the mitochondrial electron transport chain, was increased in circulating lymphocytes of COPD patients. This abnormality could be detected also in patients with other chronic inflammatory diseases, both of pulmonary—bronchial asthma—and nonpulmonary origin—chronic arthritis—suggesting that it may be a nonspecific marker of lymphocyte activation in chronic inflammatory diseases (15).

Finally, peripheral monocytes seem also to function abnormally in patients with COPD because they produce increased quantities of TNFα when stimulated in vitro (16). This is particularly evident in patients with COPD and low body weight, suggesting that an excessive production of TNFα by peripheral monocytes may play a role in the pathogenesis of weight loss in COPD (16) (see below).

C. Increased Plasma Levels of Proinflammatory Cytokines

Numerous studies have now reported increased levels of circulating cytokines and acute phase reactants in the peripheral circulation of patients with COPD, including TNFα, its receptors (TNFR-55 and TNFR-75), IL-6, IL-8, C-reactive protein, LPS-binding protein, Fas and Fas-L (17–20). These abnormalities were seen in patients considered clinically stable but were generally more pronounced during exacerbations of the disease (19).

D. Mechanisms of Systemic Inflammation in COPD

To date, the precise mechanism(s) underlying systemic inflammation in COPD has not been identified. Several not mutually exclusive candidates

can be operative: (a) tobacco smoke can cause significant extrapulmonary damage (such as endothelial dysfunction) independently of COPD (21,22); (b) cytokines produced by inflammatory lung cells (such as TNFα, IL-6, and IL-1β) may reach the systemic circulation and/or contribute to the activation of inflammatory cells during their transit through the pulmonary circulation; (c) other organs rather than the lungs can contribute to systemic inflammation in COPD. For example, the bone marrow can release increased numbers of inflammatory cells in response to inhaled pollutants (23) and skeletal muscle increases the release of circulating cytokines in COPD (19); and, finally, (d) theoretically, at least, some of the abnormalities described in the peripheral circulation of patients with COPD (e.g., the increased surface expression of several neutrophil adhesion molecules (CD11b) and down-regulation of G-protein subunit (Gαs (12))) may be a cause rather than a consequence of COPD. Due to their specific genetic background, these cells can exhibit a more vigorous response to the same degree of stimulation, including a higher expression of surface adhesion molecules, which would facilitate their recruitment to the site of inflammation (12), and an increased respiratory burst that would enhance their damaging potential (11).

III. WEIGHT LOSS

Unexplained weight loss is common in COPD, particularly (but not exclusively) in patients with severe COPD (24). Weight loss is an important prognostic factor in COPD (25,26). Its prognostic value is independent of the degree of lung function impairment present (25,26). It is a reversible prognostic factor because if body weight recovers, prognosis improves and, interestingly, this occurs in the absence of any change in lung function (25). Therefore, unexplained weight loss identifies a new systemic domain of COPD not considered by the traditional measures of lung function.

Atrophy of skeletal muscle mass is the main cause of weight loss in COPD, while loss of fat mass contributes to a lesser extent (24). The cause(s) of skeletal muscle atrophy in COPD are unclear but, very likely, they are multiple. These are discussed below.

IV. SKELETAL MUSCLE DYSFUNCTION (SMD)

Over the past few years, there has been increasing realization that skeletal muscle is abnormal in COPD (27). Killian et al. (28) were probably the first to report that many patients with COPD stop exercise because of leg fatigue, not dyspnea (29). Several publications since then confirmed that an SMD is common in COPD, and that it is a relevant contributor to the clinical picture of the disease (27,30). In particular, an SMD in COPD has two obvious

consequences: (1) it contributes significantly to weight loss (24), a poor prognostic factor in these patients (25,26); and (2) it is a main cause of exercise limitation (27) having a profound impact on their quality of life (31,32). Thus, appropriate treatment of an SMD should be a priority in the clinical management of COPD (27). Currently, this is mostly based upon rehabilitation programes, nutritional support and, perhaps, oxygen therapy (18,30,33–37). However, more specific and effective therapies need to be developed. Yet, for this purpose, a better understanding of the mechanisms leading to an SMD in COPD is crucial (38,39) (Fig. 1).

Conceptually, it may be useful to consider that an SMD in COPD is probably characterized by two different, although possibly related, phenomena: (1) net loss of muscle mass (atrophy), an intrinsic muscular phenomenon; and (2) dysfunction or malfunction of the remaining muscle. In turn, muscle malfunction may be secondary to either intrinsic muscle alterations—mitochondrial abnormalities, loss of contractile proteins—or

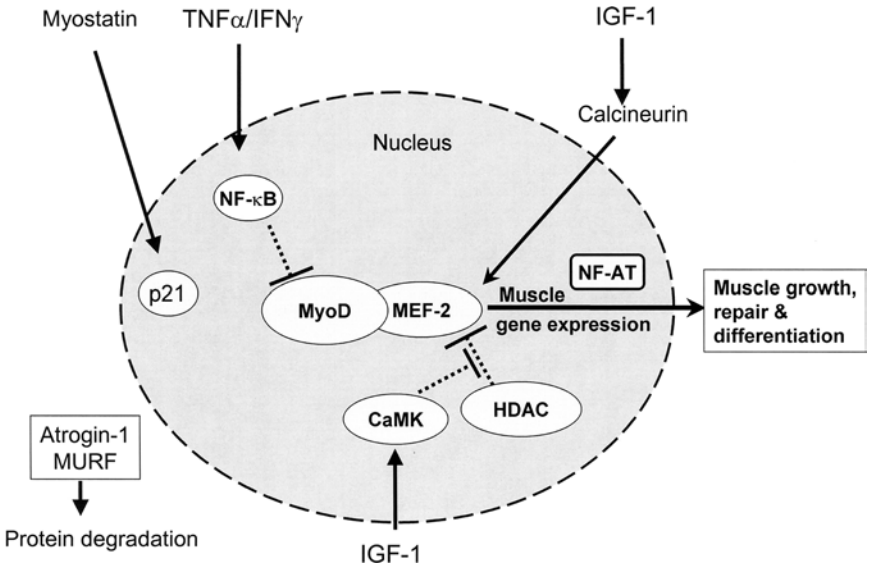

Figure 1 Signal transduction pathways potentially implicated in skeletal muscle mass loss in patients with COPD: (1) TNF-α and IFN-γ can activate death domains and NF-κB nuclear translocation leading to a marked inhibition of the activity of several skeletal muscle transcription factors, such as MyoD and MEF2 which are highly relevant for muscle growth, repair, and differentiation, whereas the cytokine myostatin can inhibit muscle regeneration via p21 expression; (2) skeletal muscle proteolysis is enhanced by the over-expression of ubiquitin-proteasome elements (atrogin-1, MURF); and (3) down-regulation of skeletal muscle growth factors as IGF-1 prevents skeletal muscle hypertrophy via inhibition of the activation of calcium-dependent kinases, NF-AT, and histone deacetylases.

Table 2 Potential Mechanisms of Skeletal Muscle Dysfunction in COPD

Sedentary lifestyle
Caloric imbalance
Tissue hypoxia
Systemic inflammation
Skeletal muscle apoptosis
Oxidative stress
Abnormal NO regulation
Tobacco
Individual susceptibility
Hormone alterations
Electrolyte alterations
Drugs

alterations in the external milieu under which the muscle works—hypoxia, hypercapnia, acidosis—resulting from the abnormalities of pulmonary gas exchange that characterizes COPD (40). Although conceptually important, the separation of these two aspects of an SMD is extremely difficult in vivo and, probably, both play some role in any given patient.

It is also important to consider that the mechanisms of an SMD in COPD are likely to be multiple (Table 2) and, importantly, that they may not be exclusive of COPD. In fact, they may play also an important role also in other chronic diseases, such as cardiac and renal failure, cancer, and AIDS (30). For instance, it is interesting to note that patients with chronic heart failure also lose skeletal muscle mass during the course of their disease (30) and that their skeletal muscle presents similar histopathological abnormalities to those reported in COPD (30), including increased apoptosis (41). Thus, it is likely that an SMD represents a final pathway common to several chronic diseases.

With these considerations in mind, the mechanisms most likely contributing to an SMD in COPD are the following (Table 2).

A. Sedentary Lifestyle

The COPD patients often adopt a sedentary lifestyle due to their shortness of breath during exercise. Physical inactivity causes net loss of muscle mass, reduces the force generation capacity of the muscle, and decreases its resistance to fatigue (42). The fact that exercise training improves muscle function in COPD patients (43–46), suggests that a sedentary lifestyle is likely to be an important contributor to an SMD. Yet, other observations indicate that other factors are also likely to play a role. For instance, the increased activity of cytochrome oxidase observed in the skeletal muscle of patients with COPD cannot be explained by sedentary lifestyle, which is

characterized by decreased—not increased—cytochrome oxidase activity (47). Likewise, exercise in patients with COPD enhances the release of amino acids from skeletal muscle, particularly alanine and glutamine (48). This is at variance with the normal training response and suggests the presence of intrinsic muscle abnormalities of the intermediate amino acid metabolism (49).

B. Caloric Imbalance

When metabolic demands are not met by caloric intake, weight loss ensues (50). Caloric intake appears to be normal (not decreased) in most patients with COPD except, perhaps, during exacerbations of the disease (50). In contrast, most patients with COPD show an increased basal metabolic rate, which can, therefore, contribute to weight loss (50). Its cause(s) are also unclear but can include—among others—(a) an increased oxygen consumption of the respiratory muscles due to the increased work of breathing that characterizes the disease (51); (b) drugs commonly used in the treatment of COPD (e.g., $\beta 2$ agonists) (52); (c) systemic inflammation, as shown by the relationship between metabolic derangement and increased levels of inflammatory mediators in COPD (20); and (d) tissue hypoxia since, in our laboratory, we have found a direct relationship between the activity of cytochrome oxidase in skeletal muscle—the mitochondrial enzyme that consumes oxygen—and the degree of arterial hypoxemia present in COPD (47).

C. Tissue Hypoxia

Several observations support a potential pathogenic role for tissue hypoxia in the development of an SMD in COPD: (a) chronic hypoxia suppresses protein synthesis in muscle cells, causes net loss of amino acids, and reduces the expression of myosin heavy chain isoforms (53,54); (b) healthy subjects at high altitude (hypobaric hypoxia) lose muscle mass (55,56); (c) skeletal muscle from patients with COPD and chronic respiratory failure present structural—decrease of type I fibers (57,58)—and functional alterations—up-regulation of mitochondrial cytochrome oxidase (47)—proportional to the severity of arterial hypoxemia; and, finally, (d) as discussed above, we observed a direct relationship between the activity of cytochrome oxidase in skeletal muscle and the level of arterial hypoxemia present in COPD (47).

D. Systemic Inflammation

Systemic inflammation is likely to be an important pathogenic mechanism of an SMD in COPD. In this context, TNFα is probably of particular relevance because it can affect muscle cells in several ways and there is evidence supporting that TNFα plasma levels as well as the concentration of its soluble receptors (17,18,20,59,60) are increased in patients with COPD and that circulating monocytes harvested from patients with COPD produce higher amounts of

this cytokine than healthy controls (16); (c) several authors have now shown increased plasma; and, finally, (d) TNFα can affect muscle cells in several ways (61). In differentiated myocites studied in vitro, TNFα induces proteolysis through the ubiquitin-proteasome complex (U/P) by an NF-κB-dependent mechanism (61). In fact, NF-κB plays an important role in activating specific transcriptional mechanisms of atrophy (62). Several studies have now shown that a disregulation of the U/P system contributes to the loss of muscular mass caused by sepsis or tumors in rats (63). In this context, recently a number of genes, whose expression is characteristic of the muscle wasting process, have been identified, including ubiquitin ligases as atrogin-1 and MURF (64) and myostatin (65). Whether this occurs also in COPD patients has not yet been investigated. Alternatively, TNFα can induce the expression of a variety of genes, such as the inducible form of the nitric oxide synthase (iNOS), the TNFα gene itself or those of many other proinflammatory cytokines, that would create a closed loop and contribute to the persistence and amplification of the inflammatory cascade (61). Finally, TNFα can induce apoptosis in several cell systems (66). We have recently detected the presence of apoptosis markers as positivity for nuclear transferase-mediated dUTP nick end labeling (TUNEL) staining and the presence of poly-(ADP-ribose) -polymerase (PARP) proteolytic fragments in skeletal muscle of patients with COPD and low body weight indicating that apoptosis may play a role in the process of unintentional weight loss in this disease (67). Increased levels of circulating TNFα and increased apoptosis of skeletal muscle cells have also been described recently in patients with chronic heart failure (68,69), suggesting that this mechanism may be operating in other chronic diseases and may not be unique to COPD (30). Given that cytochrome c release from the mitochondria is an early event in apoptosis (70,71) and that the activity of cytochrome oxidase is increased in these patients (47), it is possible that mitochondrial abnormalities can play a mechanistic role in this context. This will have to be examined carefully in the future, because a better understanding of the molecular pathways controlling this phenomenon may lead to the development of new therapeutic alternatives for these patients (72).

E. Oxidative Stress

As discussed above, patients with COPD present oxidative stress in their systemic circulation, particularly during exacerbations of the disease (7) that could also be relevant for the pathogenesis of an SMD (38). Oxidative stress causes muscle fatigue (73) and facilitates proteolysis (63,74). This would be particularly relevant since the regulation of glutathione (GSH), the most important intracellular antioxidant (38), is abnormal in skeletal muscle of patients with COPD (75). Finally, oxidative stress is an important contributor to the normal process of aging characterized by, among other things, loss of muscle mass (76,77). Whether or not a premature and/or accelerated

aging process occurs in COPD patients with an SMD has not been explored before, but we are currently investigating this possibility (78).

F. Nitric Oxide

Nitric oxide (NO) is a free radical molecule synthesized from the amino acid L-arginine by the action of three NO synthases (NOS) (79), all of which are expressed in human muscle (80). Two NOS isoforms—the so-called type I, neuronal NOS (nNOS) or brain NOS (bNOS) and type III or endothelial NOS (eNOS)—are expressed constitutively, while the third isoform—type II or inducible NOS (iNOS)—is expressed in response to a variety of stimuli, including cytokines, oxidants, and/or hypoxia (80). The role of NO in the pathogenesis of an SMD in COPD is unclear. Yet, systemic inflammation can upregulate the expression of iNOS in skeletal muscle (81). Preliminary results from our laboratory suggest that this actually occurs in COPD patients who loose weight (82). In turn, the increased NO production resulting from iNOS up-regulation can cause protein nitrotyrosination and facilitate protein degradation through the U/P system (63), and/or enhance skeletal muscle apoptosis (83). Results from our laboratory indicate that both do indeed occur in patients with COPD and low body weight (67,82). Finally, iNOS induction can also cause contractile failure (84), thus potentially limiting exercise tolerance in these patients.

G. Tobacco Smoke

Tobacco smoke is the main risk factor for COPD (85). Tobacco smoke reaches the systemic circulation, as shown by the increased prevalence of coronary artery disease and endothelial dysfunction in smokers (21,22) and contains many substances potentially harmful for the skeletal muscle. For instance, nicotine alters the expression of important growth factors, such as TGF-β1, involved in the maintenance of the muscular mass (86) and competes with acetylcholine for its receptor at the neuromuscuolar junction, thus having the potential to affect directly muscle contraction (87). Therefore, it is possible that tobacco smoke by itself may also contribute to an SMD in COPD.

H. Genetic Susceptibility

Not all smokers develop COPD (85). Likewise, not all COPD patients lose muscle mass during the course of their disease (24). Although this may be related to the severity (24) or phenotype of the disease (88), a genetic component similar to that suggested explaining the development of COPD in only a fraction of smokers (89,90) cannot be excluded. The potential genes involved in this process are unknown. Some potential candidate genes include the angiotensin-converting enzyme (ACE) gene, those of several

transcription factors (MyoD, MEF-2), and those related to the process of histone acetylation–deacetylation (CBP/p300; HDAC-5). The ACE gene is known to influence the muscle response to training in athletes (91) or the development of right ventricular hypertrophy in patients with COPD (92). Further, a very recent report has shown that the use of ACE inhibitors can reduce the normal decline of muscle mass that occurs in aging and can improve exercise capacity (93), thus raising the possibility of using these drugs therapeutically in patients with COPD and weight loss. The transcription factors MyoD and MEF-2, as well as genes related to the histone acetylation–deacetylation process (CBP/p300; HDAC-5), have very recently been shown to play a fundamental role in the failure of muscle cells to regenerate after injury in patients with cancer cachexia (94,95). Whether they can play any role in the pathogenesis of an SMD in some patients with COPD has not been explored. The microarray technology may allow the investigation of differential gene expression in skeletal muscle of patients with COPD with and without weight loss (96).

I. Other Mechanisms

There are other potential mechanisms that, alone or in combination, could contribute to an SMD in patients with COPD. These include: (a) the abnormal regulation of several hormone pathways, such as low testosterone and growth hormone levels (97,98) and reduced plasma leptin concentration (99–101). These pathways are important in the control of muscle mass and body weight (48,49); (b) abnormal plasma electrolyte values, such as low concentrations of potassium, phosphorus, calcium, and magnesium, are not uncommon in patients with COPD and can also cause contractile dysfunction and muscle weakness (102–105); and (c) many of the drugs used in the treatment of COPD can interfere with skeletal muscle function. For instance, β_2-adrenergic drugs increase oxygen consumption (52), a condition that by itself can cause oxidative stress; treatment with oral corticoids can clearly cause skeletal muscle weakness in patients with COPD (106–109) and, more importantly, it also seems to jeopardize their prognosis (110).

V. OTHER POTENTIAL SYSTEMIC EFFECTS OF COPD

So far, systemic inflammation, weight loss, and skeletal muscle dysfunction are the most commonly considered systemic effects of COPD. However, it is possible that other organ systems might be also affected by the systemic influences of COPD.

A. Cardiovascular Effects

Cardiovascular disease is common in COPD. This is, most likely, because tobacco smoking is a common risk factor for both disease entities. Yet, in

the absence coronary artery disease, it is presently unclear whether left ventricular function is normal in stable patients with COPD (111). These patients show an abnormally low peak oxygen uptake and, correspondingly, a low peak cardiac output, which is equivalent to that of a healthy subject at that level of exercise (111). This observation suggests that either the regulation of cardiac output during exercise in lung disease may remain so tight that, despite the capacity for a higher cardiac output, it matches the level of exercise achieved (111) or, alternatively, that despite the absence of over heart failure left ventricular function may be compromised in COPD, and a higher cardiac output could not be achieved. Although this possibility is speculative, it may merit future studies because similar mechanisms to those described for the skeletal muscle may be operative for the myocardium (112–115).

Endothelium function is abnormal in the pulmonary vessels of patients with COPD (116). Some studies have shown that it is also abnormal in other systemic vascular territories, such as the renal circulation (117,118). Whether or not this abnormality may also occur in other systemic vascular territories is not known at present.

B. Nervous System Effects

Using nuclear magnetic resonance—spectroscopy—it has shown that the bioenergetic metabolism of the brain is altered in COPD patients (119). Whether this represents a physiological adaptation to chronic hypoxia, as it occurs at altitude (120), or it may be considered another systemic effect of COPD mediated by other unknown mechanisms is unclear.

Depression is highly prevalent in COPD (121–123). Although this may represent a physiological response to a chronic debilitating disease, it is equally plausible that it may have some biological basis. In this context, it is particularly interesting to note that TNFα and other cytokines and molecules such as NO have been implicated in the pathogenesis of depression in several experimental models (124–126) and that, as discussed above, patients with COPD present evidence of systemic inflammation. A better delineation of these questions may open new therapeutic possibilities in COPD.

Finally, the autonomic nervous system (ANS) may also be altered in patients with COPD, as suggested by Takabatake et al. (127) who showed indirect evidence of abnormal ANS control in patients with COPD, particularly those with low body weight. Interestingly, these alterations seem related to a deregulation of the normal circadian rhythm of leptin (127). Leptin has important effects on neuroendocrine function, apetite regulation, body weight control, and thermogenesis in humans (127) and, importantly, its plasma levels are reduced in COPD (99–101).

C. Osteoporosis

The prevalence of osteoporosis is increased in patients with COPD (128,129). This can be due to multiple causes, including malnutrition, sedentarism, smoking, steroid treatment, and systemic inflammation (129–131). Thus, excessive osteoporosis in relation to age can also be considered a systemic effect of COPD (88). In fact, it is interesting to note that emphysema and osteoporosis are both characterized by net loss of lung or bone tissue mass and, pictorially, an osteoporotic bone looks quite similar to an emphysematous lung. It is therefore tempting to speculate that, perhaps, both conditions share common mechanisms explaining the accelerated loss of tissue mass or its defective repair.

D. Anemia

Many chronic diseases are characterized by some degree of anemia (132). This has been seldom studied in COPD, but there is some evidence in the literature to suggest that mild anemia may not be uncommon in these patients (133). The pathogenesis of this phenomenon is unclear but it is likely to be related to the systemic inflammation present in COPD, as in other chronic conditions (132). A better understanding of anemia in COPD is potentially important because its therapeutic correction may have a significant clinical impact. After all, oxygen therapy is only one way to increase oxygen delivery to tissues. Increasing hemoglobin values (using erythropoietin therapy, for instance Ref. 132) is another strategy.

VI. CONCLUSIONS

Available evidence indicates that COPD is associated to important systemic effects. This chapter reviews the more commonly accepted systemic effects of COPD and identifies some potentially new ones. It also discusses the mechanisms underlying all of them. However, there are more questions than answers. Therefore, a better understanding of the pathogenesis of these systemic effects of COPD may allow the development of new therapeutic strategies that, eventually, can contribute to improve the health status and prognosis of the patients suffering this devastating disease.

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17

Mechanisms of Exacerbations of Chronic Obstructive Pulmonary Disease

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I. INTRODUCTION

There is an increasing interest in the causes and mechanisms of exacerbations of chronic obstructive pulmonary disease (COPD), as exacerbations are an important cause of the considerable morbidity and mortality that occur in COPD (1). Exacerbations are defined by a worsening symptom from the usual stable state, especially the symptoms of dyspnoea, increased sputum amount, and purulence. COPD exacerbations are associated with increased airway inflammation, with a particular increase in neutrophils.

Some patients are prone to frequent exacerbations that are an important cause of hospital admission and readmission, and these frequent exacerbations may have considerable impact on quality of life, activities of daily living, and disease progression (1,2). An understanding of the mechanisms of exacerbations is important as therapies need to be developed that can reduce exacerbation frequency. Exacerbations are also associated with a number of triggers e.g., respiratory viruses or bacteria and these are important factors in modulating the airway inflammation that occurs at exacerbation.

II. AIRWAY INFLAMMATION AT EXACERBATION

COPD exacerbations are associated with airway inflammation (3), though there has been relatively little information available on the nature of inflammatory markers especially when studied close to an exacerbation, as performing bronchial biopsies at exacerbation are difficult in patients with moderate-to-severe COPD. In stable COPD, there is an increase in the CD8+ lymphocytes and macrophages in the bronchial mucosa and an increase in neutrophils with more severe disease. In one study, where biopsies were performed at exacerbation in patients with chronic bronchitis, increased airway eosinophilia was found, though the patients studied had only mild COPD (4). In these patients at exacerbation, there were more modest increases observed in neutrophils, T-lymphocytes (CD3), and TNF α positive cells. However, in patients with more severe COPD, there are increases seen in airway neutrophils when stable that increase further at exacerbation. In a recent study, Qiu et al. (5) studied biopsies from patients, with severe COPD, who were treated with tracheal intubation and showed that there was considerable airway neutrophila and neutrophil elastase expression in the biopsies obtained. Neutrophil chemoattraction occurs through the mediation of a number of neutrophil selective chemokines and in this study Qiu et al. studied gene expression for a number of CXC cytokines in the airway mucosa. The authors found that at exacerbations CXCL8 (interleukin-8), CXCR2, and CXCL5 (epithelial-derived neutrophil attractant-78) were upregulated at exacerbation compared to stable COPD (Fig. 1). They also describe an association between the neutrophil number in the biopsies at exacerbation and chemokines especially CXCL5, which they found to be the dominant chemokine in the airway and CXCL8. Furthermore, they showed the importance of CXCR2 and that the increase in neutrophils at exacerbation was related to the increase in CXCR2 expression but not to CXCR1 expression. They showed that CXCL8 correlated with CXCR1 and CXCL5 was related to CXCR2 and these chemokines had important roles in neutrophil recruitment at exacerbations. However, there are some limitations to this study as the patients were intubated and the results may have been complicated by secondary infection.

Sputum induction allows study of these patients at exacerbation and it has been shown that it is a safe and well-tolerated technique in COPD patients (6). In the East London COPD study, there was a relation between exacerbation frequency and sputum cytokines, in that there was increased sputum interleukin (IL) -6 and IL-8 found in patients at baseline when stable with a history of frequent exacerbations compared to those with infrequent exacerbations (3) (Fig. 2). Exacerbations that were triggered by viral infections were associated with increased airway inflammatory markers compared to those when viruses were not detected at exacerbations. (3,7,8) Rhinovirus is commonly detected at exacerbation and rhinovirus

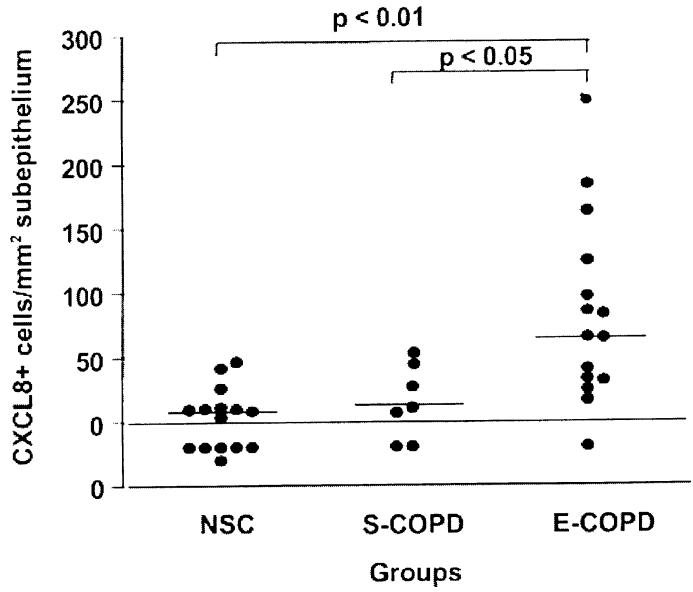

Figure 1 Counts of CXCL8 mRNA-positive cells in the subepithelium of biopsies taken from intubated nonsmoker surgical control subjects (NSC), patients with stable COPD (S-COPD), and those intubated for severe exacerbations of COPD (E-COPD). (From Ref. 5.)

has been shown to increase cytokine production in an epithelial cell line (9). Thus, repeated viral infection may lead to upregulation of cytokine airway expression and lead to the increased airway inflammatory load characteristic of patients with frequent exacerbations.

Increases in various inflammatory markers have been found at COPD exacerbation such as inflammatory cytokine eg IL-6, IL-8, endothelin-1, the neutrophil chemoattractant LTB4, and neutrophil elastase (3,10,11). At exacerbation, increases in induced sputum IL-6 were higher when exacerbations were associated with symptoms of the common cold (3). However, there was considerable variability in inflammatory markers with exacerbation, suggesting marked heterogeneity in the degree of the inflammatory response at exacerbation. In the study performed by Bhowmik et al. (3), there was no increase seen in the eosinophil count in induced sputum at exacerbation, even though the patients in that study were sampled early at exacerbation at the time of onset of symptoms. Compared to the study by Saetta et al. (4), where patients had mild COPD, the patients had more severe and irreversible airflow obstruction with an FEV_1 at 39% predicted. Also, the majority of the patients were taking inhaled steroids and this could have reduced any eosinophils that may have been present. It is likely that the

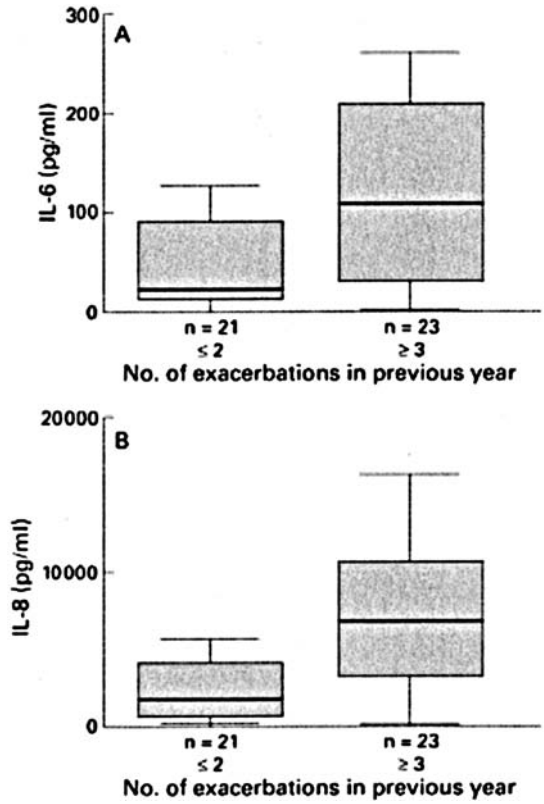

Figure 2 Induced sputum levels of (A) IL-6 and (B) IL-8 in patients categorized as frequent exacerbators and infrequent exacerbators. Data expressed as medians (IQR). (From Ref. 3.)

inflammatory response at exacerbation is different in nature in patients with moderate-to-severe COPD than in patients with milder disease.

The genes for a number of airway inflammatory markers are regulated by the transcription factor nuclear factor κB (NF-κB). Caramori et al. (12) studied the nuclear localization of p65 as a marker of NF-κB activation in induced sputum at COPD exacerbation. They found an absence of nuclear staining for p65 in sputum neutophils, both during exacerbations and baseline, but in contrast they found that the p65 staining increased significantly in sputum macrophages. Thus, NF-κB seems to be involved in activation of sputum macrophages during COPD exacerbations, but not in the activation of the increased neutrophils found in the sputum at exacerbations.

In addition to the increase in airway inflammatory markers at COPD exacerbation, there are also increases in oxidative stress at exacerbation. Various markers of oxidative stress have been shown to rise with exacerbation

such as hydrogen peroxide and 8-isoprostane. (13). In a recent study, Biernacki et al. (14) showed that at exacerbations, that were all associated with purulent sputum, 8-isoprostane levels in exhaled breath condensate increased markedly and then fell with antibiotic therapy at 2 weeks, though at this time levels were not back to baseline. At a further follow up at 2 months, there was a further fall in exhaled breath 8-isoprostane, suggesting that oxidative stress takes a significant time to recover after an exacerbation and this may be another important factor in the reason that some exacerbations take considerable time to recover or they may never recover to baseline levels (Fig. 3).

In patients with alphal-antitrypsin deficiency, there is some evidence for greater inflammatory load at the start of the exacerbation with higher elastase activity and lower levels of sputum alphal-antitrypsin and secretory leukoprotease inhibitor, than in nondeficient COPD patients (15). Biochemical markers were reduced by 3 days after antibiotic therapy and took a variable time to return to baseline. However, there was no difference in the time

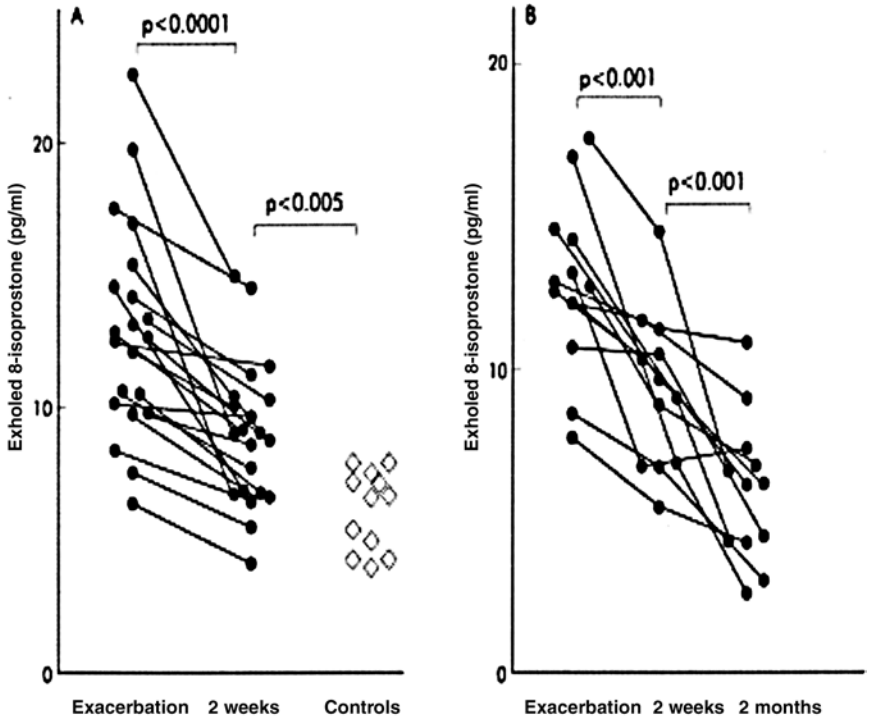

Figure 3 (A) Exhaled 8-isprostane during a COPD exacerbation and after 2 weeks, compared to controls. (B) Exhaled 8-isprostane in 12 patients at COPD exacerbation, after 2 weeks and after 2 months. (From Ref. 14.)

course of the reduction in inflammatory markers in the two groups, though there was considerable variation in levels observed. Although there is a suggestion that exacerbations in alphal-antitrypsin patients may be more severe, peak flow changes with therapy did not differ and this suggests that response to therapy is similar in the two patient groups. Whether the increased inflammatory response at exacerbation in alphal-antitrypsin deficiency plays a part in the accelerated decline in FEV1 requires further studies with larger patient numbers.

III. VIRAL INFECTIONS

COPD exacerbations are frequently triggered by upper respiratory tract infections and these are commoner in the winter months with colder temperatures (16), when there are more respiratory viral infections prevalent in the community. Patients may also be more prone to exacerbations in the winter months as lung function in COPD patients shows small but significant falls with reduction in outdoor temperature during winter (16).

Further evidence that respiratory viral infections are important triggers of exacerbations comes from the association of colds with exacerbations. In a prospective analysis of 504 exacerbations, where daily monitoring was performed, larger falls in peak flow were associated with symptoms of dyspnoea, presence of colds, and related to longer recovery time from exacerbations (17). We have reported that up to 64% of exacerbations were associated with symptomatic colds as assessed using daily diary card monitoring and thus it is likely that these exacerbations were precipitated by viruses (8). The commonest virus associated with COPD exacerbations are rhinoviruses and other viruses include coronavirus, RSV (respiratory syncytial virus), influenza, parainfluenza, and adenovirus.

Rhinovirus is the virus that is responsible for the common cold and is currently the most important cause of COPD exacerbation. Since the introduction of influenza immunization for patients with chronic lung disease, influenza has become a less prominent cause of exacerbation, though this is still likely to be an important factor at times of influenza epidemics (18). Together with enteroviruses, rhinoviruses belong to the picornavirus group of RNA viruses. Rhinoviruses are spread directly from one person to another by infected respiratory secretions. Although rhinovirus has been recognized as an important cause of asthmatic exacerbations (19,20), till recently rhinovirus has not been considered to be of much significance during exacerbations of COPD, as the techniques for detection used only isolation by cell culture and serology. This virus has fastidious growth requirements and over 100 serotypes making detection by culture or serological methods very difficult.

Early studies using serological and cell culture diagnostic methods reported relatively small effects of rhinovirus at COPD exacerbations

(21,22). Greenberg et al. (23) studied viral etiologies of COPD exacerbations and found using viral culture and serology that 27% of COPD exacerbations were associated with respiratory viruses, while in 44% of acute respiratory illnesses in control subjects were associated with viruses. In the COPD patients, rhinoviruses accounted for 43% of the virus infections and thus responsible for about 12% of exacerbations. The advent of PCR techniques for viral detection enabled a more detailed evaluation of the role of viruses at asthmatic and COPD exacerbations. Studies in childhood asthma have shown that rhinovirus can be detected by polymerase chain reaction from a large number of these exacerbations (20).

Seemungal et al. (8) performed a study taking samples of nasophrayngeal aspirates or throat swabs at exacerbation and also when patients were stable. Up to 40% of COPD exacerbations were associated with viral infections, though this may be an underestimate due to difficulties in sampling at the very onset of an exacerbation. Rhinovirus was the commonest respiratory virus detected and found in 58% of viral exacerbations. The other viruses detected included coronavirus (11% of virus exacerbations), influenza A and B (8), Parainfluenza, Adenovirus, and *Chlamydia pneumoniae* were each detected in one exacerbation. The relatively low levels of influenza were related to the fact that 74% of the patients had received influenza immunization. In another study of viruses at COPD exacerbations in patients admitted to hospital, respiratory viruses were detected by PCR in 56% of exacerbations and again rhinovirus was the commonest virus to be detected (24). Seemungal et al. (8) also showed that respiratory viruses were associated with a longer median symptom recovery time at exacerbation compared to the recovery time for nonviral exacerbations. (Fig. 4). Thus, viruses are associated with more severe exacerbations and therefore with greater morbidity. Measures to prevent viral infection may lead to a reduction in exacerbation frequency, exacerbations severity, and reduction in hospital admission and thus have important health economic consequences.

Using the median number of exacerbations as a cut-off point, we have previously classified COPD patients as frequent and infrequent exacerbators (1). Quality of life was significantly worse in the frequent, compared to the infrequent exacerbators. Factors predictive of frequent exacerbations included the exacerbation frequency in the previous year. This suggests that exacerbation frequency is an important determinant of health status in COPD and is thus one of the important outcome measure in COPD. In our study of Seemungal et al. (8) of respiratory virus detection by PCR, at least one virus was detected in 64% of patients and these patients had a higher exacerbation frequency than patients where viruses were not detected. Thus, patients with a history of frequent exacerbations may be more susceptible to respiratory viral infections and further work is required to study the nature of this susceptibility.

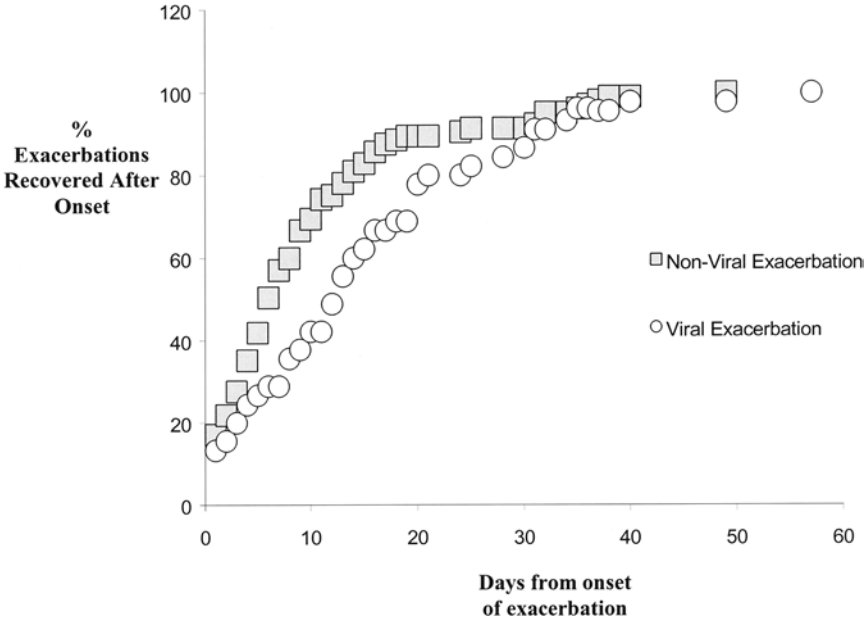

Figure 4 Graph showing the cumulative percentage of viral and nonviral exacerbations recovering symptomatically with respect to time after onset during 150 COPD exacerbations ($p = 0.006$). (From Ref. 8.)

We also found that 19 exacerbations were associated with RSV, though more patients had RSV detected in the stable state than at exacerbation (8). In none of these samples was the RSV detected by culture or serology and the detection disappeared when the sensitivity of the PCR was reduced suggesting that the colonization with virus was low grade. However, we found that patients in whom RSV was detected were more likely to have elevated systemic inflammatory markers. This implies that RSV may be a cause of chronic infection in COPD and further evaluation of the role of RSV at COPD exacerbation is required. Viruses apart from RSV were detected in 16.2% of patients with stable COPD by PCR and rhinoviruses were the most common virus detected in the stable state as well as exacerbation. Recent work by Retmales et al. (25) shows that latent expression of adenoviral E1 A protein in alveolar epithelial cells may amplify the effects of cigarette smoke-induced lung inflammation. Thus, chronic viral infection may be linked to disease severity in COPD and further work is required on the relation between viruses detected in the stable state and at exacerbation.

There is now an increasing evidence from experimental rhinovirus infections that respiratory viruses can infect the lower airway. (26,27). Seemungal

et al. (7) showed that the rhinovirus can be recovered from induced sputum more frequently using PCR techniques than from nasal aspirates at exacerbation, suggesting that wild type rhinovirus can infect the lower airway and contribute to inflammatory changes at exacerbation. They also found that exacerbations associated with the presence of rhinovirus in induced sputum had larger increases in airway IL-6 levels, compared to exacerbations where rhinovirus was not detected. This suggests that viruses increase the severity of lower airway inflammation at exacerbation. This finding is in agreement with the data that respiratory viruses produce longer and more severe exacerbations and have a major impact on health care utilization (3,8).

As respiratory viruses are associated with more severe exacerbations and increased airway inflammation, the mechanisms of virus-associated exacerbations require discussion. The major group of rhinovirus (accounting for 90% of total rhinovirus types) attach to airway epithelium through ICAM-1, inducing ICAM-1 expression. This then promotes inflammatory cell recruitment and activation as seen in the inflammatory response at exacerbations (28). The minor rhinovirus group uses members of the LDL-receptor family as cell surface receptors, though ICAM-1 surface expression may also be upregulated (17). There is some evidence for upregulation of ICAM-1 in the bronchial mucosa of patients with chronic bronchitis (29), and thus ICAM-1 is an important potential therapeutic target in COPD exacerbations associated with rhinoviruses. Experimental rhinovirus infection has been shown to increase sputum IL-6 in normal subjects and asthmatics (30–32). Lower airway IL-8 has been shown to increase with experimental rhinovirus infection in normal and asthmatic patients in some studies (31), but not in others (32).

Viral infections have been associated with increased oxidant stress that is increased at COPD exacerbation (33). Rhinovirus infection of human respiratory epithelial cells increases production of reactive oxygen species and stimulates the activation of NF-κB important in the regulation of the IL-8 gene (34). In patients with experimental rhinoviral infections, nasal IL-8 levels have been related to common cold symptoms (35). Viral infections can also induce the expression of stress-response genes e.g., hemeoxygenase-1 and genes encoding antioxidant enzymes e.g., glutathione peroxidase, MnSOD (36), and these responses may be important in potentiating the effects of the virally mediated inflammation at COPD exacerbation. We have also shown that exacerbations are associated with increased airway and systemic endothelin-l levels (10). Endothelin-1 is an important bronchoconstrictor peptide that has been found to be proinflammatory and mucogenic and has been also implicated in the pathogenesis of virally mediated inflammation (37). Sputum ET-1 levels increase at COPD exacerbation and these increases are related to sputum IL-6 levels. Further work with specific ET receptor antagonists may provide a new therapeutic option for virus-induced inflammation associated with COPD exacerbations.

Respiratory viral infections are also associated with a systemic inflammatory response. Plasma fibrinogen is an independent risk factor for cardiovascular disease (38) and we have shown that plasma fibrinogen is increased in COPD, suggesting that COPD patients with moderate-to-severe disease more susceptible to ischaemic events (39). At exacerbation, we found further increased levels of plasma fibrinogen and IL-6 that are produced by blood monocytes and stimulate the production of fibrinogen in the liver (40). We found that plasma fibrinogen levels were higher in the presence of colds and respiratory viral infections at COPD exacerbation (8,40). This suggests that respiratory viral infection may predispose to an increased risk from vascular disease. Epidemiological studies have suggested that infections especially those of the respiratory tract may be involved in the onset of myocardial infarction and stroke (41) and thus patients who are frequent exacerbators with their recurrent infections may be particularly susceptible to cardiovascular disease.

IV. ROLE OF BACTERIAL INFECTIONS

The precise role of bacteria at COPD exacerbation has been difficult to evaluate as airway bacterial colonization in the stable state has been found in approximately 30% of COPD patients. The commonest organism isolated is *Haemophilus influenzae*, but others isolated include *Streptococcus pneumoniae*, *Branhamella cattarhalis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa.* Bacterial colonization has been shown to be related to the degree of airflow obstruction, current cigarette smoking status, and also associated with an increased exacerbation frequency (42–45). Soler et al. (43) showed that the presence of potentially pathogenic organisms in bronchoalveolar lavage from COPD patients at bronchoscopy was associated with a greater degree of neutrophilia and higher TNFα levels. Hill et al. (46) in a larger study showed that the airway bacterial load was related to inflammatory markers. They also found that the bacterial species was related to the degree of inflammation, with *P. aeruginosa* colonization showing greater myeloperoxidase activity (an indirect measure of neutrophil activation).

Evidence for the involvement of bacteria at COPD exacerbation has come from studies of antibiotic therapy. Acute exacerbations of COPD often present with increased sputum purulence and volume and antibiotics have traditionally been used as first line therapy in such exacerbations. A study investigating the benefit of antibiotics in over 300 acute exacerbations demonstrated a greater treatment success rate in patients treated with antibiotics, especially if their initial presentation was with the symptoms of increased dyspnoea, sputum volume, and purulence (47). Patients with mild COPD obtained less benefit from antibiotic therapy. A meta-analysis of trials of antibiotic therapy in COPD identified only nine studies of

significant duration and concluded that antibiotic therapy offered a small but significant benefit in outcome in acute exacerbations (48).

Recent studies have re-examined the effects of bacteria at acute exacerbation with more detailed evaluation of the nature of the COPD exacerbation and bacterial culture. Stockley et al. (49) showed that COPD exacerbations associated with purulent sputum are more likely to produce positive bacterial cultures than exacerbations where the sputum production was mucoid. Sethi et al. (50) have shown that exacerbations associated with *H. Influenzae* and *B. catarrhalis* are associated with significantly higher levels of airway inflammatory markers and neutrophil elastase, compared to pathogen-negative exacerbations. Miravitlles et al. (51) also showed that the patients with a highest degree of functional impairment were more likely to have *P. aeruginosa* and *H. influenzae* isolated, though this group of patients also are more likely to have airway bacterial colonization. However, the degree of airway bacterial load may be a more important determinant of airway infection at exacerbation rather than the type of bacteria isolated, as different types of bacteria and different strains may be present in individual patients at exacerbation, especially those with more severe COPD. With antibiotic therapy, bacterial load and airway inflammation decrease and the rate of resolution of the airway inflammatory changes and thus exacerbation recovery has been related to the clearance of bacteria from the sputum (52).

Bandi et al. (53) have reported that different strains of *H. influenzae* were recovered from the upper and lower airway in patients with chronic bronchitis. At exacerbation, there was a low recovery of *H. influenzae* in the lower airway due to early administration of antibiotics but, in 87% of biopsies taken from acute exacerbations in intubated patients, *H. influenzae* could be detected intracellularly. Thus, during an exacerbation, there is intracellular invasion of *H. influenzae* and this will contribute to the increased airway inflammation associated with exacerbation. However, in view of the presence of airway bacterial colonization, detection of bacteria at exacerbation does not prove the bacterial etiology of the exacerbation and important virus–bacterial interactions may exist and require further study. Recently, Sethi et al. (54) have suggested that isolation of a new bacterial strain in COPD patients who were regularly sampled was associated with an increased risk of an exacerbation, though this also does not conclusively prove that bacteria are direct causes of exacerbations.

V. OTHER INFECTIVE AGENTS—*CHLAMYDIA PNEUMONIAE*

There has been some controversy about the role of *C. pneumoniae* at COPD exacerbation. Using IgM and IgG antibody titres, *C. Pneumoniae* has been identified as the etiologic factor in 5% of COPD exacerbations in outpatients (55). This is similar to the results obtained by Blasi et al. (56) who identified

C. Pneumoniae in 4% of COPD exacerbations, also using serological testing. Mogulkoc et al. (57) in a relatively small sample of patients with exacerbations detected high IgG titers to *C. pneumoniae* in 7 out of 49 exacerbations, suggesting that *C. pneumoniae* was associated with about 16% of COPD exacerbations. Karnak et al. (58) detected serological evidence of recent *C. pneumoniae* infection in 34% of COPD patients having acute exacerbations, though microbiological examination of the sputum found potentially pathogenic microorganisms in 60% of the COPD patients suggesting that *C. pneumoniae* was not the sole agent responsible for the exacerbation.

However, one of the problems with relating *C. pneumoniae* infection to exacerbation is that many COPD patients have had exposure to previous *C. pneumoniae* infection and thus chlamydial serology is not the best technique to evaluate the cause of COPD exacerbation. In addition, chronic infection with *C. pneumoniae* may be a feature of COPD. Using PCR techniques, Blasi et al. (59) have shown that patients with chronic colonization with *C. pneumoniae* showed faster disease progression. In addition, COPD patients who were positive by PCR for *C. pneumoniae* in their blood monocytes showed an increased exacerbation frequency compared to *C. pneumoniae* negative patients, suggesting an important role for *C. pneumoniae* in modulating exacerbation frequency. Seemungal et al. (60) in our group showed no relationship between chlamydial serology and exacerbation frequency or disease progression, thus the significance of persistent antibodies against *C. pneumoniae* is not known. Seemungal et al. (60) found no evidence of *C. pneumoniae* DNA in induced sputum in stable patients though did detect *C. pneumoniae* at exacerbation. There was no relationship between *C. pneumoniae* detection at excerbation and exacerbation frequency or disease severity. More importantly unlike the case of rhinovirus-triggered exacerbations, we found no relation between *C. pneumoniae* detection and inflammatory markers (60).

VI. CONCLUSIONS

The mechanisms of COPD exacerbation are complex and associated with an increase of airway inflammation that is predominantly neutrophilic and increased oxidative stress. Respiratory viruses, especially infections with rhinovirus, the cause of the common cold, are the most important triggers of exacerbations, and are also associated with more severe exacerbations. Although bacteria have been implicated in COPD exacerbations for many years and antibiotics are used for therapy, there is much less information available about their actual role at exacerbation. Further research is required on the nature of interactions between viruses and bacteria. Respiratory viral infections are an important target for therapy in COPD and prevention of viral infection will reduce exacerbation frequency. Recent

studies of the mechanisms of exacerbations have identified a number of therapeutic targets. Reduction of COPD exacerbation will have an important impact on the considerable morbidity and mortality associated with COPD.

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18

Mechanisms of Pulmonary Vascular Changes

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I. INTRODUCTION

Pulmonary circulation abnormalities are common in COPD (1). Presumably, pulmonary hypertension associated with COPD is the most frequent form of presentation of this disorder. The field of vascular biology has experienced a tremendous evolution in the last decades, since the seminal studies by Furchgott and Zawadzki (2) identified the key role of endothelium in the regulation of vascular homeostasis. Concepts gathered in the systemic circulation have contributed to better understand changes occurring in pulmonary vessels. This has promoted a switch in the notion of the pathogenesis of pulmonary hypertension from a vasoconstrictive phenomenon to a cell proliferative disorder. This conception has facilitated the development of new therapeutic strategies for some of the most severe forms of pulmonary hypertension (3).

Some of the concepts used to explain the pathogenesis of the most severe forms of pulmonary hypertension have been applied to COPD, yielding to new proposals for its pathobiology. In the present chapter, we will review the current knowledge on the mechanisms of pulmonary vascular changes associated with COPD.

II. PULMONARY VASCULAR REMODELING IN COPD

In addition to alterations in vascular tone, vessels can undergo profound fibrocellular changes that lead to an enlargement of the vessel wall; an active process broadly defined as vascular remodeling. In the pulmonary vascular bed, remodeling affects preferentially small and precapillary arteries and is the principal pathological feature of pulmonary hypertension. Morphological changes in pulmonary arteries have been identified at different degrees of COPD severity.

A. Mild-to-Moderate COPD

Morphological characteristics of pulmonary muscular arteries in patients with mild-to-moderate COPD have been assessed in lung specimens obtained by surgical resection of lung carcinoma. These specimens provide information on the early changes occurring in patients with a mild degree of lung function impairment, since the patients must tolerate the resection of a lung or lobe. These cases provide the opportunity to establish correlations between functional measurements performed before surgery and structural changes assessed in resected specimens.

Morphometric studies of pulmonary vessels in these patients have consistently shown an enlargement of the intimal layer, with reduction of the

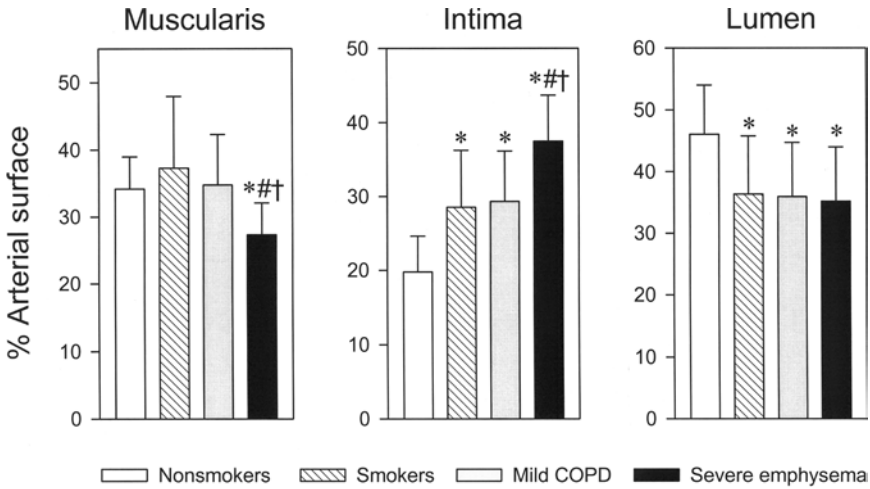

Figure 1 Morphometric measurements in pulmonary muscular arteries of nonsmokers (white bars), smokers with normal lung function (dashed), patients with mild COPD (gray) and patients with severe emphysema (black). Bars indicate mean values of the proportion of total arterial surface occupied by muscularis, intima, and lumen. $^{*}p < 0.05$ compared with nonsmokers; $^{\#}p < 0.05$ compared with smokers; $^{\dagger}p < 0.05$ compared with mild COPD.

lumen size, in pulmonary muscular arteries (4–8) (Fig. 1). This enlargement is apparent when comparing COPD patients with nonsmokers of similar age (6), thereby ruling out a potential effect of aging (9). Intimal enlargement occurs in arteries of different sizes, although it is more pronounced in small arteries with an external diameter less than 500 μm (4,5).

Muscularization of small arterioles, which in normal conditions lack a definite muscular layer, is another characteristic feature of pulmonary vessels in patients with mild-to-moderate COPD. Morphometric studies have shown an increased number of pulmonary muscular arteries with small diameters (< 200 μm) as compared with control subjects (10).

Changes in the media of pulmonary muscular arteries are less conspicuous and the majority of morphometric studies have failed to show differences in the thickness of the muscular layer when comparing patients with mild-to-moderate COPD with control subjects (4–7) (Fig. 1). Therefore, muscular hypertrophy is not a characteristic feature of pulmonary vascular remodeling in mild COPD.

The degree of pulmonary vascular changes in mild-to-moderate COPD correlates with the severity of emphysema and small airway abnormalities (4,7,8).

B. Severe COPD

In a classic post-mortem study conducted in patients with end-stage COPD and cor pulmonale, Wilkinson et al. (11) showed prominent changes in pulmonary muscular arteries consisting of an active deposition of longitudinal muscle, fibrosis, and elastosis of the intima. In the arterioles, they showed the development of a medial coat of circular smooth muscle, bounded by a new elastic lamina. There was also deposition of longitudinal muscle and fibrosis of the intima (11,12).

In another post-mortem study conducted in patients with COPD and pulmonary hypertension who died while enrolled in the National Institutes of Health Nocturnal Oxygen Therapy Trial (NOTT) study, Wright et al. (12) showed a significant increase of intimal thickness in pulmonary muscular arteries, compared with control subjects. The degree of intimal enlargement was unrelated to the severity of pulmonary hypertension. The thickness of the tunica media was slightly increased in patients with moderate-to-severe pulmonary hypertension. Interestingly, the degree of pulmonary hypertension was not related to changes in vascular structure since in patients with mild and severe pulmonary hypertension intimal and medial thickness were similar (12).

The study of lung tissue specimens obtained at lung volume reduction surgery (LVRS) has provided the opportunity to evaluate pulmonary vessels in patients with severe emphysema, who had functional measurements obtained before surgery, thus allowing to establish structure–function correlations in patients with advanced COPD. Yet, the morphologic assessment is restricted to tissue in the vicinity of areas of emphysematous destruction.

In a morphometric analysis of pulmonary muscular arteries in these specimens, Santos et al. (13) showed a significant enlargement of the intima, compared with patients with mild COPD and control nonsmokers. Conversely, medial thickness was slightly reduced in patients with severe emphysema, when compared with patients with mild disease (13) (Fig. 1).

Contrasting with these findings, Turato et al. (14) did not find differences in intimal and medial thickness between patients with mild-to-moderate COPD who underwent lung resection and patients with severe emphysema who underwent LVRS.

C. Smokers with Normal Lung Function

Structural changes in pulmonary arteries are not restricted to patients with COPD. Morphometric studies performed by the authors (6) and others (15) have shown that, compared with nonsmokers, pulmonary muscular arteries of heavy smokers who have normal lung function show intimal thickening, the magnitude of which does not differ significantly from that in patients with mild COPD (6) (Fig. 1). These observations are in agreement with a former post-mortem study conducted by Hale et al. (8) showing that cigarette smoking was associated with morphologic changes in pulmonary muscular arteries that evolve in parallel with small airway disease and emphysema.

D. Correlation with Lung Function and Pulmonary Hemodynamics

In patients with mild-to-moderate COPD, the severity of pulmonary vascular remodeling is weakly correlated with the degree of airflow obstruction and the magnitude of hypoxemia (4,6). Nevertheless, morphometric measurements of pulmonary muscular arteries in patients with mild airflow obstruction without hypoxemia may not differ from measurements in patients with severe airflow obstruction with hypoxemia (5,13). Furthermore, in patients with severe disease, no correlation has been shown between vascular changes and arterial blood gas measurements or the degree of pulmonary hypertension (11,12).

The authors have shown that in patients with mild-to-moderate COPD, the thickness of the intima was correlated with the impairment of ventilation–perfusion distributions, and that intimal thickening was associated with reduced efficiency of hypoxic vasoconstriction in maintaining adequate ventilation–perfusion matching (4).

III. CHANGES IN VASCULAR CELLS

A. Endothelial Cells

Endothelial cells play a pivotal role in regulating vascular homeostasis. Changes in endothelial cell phenotype and function are thought to be at

the origin of both systemic and pulmonary hypertensive disorders. In primary (or idiopathic) pulmonary hypertension (PPH), proliferation of endothelial cells is prominent and causes enlargement of the arterial wall and narrowing and obliteration of the vessel lumen (16). Lee et al. (17) evaluated the clonal nature of endothelial cells proliferating in the plexiform lesions of patients with pulmonary arterial hypertension by using the human androgen receptor gene assay. They showed that in plexiform lesions of PPH endothelial cell proliferation was essentially monoclonal, whereas in the associated forms of pulmonary arterial hypertension (congenital heart defects, scleroderma) it was polyclonal (17). These findings suggest that a somatic genetic alteration similar to that present in neoplastic disorders might be responsible for endothelial cell proliferation in PPH. By contrast, in ''secondary'' pulmonary hypertension, the polyclonal nature of endothelial cell proliferation seems to be the result of a reaction in response to a variety of stimulus (shear stress, inflammation).

Contrasting with severe forms of pulmonary arterial hypertension, endothelial cell proliferation is not a characteristic feature of pulmonary vascular remodeling in COPD. Santos et al. (18) characterized the cells present in hyperplasic intimas of pulmonary muscular arteries in COPD. By using a monoclonal antibody against Factor VIII, they showed that in COPD endothelial cells outline the innermost portion of pulmonary muscular arteries in a single cell layer (18). No proliferation of endothelial cells was detected within the intimal layer, thereby excluding endothelial cell proliferation as the cause of intimal enlargement, at variance with what occurs in PPH.

B. Endothelial Dysfunction

The absence of endothelial cell proliferation does not exclude alterations in endothelial function. Indeed, endothelial dysfunction in pulmonary arteries has been shown at different degrees of COPD severity (6,19). In pulmonary arteries of explanted lungs from patients with end-stage COPD who underwent lung transplantation, Dinh-Xuan et al. (19) showed a significant reduction of endothelium-dependent vasodilation induced by acetylcholine, when compared with control subjects. In a similar study conducted in pulmonary arteries from lung specimens of patients with mild-to-moderate COPD, the authors also showed reduction of endothelium-dependent vasorelaxation induced by both acetylcholine and ADP, when compared with nonsmokers (6). These studies denote that endothelial dysfunction of pulmonary arteries is a common feature of COPD and that it is present not only in advanced disease, when pulmonary hypertension is usually identified, but also in patients with mild disease, when pulmonary vascular involvement is not clinically apparent.

Endothelial function has also been assessed in pulmonary arteries of smokers with normal lung function (6). In these subjects, the magnitude of endothelium-dependent vascular relaxation is intermediate between that of patients with mild-to-moderate COPD and that of nonsmokers, suggesting that cigarette smoking per se might exert a detrimental effect on endothelial function of pulmonary arteries (6).

Impairment of endothelial function may be associated to or result from changes in the expression and release of vasoactive mediators. Endothelium-derived nitric oxide (NO) is a potent endogenous vasodilator with antiproliferative properties in the vessel wall. NO is synthesized from L-arginine by NO synthase, which is expressed constitutively in endothelial cells (eNOS or type III NOS). Giaid and Saleh (20) showed a significant reduction of eNOS expression in pulmonary arteries in severe forms of both primary and secondary pulmonary hypertension (including 5 patients with COPD out of 24), thereby suggesting that downregulation of NO might contribute to the development of pulmonary hypertension. Reduced expression of eNOS has also been shown in pulmonary arteries of smokers without or with only minimal airflow obstruction (21). More recent data demonstrate that eNOS expression in pulmonary arteries is reduced in patients with different degrees of COPD severity, being the patients with the greatest severity those with the most reduced expression of eNOS (22).

Prostacyclin is the principal arachidonic acid metabolite of endothelial cells, produced by the action of prostacyclin synthase (PGI_2-S). It is a powerful vasodilator and inhibits cell growth (23). Tuder et al. (24) demonstrated loss of expression of PGI_2-S in endothelial cells of pulmonary arteries of different size in patients with PPH and in associated forms of pulmonary arterial hypertension. At present, there is no information about the expression and activity of PGI_2-S in pulmonary arteries of COPD patients. However, in a hypoxic model of pulmonary hypertension in piglets, Fike et al. (25) showed reduced production of prostacyclin in pulmonary arteries.

Endothelin-1 (ET-1) is a potent vasoconstrictor released by endothelial cells that also exert a mitogenic effect on arterial smooth muscle cells. Part of the activity of ET-1 on smooth muscle cells is mediated via an increase in reactive oxygen species production (26). Since oxidative stress is increased in COPD, it is possible that ET-1 might play a role in the pathogenesis of pulmonary vascular changes in this disease. Giaid et al. (27) showed that the expression of ET-1 in pulmonary arteries was increased in both primary and secondary forms of pulmonary hypertension (including 2 patients with COPD out of 28). Studies conducted in smokers with normal lung function, patients with mild-to-moderate COPD, and patients with severe emphysema, have failed to show differences in the expression of ET-1 in pulmonary arteries when compared with nonsmokers (21,22). Although, this could be due to the fact that patients evaluated in these series did not have pulmonary hypertension. Furthermore, studies performed in pulmonary

hypertensive sheeps have demonstrated regional variability in the expression of proET-1 gene in pulmonary arteries (28). To what extent regional variations may explain the lack of expression of ET-1 in arteries evaluated in COPD patients is uncertain.

C. Smooth Muscle Cells

The majority of cells proliferating in hyperplasic intimas of pulmonary muscular arteries in patients with mild-to-moderate COPD are smooth muscle cells (SMCs), as shown by positive immunoreaction to α-smooth muscle actin (18) (Fig. 2). Interestingly, comparative analyses of serial sections showed that some of the SMCs in the intima did not express desmin filaments, whereas all cells expressed vimentin filaments (18) (Fig. 2). The pattern of expression of both intermediate filaments may discriminate between a synthetic phenotype of SMCs and the contractile phenotype observed in mature cells (29,30). Accordingly, vimentin-positive, desmin-negative SMCs represent a subpopulation of less differentiated SMCs that may possess synthetic capacity and take part in an ongoing process of vascular remodeling (31). These findings are consistent with previous observations in patients with advanced COPD showing muscle deposition in pulmonary muscular arteries and formation of a definite muscle layer in small arterioles (11,12). Newly formed smooth muscle bounds adopt a longitudinal disposition that differs from the circumferential disposition of normal smooth muscle.

The origin of SMCs proliferating in the intima of muscular arteries and in small arterioles, which in normal conditions lack of muscular cells, is uncertain. One possibility is that after endothelial injury, SMCs migrate

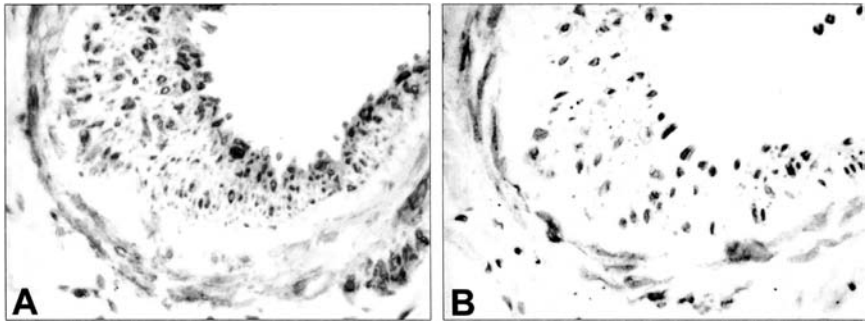

Figure 2 Photomicrographs of a pulmonary muscular artery from a patient with mild COPD with prominent intimal hyperplasia. Serial sections were immunostained for (A) α-smooth muscle actin and (B) desmin, a contractile filament. Note that a number of smooth muscle cells proliferating in the intima did not express desmin filaments, indicating a less differentiated phenotype. (Original magnification ×400.)

from the media through the internal elastic lamina, dedifferentiate, and proliferate into the intima. Yet, very few studies have documented SMCs migrating across the internal elastic lamina, from the media into the intima (32). A second possibility is that intimal SMCs may arise from a preexisting precursor cell that after specific stimulus proliferates and differentiates into SMCs. Studies conducted in an hypoxic rat model have shown that SMCs developing in small arteries originate from pericytes, a cell type found in the walls of nonmuscularized arterioles, and from intermediate cells in partially muscularized arteries (33,34). A third possibility is that SMCs might originate from an external source. Recent studies have shown that bone marrow cells have the potential to give rise to vascular progenitor cells that have the capacity to mobilize, home to sites of vascular injury and differentiate into endothelial or SMCs, contributing to vascular repair, remodeling, and lesion formation (35). Experimental studies, combining bone marrow transplantation and models of vascular injury, have demonstrated that recipients' bone marrow cells may contribute to SMC proliferation and neointimal formation. This mechanism has been shown in graft-associated vasculopaty, arterial remodeling after mechanical injury, and atherosclerotic plaque formation (36). In a recent study conducted in a bovine model of hypoxia-induced pulmonary hypertension, Davie et al. (37) showed that cells expressing the transmembrane tyrosine kinase receptor for stem cell factor, c-kit, are mobilized from the bone marrow in response to hypoxia, and that cells expressing c-kit are present in the remodeled vessel walls. To what extent this mechanism might contribute to pulmonary vascular remodeling in COPD has not been determined yet.

D. Potassium Channels and Smooth Muscle Cell Function

Membrane potassium (K^+) channels in vascular SMCs play an essential role in the regulation of membrane potential and, therefore, vascular tone (38). Closure of K^+ channels reduces K^+ efflux and causes membrane depolarization, which opens voltage-gated Ca^{2+} channels, leading to an increase in intracellular Ca^{2+} concentration and vasoconstriction. Using patch-clamp electrophysiology, three families of K^+ channels have been identified in vascular SMCs: delayed-rectifier K^+ channels (K_{DR}); large conductance, calcium activated K^+ channels (K_{Ca}); and ATP-sensitive K^+ channels (K_{ATP}). Potassium channels are important in pulmonary circulation because they have been closely related with hypoxic vasoconstriction, since hypoxia can cause both an inhibition of whole cell K^+ current and membrane depolarization in isolated pulmonary artery smooth muscle cells (PASMCs) (39). In addition to these pathways, NO has been shown to act directly on some K^+ channels (40).

The mechanisms by which changes in oxygen are sensed and K^+ channel activity is modified have not been identified. Some studies have suggested that changes in the redox status of PASMCs cytoplasm to a more reduced

state might induce vasoconstriction in response to hypoxia, since the activity or gating of K^+ channels can be redox modulated (41–43). Interestingly, hypoxia increases the ratios of reduced to oxidized forms of the cytosolic redox pairs, such as NADH/NAD, NADPH/NADP, and/or glutathione (GSH/GSSG) (41,44), thus shifting the cells to a more reduced state.

Hypoxia may target two types of PASMC K^+ channels leading to hypoxic vasoconstriction, K_{Ca} (45) and one or more members of the delayed rectifier type (Kv) (46). Vascular SMCs show different responses to hypoxia according to the size and type of vessel from which they are isolated. Small resistance pulmonary arteries constrict strongly in response to hypoxia, whereas large conduit pulmonary arteries exhibit lower contractile responses. One proposed mechanism for this differential response is K^+ channel diversity. Frid et al. (47) found at least three populations of PASMCs in the media of calf conduit pulmonary arteries, based on the distribution of contractile and cytoskeletal proteins. They also showed that exposure of calves to hypoxia caused proliferation in only one of the populations (47). Electrophysiological studies in rat (48,49) and rabbit (50) pulmonary vasculatures also demonstrate K^+ channel current heterogeneity between cells isolated from conduit arteries and those isolated from resistance vessels. Therefore, regional heterogeneity within the pulmonary circulation in response to hypoxia could result from the differential expression of K^+ channel subtypes between conduit and resistance vessels. There are no specific studies conducted in COPD patients investigating whether the variability in response to hypoxia could be due to changes in K^+ channel distribution. However, considering that intimal enlargement of resistance pulmonary arteries in COPD is produced by a phenotypically heterogeneous proliferation of SMCs (18), it is reasonable to speculate that the phenotype of proliferating SMCs might condition the vascular response to hypoxia as a result of changes in K^+ channel distribution.

In human PASMCs, chronic hypoxia does not affect baseline activity of K_{DR} channels, whereas it can cause a decrease in K_{Ca} activity (51). Such hypoxia-induced decrease in K_{Ca} activity could be due to a decrease in K_{Ca} sensitivity to membrane potential and to cytosolic calcium concentration. By contrast, in rat small pulmonary artery SMCs, chronic hypoxia decreases the activity of both K_{DR} and ATP-dependent K^+ currents (52). Perturbation of K_{DR} channels might be important in the pathogenesis of pulmonary hypertension since it has been demonstrated that Kv1.5 expression is selectively reduced in primary pulmonary hypertension (53).

E. Serotonin Transporter

Investigations on serotonin, 5-hydroxytryptamine (5-HT), and its transporter (5-HTT) are of special interest because they could play a key role in the pathogenesis of PASMC proliferation and because a 5-HTT polymorphism

confers susceptibility to hypoxic pulmonary hypertension. 5-HT may promote the development of hypoxic pulmonary hypertension by stimulating SMC growth (54). In fact, exposure of PASMCs to hypoxia results in a rapid increase in 5-HTT expression and activity, together with a marked enhancement in the growth-promoting effect of 5-HT (55,56). Increased 5-HTT gene expression also occurs in remodeled pulmonary arteries from animals developing pulmonary hypertension related to chronic hypoxia exposure (55).

Several observations support the development of pulmonary hypertension through 5-HT. The mitogen effect of 5-HT on SMCs is dose-dependently inhibited by selective inhibitors of 5-HT transport such as paroxetine and fluoxetine (55–57), but not by the 5-HT2A receptor antagonist ketanserin. In mice lacking, the 5-HTT gene and exposed to hypoxia for 2 weeks, wall thickness and the number of muscularized pulmonary arteries were decreased as compared with wild-type controls (58). These animals also developed less severe hypoxic pulmonary hypertension than wild-type controls.

The mechanism by which 5-HT exerts its mitogenic effect after being transported inside SMCs remains speculative. The mitogenic action of 5-HT is initiated through its binding to a cell surface receptor and transport into the cell, notably the 5-HT2A type (56,59) where tyrosine phosphorylation of a GTPase-activating protein appears as a downstream intermediate in the signaling pathway. The involvement of superoxide anions formation in association with 5-HT transport could also to play a role in the mitogenic effects of 5-HT (60).

IV. INFLAMMATORY CELLS

COPD is defined as an inflammatory disease of the lung in response to inhaled toxic agents, usually cigarette smoking (61). Accordingly, it is conceivable that inflammatory cells might contribute to the alterations of pulmonary vessels. Indeed, the extent of pulmonary vascular remodeling correlates with the severity of the inflammatory cell infiltrate in small airways (4,10). The authors have shown that patients with COPD have an increased number of inflammatory cells infiltrating the walls of pulmonary vessels (62). The number of inflammatory cells in the adventitia of pulmonary muscular arteries of patient with mild-to-moderate COPD was compared with that in nonsmokers and smokers with normal lung function. There was an increased number of leukocytes in pulmonary arteries of the COPD group as compared with the other two groups (62). This inflammatory infiltrate was largely constituted by activated T lymphocytes with a predominance of the $CD8^+$ T cell subset (62). The number of neutrophils, macrophages, and B-lymphocytes was minimal in the different groups and did not differ among them. The finding of increased number of $CD8^+$ T-lymphocytes in pulmonary arteries of COPD patients was subsequently corroborated by Saetta et al. (15) and is consistent with a

number of studies showing increased number of $CD8^+$ T-lymphocytes in large and small airways, as well as in alveolar septa, in patients with different degrees of COPD severity (63,64).

It is of note that inflammatory cells in pulmonary arteries are confined to the adventitia and that they are rarely identified either in the muscularis or in the intima (62). This suggests that it is unlikely that these cells might originate from circulating blood in pulmonary arteries since both internal and external elastic laminas may represent an anatomic barrier to their migration from the arterial lumen to the adventitia. Presumably, adventitial inflammatory cells might originate from nutritional vessels (vasa vasorum) derived from the bronchial circulation. Indeed, in a bovine model of hypoxia-induced pulmonary hypertension, it has been shown that circulating blood precursor cells migrate to the pulmonary vessel wall through expanded adventitial vasa vasorum (37).

In patients with mild-to-moderate COPD, the intensity of the inflammatory cell infiltrate in pulmonary arteries correlates with the degree of airflow obstruction, suggesting that as the disease progresses the inflammatory reaction in pulmonary arteries may become more severe (62). Nevertheless, in a recent study conducted in specimens obtained at lung volume reduction surgery, no differences in the number of inflammatory cells in pulmonary arteries were shown between patients with severe emphysema and patients with mild COPD (14). This finding is at variance with what occurs in lung parenchyma (65) and small airways (14), where increased COPD severity is associated with greater inflammatory infiltrate. The intensity of the inflammatory infiltrate shown in the wall of pulmonary arteries is directly related to the thickness of the intimal layer and inversely related to the endothelial function (62), thus suggesting a potential role of inflammatory mediators in the pathogenesis of the structural and functional changes that occur in the pulmonary circulation of COPD.

Compared with nonsmokers, smokers with normal lung function exhibit an increased number of $CD8^+$ T-cells in the arterial adventitia, with a reduction of the $CD4^+/CD8^+$ ratio that does not differ from patients with COPD (62). This finding is in keeping with the observation that smokers with and without airflow obstruction have reduced $CD4^+/CD8^+$ ratio in peripheral blood (66) and bronchoalveolar lavage samples (67). Overall, this suggests that cigarette smoking might induce inflammatory changes in pulmonary arteries at stages in which there are no detectable alterations in the lung function examination.

V. GROWTH FACTORS

Growth factors are intercellular signaling molecules that regulate cell proliferation by autocrine and paracrine mechanisms. Their role in the

pathobiology of pulmonary hypertensive disorders is currently under extensive investigation.

A. Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is a glycoprotein with important actions on cell function, which is abundantly expressed in the lung. Immunohistochemistry of lung tissue sections has revealed VEGF protein in endothelial cells, vascular SMCs, macrophages, and airway epithelial cells (13). This molecule exerts a critical role in vascular homeostasis, since it stimulates endothelial cell growth and proliferation, and promotes angiogenesis under physiologic and pathologic conditions. In addition, VEGF is critical for endothelial cell survival by inhibiting apoptosis (68), stimulates vascular SMC proliferation (69), exerts a vasodilator effect, and increases vascular permeability (70). Indeed, VEGF was previously known as vascular permeability factor (VPF).

VEGF has been involved in pulmonary vascular remodeling in primary pulmonary hypertension, which is characterized by endothelial and SMC proliferation (71). Santos et al. (13) evaluated the expression of VEGF in COPD. Patients with mild-to-moderate COPD showed increased expression of VEGF in pulmonary muscular arteries, as compared with control nonsmokers. The immunohistochemical analysis showed strong VEGF expression in endothelial cells and also in SMCs proliferating in hyperplasic intimas. The number of arteries showing positive immunoreaction to VEGF in the arterial wall was greater, and the intensity of the immunoreaction stronger, in patients with mild-to-moderate COPD than in control nonsmokers (13). In that study, it was also shown an enhanced expression of VEGF in pulmonary muscular arteries of smokers with normal lung function, suggesting that upregulation of VEGF could be more likely related to pulmonary vascular changes associated with cigarette smoking rather than to COPD per se. Indeed, in patients with advanced COPD and severe emphysema, the expression of VEGF in pulmonary arteries was lower than in patients with mild-to-moderate disease and did not differ from control nonsmokers (13). This could be associated with a generalized downregulation of VEGF in patients with emphysema that might lead to endothelial cell apoptosis, thereby contributing to the pathogenesis of emphysematous lung destruction, as suggested by Kasahara et al. (72). In the study by Santos et al. (13), the expression of VEGF in SMCs correlated with the thickness of the arterial wall, suggesting a potential role of VEGF in the pathogenesis of pulmonary vascular remodeling. Indeed, VEGF has been suggested to exert a pathogenic role in primary pulmonary hypertension due to its known effects inducing endothelial cell proliferation (73). In COPD, the role of VEGF is less apparent since endothelial cells conform a monolayer, as in normal conditions, whereas cells proliferating in pulmonary arteries are

SMCs. Nevertheless, it has been recently appreciated that VEGF also exerts an effect on SMCs. In vitro studies indicate that VEGF may induce and modulate vascular SMC proliferation and migration through the upregulation of matrix metalloproteinases (69,74), and that VEGF receptors 1 and 2 are expressed in vascular SMCs (74,75). VEGF has been recently implicated in SMC proliferation in systemic arteries. Experimental studies in animal models show that VEGF may promote SMC proliferation and neointimal formation after vascular injury (76), and that recombinant VEGF enhances the progression of the atherosclerotic plaque (77,78). In humans, VEGF has been related with the progression of coronary atherosclerosis (79). Accordingly, it is conceivable that VEGF might contribute to the structural remodeling of pulmonary arteries in early stages of COPD, presumably by enhancing the proliferation and intimal migration of SMCs.

Part of the biological actions of VEGF is related to NO-dependent mechanisms (80). Inhibition of NO synthesis with L-NAME (*N*-nitro-L-arginine methyl ester) upregulates the gene expression of VEGF and its receptors (81). Further, long-term administration of L-NAME induces coronary vascular arteriosclerosis (82) and significant increase of VEGF gene expression (83). Furthermore, VEGF receptor blockade is associated with reduced proliferative changes in coronary arteries (83). Overall, these findings suggest that endothelial dysfunction and decreased NO synthesis, as shown in patients with mil-to-moderate COPD (6,21), might promote VEGF activity.

B. Transforming Growth Factor

The transforming growth factor-beta (TGF-β) family is a multifunctional group of cytokines that play a central role in cell wound healing and tissue repair. Among other properties, TGF-β proteins modulate cellular proliferation and induce differentiation and synthesis of extracellular matrix protein. At present, the TGF-β family consists of more than 25 isoforms that, based on sequence analysis of bioactive domains, can be grouped into at least eight subfamilies (84). Members of TGF-β family signal by simultaneously contacting two transmembrane serine/threonine kinases, known as type I and type II receptors. Both receptors are required for TGF-β action.

In normal lungs, TGF-β protein and mRNA expression have been identified in bronchial epithelial cells, alveolar macrophages, and SMCs (85–88). The release and activation of TGF-β stimulates the production of various extracellular matrix proteins and inhibits their degradation. In COPD and asthma, TGF-β has been implicated in connective tissue deposition (89,90) and airway macrophage recruitment (91).

TGF-β has been identified as a potential mediator of vascular remodeling in pulmonary hypertension (92). In fact, mechanical stretch

increases TGF-β production in cultured vascular SMCs (93), a potential mechanism by which increased flow and/or pressure might result in vascular remodeling. In addition, TGF-β1 stimulates the production of ET-1 in bovine pulmonary artery endothelial ceils and in isolated perfused rat lungs, thereby suggesting alternative mechanism of pulmonary remodeling (94).

VI. PATHOGENETIC MECHANISMS

A. Genetic Factors

Current understanding of the pathogenesis of pulmonary hypertension has improved dramatically with the identification of germline mutations in patients with familial primary pulmonary hypertension (FPPH). Genetic analysis in these individuals has contributed to identify genes that are crucial for the pathogenesis of pulmonary hypertension, not only in the inherited form but also in sporadic forms and those associated with other disease conditions.

1. Bone Morphogenetic Protein Receptor

In the year 2000, two groups almost simultaneously reported that about 50% of individuals with FPPH had mutations in the gene encoding for the bone morphogenetic protein receptor type II (BMPR2) (95,96). This is a receptor for a family of secreted growth factors named bone morphogenetic proteins (BMPs), which are part of the TGF-β family (84). Originally identified as proteins regulating growth and differentiation of bone and cartilage, BMPs also regulate growth, differentiation, and apoptosis of various cell types (97). Mutations identified in patients with FPPH interrupt the BMP-mediated signaling pathways, resulting in a predisposition to proliferation of SMCs in pulmonary arteries (98). Mutations of the BMPR2 gene have also been identified in patients with sporadic PPH (99). Whereas some of these cases represent patients with an unidentified inherited form of the disease (99,100), in others the mutation appears de novo (99). Mutations in the BMPR2 gene have also been identified in pulmonary arterial hypertension associated with anorexigen use (101). These studies indicate that the TGF-β family plays a critical role in the maintenance of pulmonary vessel integrity, and that alterations in the TGF/BMP signaling pathway are crucial for the development of pulmonary hypertension. Interestingly, asymptomatic members of FPPH families that carry mutations in the PPH gene may develop pulmonary hypertension during exercise (102). This suggests that alterations in the PPH gene are associated with phenotypical changes, either an abnormal vascular reactivity or subclinical vascular lesions, even in subjects who do not manifest the full clinical picture of severe pulmonary hypertension.

Recently, alterations in the BMP signaling pathway have been identified in secondary forms of pulmonary hypertension (thromboembolic, scleroderma, mitral valve disease) (103). In these patients, Du et al. (103) reported upregulation of angiopoietin-1 in SMCs of small pulmonary arteries and increased phosphorylation of its receptor (TIE2) in endothelial cells, as well as nearly complete downregulation of the BMP receptor type 1A (BMPR1 A) in endothelium. Endothelial cell culture studies demonstrated that angiopoietin-1 downregulates BMPR1A gene product (103). These findings emphasize that inactivation of the BMP receptor complex, either by a mutation or by downregulation of its steady-state levels, is critical for the pathogenesis of pulmonary hypertension, not only in the primary (idiopathic) form of the disease but also when it is associated with other disease conditions.

To what extent alterations in the TGF/BMP signaling pathway are important in the pathogenesis of pulmonary vascular abnormalities occurring in COPD is currently unsettled. Yet, considering the significant observations made in the idiopathic form and other secondary forms of pulmonary hypertension this pathway deserves to be explored in COPD.

2. Serotonin Transporter

Human 5-HTT gene transcription is modulated by a common polymorphism in its upstream regulatory region. The short variant (S) of this polymorphism reduces the transcriptional efficiency of the 5-HTT gene promoter, resulting in decreased 5-HTT expression and 5-HT uptake (104). Eddahibi et al. (105) have shown that patients with pulmonary hypertension are frequently homozygote for the long (L) variant of the 5-HTT gene promoter, exhibiting higher platelet uptake of 5-HT, as well as greater expression of 5-HTT mRNA and immunoreactivity in lung tissue than controls. Indeed, pulmonary artery SMCs from patients with PPH with the LL polymorphism are more proliferative in response to 5-HT than SMCs from patients with PPH who do not carry the polymorphism (105).

Recent studies conducted in COPD have shown that in patients carrying the LL genotype, pulmonary hypertension is more severe than in patients with the LS or SS genotype (106). Compared with control subjects, platelet 5-HTT protein is increased in COPD patients in proportion to the level of hypoxemia, and strong 5-HTT immunostaining has been observed in remodeled pulmonary arteries from COPD patients. Accordingly, 5-HTT gene polymorphism seems to modulate the severity of pulmonary hypertension in hypoxemic patients with COPD.

B. Hypoxia

During decades, pulmonary hypertension associated with COPD has been considered to have a hypoxic origin. Indeed, in the current classification

of pulmonary hypertensive disorders, pulmonary hypertension associated with chronic respiratory diseases (i.e., COPD) and with hypoxia are considered together (107). This notion arises from demonstrated effects of hypoxia on pulmonary circulation. Indeed, chronic exposure of animals to hypobaric pressures or reduced inspired O_2 concentration results in SMC and adventitial fibroblast proliferation (108,109). Because pulmonary arteries contract in front of hypoxic stimulus, it has been considered that SMC proliferation in pulmonary vessels of COPD patients represents an adaptation to this chronic stimulus. In fact, genes potentially involved in this vascular remodeling process, such as TGF-β, platelet-derived growth factor (PDGF) and intercellular adhesion molecule-1 (ICAM-1) might be activated by increased shear stress during vasoconstriction (110).

The condition that more specifically represents chronic adaptation to hypoxia is that of subjects living at high altitude. Morphometric studies conducted by Arias-Stella and Saldaña (111) in natives living in Cerro del Pasco (4330 m altitude) showed that the most prominent change in pulmonary arteries was an increased thickness of the muscular layer. Furthermore, Heath et al. (112) showed that in highlanders the most characteristic feature in pulmonary arteries was medial thickening, whereas intimal measurements did not differ from control subjects. These morphological changes differ from those shown in COPD patients, where, as discussed above, the most prominent changes occur in the intima, whereas medial hypertrophy is rarely seen, even in subjects with advanced disease and profound hypoxemia (5,12). Indeed, in some occasions, medial thickness of patients with advanced disease and chronic respiratory failure is lower than in patients with moderate disease (13) (Fig. 1).

It is of note that in COPD, there is great variability in the individual responses of the pulmonary circulation to acute changes in inspired oxygen concentration (4,113,114). Hemodynamic measurements conducted in COPD patients while breathing an hypoxic mixture have shown different responses in pulmonary vascular resistance (marked increase, no change, or even decrease) that were unrelated to the degree of airflow obstruction, hypoxemia, or pulmonary artery pressure while breathing room air (113). The ultimate goal of hypoxic pulmonary vasoconstriction (HPV) is to maintain an adequate matching between ventilation and perfusion. Using measurements of ventilation–perfusion distributions at different inspired O_2 fractions, the authors also showed great variability in the magnitude of HPV that again was unrelated to the gas exchange status at baseline (4). Furthermore, there is some evidence that the degree of vasoconstriction induced by hypoxic stimulus is modulated by the extent of remodeling in pulmonary arteries (4) and by the endothelial function (115). Overall, these findings denote that persistent vasoconstriction in response to chronic hypoxic stimulus does not fully explain pulmonary vascular changes in COPD.

Hypoxia may elicit proliferation of SMCs in pulmonary arteries by pathways unrelated to vessel contraction. Indeed, isolated vascular SMCs in culture proliferate when exposed to hypoxia by mechanisms that are related to potassium currents and, therefore, independent of the vascular tone or the presence of other vascular cells (i.e., endothelium) (116). Furthermore, hypoxia may induce the endothelial synthesis and release of mediators that are capable to induce proliferation of SMCs such as ET-1 (117) or serotonin (58), and inhibit antiproliferative mediators, such as NO or prostacyclin (110).

Hypoxia is a major factor involved in the induction of VEGF gene expression, which may promote pulmonary vascular remodeling in chronic lung diseases (81,118). Indeed, hypoxia-inducible factor-1 (HIF-1) activates VEGF gene expression and upregulates VEGF receptor-1 (119,120). Nevertheless, the effect of hypoxia by VEGF-dependent mechanisms is complex since it might exert opposite effects on cell proliferation. Inhibition of VEGF receptor-2 causes muscularization of arterioles in nonhypoxic rat lung (110), an effect attributed to the impairment of the endothelial synthesis of NO and prostacyclin by VEGF-dependent mechanisms. On the other hand, exposure to hypoxia of rats treated with a VEGF receptor-2 inhibitor results in endothelial cell proliferation and severe pulmonary hypertension (121).

The role of hypoxia in the pathogenesis of pulmonary vascular changes in COPD is further puzzled by clinical and histological observations after hypoxemia correction. In subjects living at high altitudes or in animals kept in a hypoxic environment, restoration of normal oxygen levels results in normalization of pulmonary artery pressure and regression of muscularization of pulmonary vessels (122). By contrast, long-term oxygen therapy does not reverse completely pulmonary hypertension in COPD (123), and prominent vascular changes persist in subjects who have received this treatment for years (11). In addition, structural abnormalities in pulmonary arteries can be observed, at least in part, in patients with mild COPD who do not have hypoxemia and also in smokers with normal lung function (4,6,62).

Overall, these observations suggest that mechanisms other than hypoxemia might contribute to pulmonary vascular changes in COPD.

C. Inflammation

The idea of an inflammatory process as a mechanism of vascular remodeling arises from studies demonstrating a correlation between the severity of the inflammatory infiltrate in small airways and the structural abnormalities of pulmonary muscular arteries (4,8). Recent morphologic studies have shown an increased number of $CD8^+$ T-lymphocytes infiltrating the adventitia of pulmonary muscular arteries in patients with mild-to-moderate

COPD (15,62). The potential role of inflammation in the pathogenesis of pulmonary vascular abnormalities associated with COPD has not been established yet.

Inflammatory cells are a source of cytokines and growth factors that may target the endothelial cells and contribute to the development of structural and functional abnormalities of the vessel wall (124). Different inflammatory mediators, namely prostaglandin E_2, interleukin (IL) -6, and IL-1 are capable to induce VEGF mRNA and protein expression (125,126). In addition, macrophages and T-lymphocytes express VEGF receptors (127) and VEGF induces monocyte activation and migration (128,129). Accordingly, it might be speculated that increased VEGF activity shown in pulmonary arteries at early stages of COPD (13) could be related, to some extent, with an underlying inflammatory process. This suggestion is consistent with the fact that the number of inflammatory cells infiltrating the wall of pulmonary arteries is inversely related to the endothelial function and directly related to the enlargement of the intimal layer (62).

D. Cigarette Smoking

Studies performed by the authors have shown prominent changes in pulmonary arteries of smokers with normal lung function, namely SMC proliferation (6,18), impairment of endothelial function (6), reduced expression of eNOS (130), increased expression of VEGF (13), and $CD8^+$ T-cell infiltrate (62). In general, these changes are indistinguishable from those seen in pulmonary arteries of COPD patients, and clearly differ from nonsmokers. These observations strongly suggest that cigarette smoke products might exert a direct effect on vessel structure and function. Studies performed in the 1960s already pointed out this association (131), and Hale et al. (8) demonstrated structural abnormalities in pulmonary muscular arteries of smokers, as compared with nonsmokers.

Some of these observations have been replicated in a guinea pig model chronically exposed to cigarette smoke by Wright and Churg (132). In that model, exposure to cigarette smoke induces muscularization of precapillary vessels and increase of pulmonary artery pressure (133) (Fig. 3). Interestingly, these vascular abnormalities are detectable after 2 months of cigarette smoke exposure only, when there is no evidence of emphysema (133), thereby implicating that cigarette smoke-induced vascular abnormalities may antecede the development of pulmonary emphysema (134). Furthermore, using this model, it has been shown that cigarette smoke exposure induces rapid changes in gene expression of VEGF, VEGF receptor-1, ET-1, and inducible NOS (135), mediators that control vascular cell growth and vessel contraction, and that are likely involved in the pathogenesis of pulmonary vascular changes of COPD.

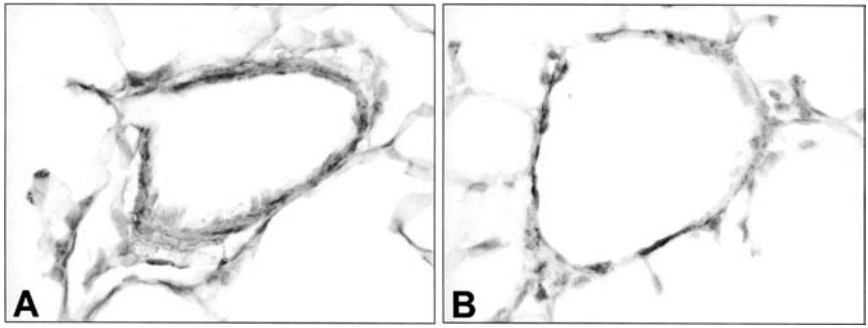

Figure 3 Photomicrographs of small arteries from guinea pigs exposed to cigarette smoke during 6 months (A), and sham-smoked (B). Note the development of a complete and thick muscular layer in arterioles of guinea pigs exposed to cigarette smoke.

It is well known that cigarette smoking is a risk factor for the development of vascular disease. Active and passive exposure to tobacco smoke produces endothelial dysfunction in both coronary and systemic arteries (136). Exposure of pulmonary artery endothelial cells to cigarette smoke extract causes an irreversible inhibition of eNOS activity, which is due to a diminished eNOS protein content and mRNA (137). Cigarette smoke contains a number of products that have the potential to induce endothelial damage. However, the specific product responsible for the inhibition of eNOS remains unsettled. In this respect, pretreatment with antioxidants does not protect eNOS from the inhibitory effect of cigarette smoke extract (137). Cigarette smoke products can also be potential triggers for VEGF since nicotine and cotinine, in doses similar to those seen in the plasma of current smokers, upregulate VEGF expression in endothelial cells (138).

VII. PATHOBIOLOGY OF PULMONARY VASCULAR CHANGES IN COPD

Several evidences discussed above suggest that the initial event in the natural history of pulmonary hypertension in COPD might be the lesion of pulmonary endothelium by cigarette-smoke products with a subsequent downregulation of eNOS expression and impairment of endothelial function (Fig. 4). It is also possible that inflammation products could also play a similar role damaging pulmonary endothelium. One of the consequences of endothelial dysfunction is the impairment of the reactivity of pulmonary arteries to hypoxia (12,114,115), thereby contributing to ventilation–perfusion mismatching and promoting the development of arterial hypoxemia. Endothelial damage results in an imbalance among factors that regulate cell growth, thereby favoring proliferation of SMCs and extracellular matrix deposition. Upregulation

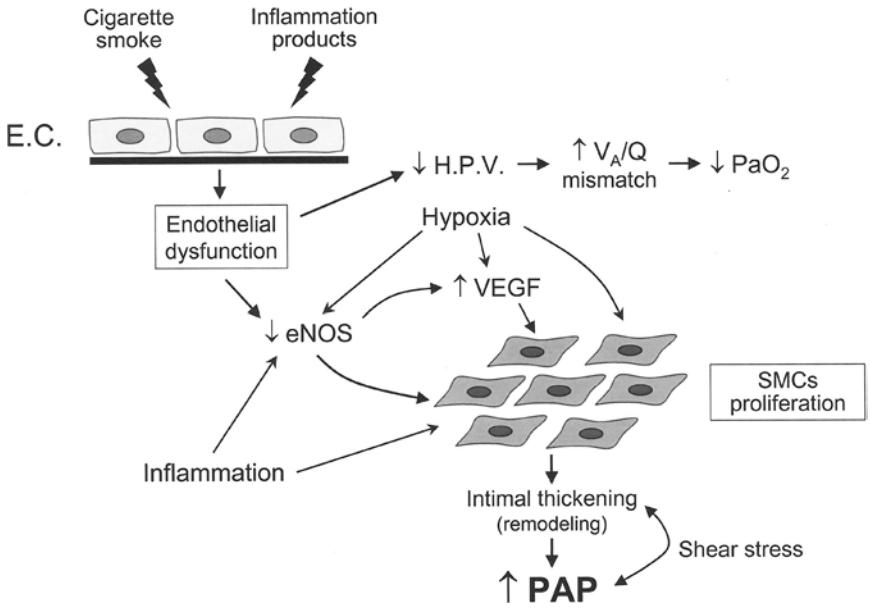

Figure 4 Pathobiology of pulmonary hypertension in COPD, where cigarette smoke products or inflammation may initiate the sequence of changes by producing endothelial dysfunction. eNOS: endothelial nitric oxide synthase; HPV: hypoxic pulmonary vasoconstriction; V_A/Q: ventilation–perfusion; VEGF: vascular endothelial growth factor; SMCs: smooth muscle cells.

of VEGF, enhanced by endothelial dysfunction, might contribute too to SMC proliferation. All these changes result in intimal hyperplasia with the ensuing reduction of arterial lumen, which increases pulmonary vascular resistance.

Arteries with endothelial dysfunction are more susceptible to the action of additional factors. Among those, sustained arterial hypoxemia and alveolar hypoxia in poorly ventilated lung units play a crucial role, since they may induce further endothelial impairment and vessel remodeling, either directly or through VEGF-dependent mechanisms, thus amplifying the initial effects of cigarette smoke products (Fig. 4). Similar effects might be produced by cytokine release by inflammatory cells, and by shear stress induced by increased vascular resistance.

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Therapeutic Implications and Future Developments

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I. INTRODUCTION

Despite the enormous global impact of COPD and the increasing investment in research, there are no current therapies that have been shown to prevent the inevitable progression of the disease. However, as exemplified in this volume, there is now an increasing understanding of the cellular and molecular mechanisms involved in COPD (1) and this has identified many new targets and will lead to the development of new therapies in the future (2,3) (Table 1).

A. The Need for Novel Therapies

There have been disappointingly few therapeutic advances in the drug therapy of COPD, in contrast to the enormous advances made in asthma management that reflect a much better understanding of the disease (4). Rational therapy depends on elucidating the cellular and molecular mechanisms that may be involved. There is a particular need to develop drugs that suppress the underlying inflammatory, fibrotic, and destructive processes that underlie COPD (Fig. 1). As discussed in Chapter 4, the inflammation of COPD is quite different from that seen in asthma, indicating that different treatments are likely to be needed (5,6). An important observation is that the inflammation of COPD patients is not suppressed by corticosteroids (7–9) and even key inflammatory

Table 1 Potential New Therapies for COPD

Target	Candidate therapies
Smoking	Drugs acting on neural nicotine addiction
Oxidants	Antioxidants (e.g., stable glutathione analogs), resveratrol, inducible nitric oxide synthase inhibitors
Leukotrienes	BLT_1 receptor antagonists (LY 29311, SB 201146, BIIL284), 5′-lipoxygenase inhibitors (zileuton, BAYxl005)
Adhesion molecules	Anti CD11/CD18, anti-ICAM-1, E-selectin inhibitors
Chemokines	CXCR2 antagonists (SB 225002), CCR2 antagonists, CXCR3 antagonists
Cytokines	TNF-α inhibitors (infliximab, etanercept), TNF-α converting enzyme (TACE) inhibitors, interleukin-10, and analogs
Phosphodiesterase-4	PDE-4 inhibitors (cilomilast, roflumilast)
Kinases and transcription factors	NF-κB inhibitors (IKK-2 inhibitors, proteasome inhibitors, IκB-α gene transfer), p38 MAP kinase inhibitors (SB203580, SB 239063), PI-3 kinase-γ inhibitors, PPAR activators (glitazones)
Mucus hypersecretion	EGF receptor kinase inhibitors (gefitinib), calcium-activated chloride channel inhibitors (niflumic acid, MSI 1956)
Fibrosis	TGF- βl receptor kinase inhibitors, PAR-2 inhibitors
Proteinases	Endogenous antiproteases: α1-AT, SLPI, TIMPs, elafin; neutrophil elastase inhibitors; cysteine proteinase inhibitors; matrix metalloproteinase inhibitors
Lung regeneration agents	Retinoic acid (all-*trans* retinoic acid), retinoic acid receptor- γ agonists, stem cells

Note: α-AT, α_1-antitrypsm; BLT_1, leukotriene B_4 receptor type 1; EGF, epidermal growth factor; ICAM-1, intercellular adhesion molecule-1; NF-κB, nuclear factor-κB; IκB, inhibitor of NF-κB; IKK, inhibitor of IκB kinase; MAP, mitogen-activated protein; MCP, monocyte chemotactic protein; PAR, protemase-activated receptor; PI-3, phosphoinositide-3; PPAR, peroxisome proliferator-activated receptor; SLPI, secretory leukoprotease inhibitor; TIMP, tissue inhibitor of matrix metalloproteinases; TNF-α, tumor necrosis factor-α; TGF-β1, transforming growth factor-β1.

cells, such as alveolar macrophages, are steroid resistant (10). This means that alternative anti-inflammatory approaches need to be developed.

B. The Challenge of Drug Development

There are several reasons why drug development in COPD may be difficult. Only recently (and summarized in this volume) has there been a major research interest in the molecular and cell biology of COPD in order to identify new therapeutic targets. Animal models of COPD for early drug testing

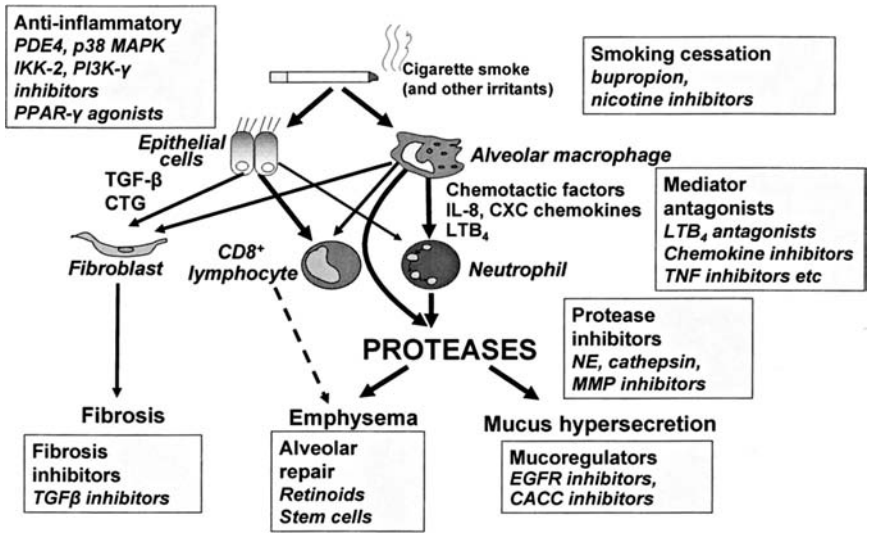

Figure 1 Targets for COPD therapy. Many new treatments for COPD are now in development based on logical targets revealed by a better understanding of cellular and molecular mechanisms involved in disease pathogenesis. More effective smoking cessation drugs are needed. Antagonists of specific mediators, such as LTB4, and chemokines, such as interleukin-8, are in clinical trial, but may be too specific. Inhibition of TNF-α is more likely to be successful, particularly in patients with systemic features. Other approaches include inhibiting proteases (elastases) such as neutrophil elastase (NE), cathepsins, or MMP. Drugs with a broader spectrum of anti-inflammatory effects, such as PDE-4 inhibitors or inhibitors of signal transduction pathways, such as inhibitors or inhibitor of nuclear factor-κB kinase (IKK2), p38 MAP kinase, or phosphoinositide-3-kinase are promising. Drugs that inhibit TGF-β may inhibit the fibrosis in small airways. Drugs targeted at mucus hypersecretion include inhibitors of epidermal growth factor receptors (EGFR) and CACC. There are also approaches to repair emphysema using retinoids (that are effective in rodent lungs) and stem cells.

are not very satisfactory and require long exposure times to cigarette smoke to induce emphysema (11). There are still many uncertainties about how to test new drugs for COPD, which may require long-term studies (over 3 years) in relatively large numbers of patients. It is difficult to give new chemical entities for such prolonged periods, so that the drug would need to show efficacy in more short-term clinical parameters, such as a reduction in exacerbations. Many patients with COPD may have comorbidities, such as ischemic heart disease and diabetes, which may exclude them from clinical trials of new therapies. There is little information about surrogate markers, for example, biomarkers in blood, sputum, or breath, to monitor the short-term efficacy and predict the long-term potential of new

treatments. However, progress is underway and there are several classes of drug that are now in preclinical and clinical development (2,3).

II. SMOKING CESSATION

Cigarette smoking is the major cause of COPD in the world and smoking cessation is the only therapeutic intervention so far shown to reduce disease progression. Nicotine addiction is the major problem and treatment should be directed at dealing with this addictive state. Nicotine replacement therapies have not proved to be very effective and do not deal with the underlying addictive state. An important advance has been the discovery that a short course of the atypical antidepressant bupropion is an effective adjunct for smoking cessation in patients with COPD (12). However, the relatively poor long-term quit rate (16% at 6 months) means that more effective approaches are needed. Bupropion is an antidepressant that has effects on noradrenergic pathways, and other drugs with a similar profile are now in development.

There is a much better understanding of the neurobiology of nicotine dependence and some of the central neurotransmitters that are involved (13). Neural mechanisms involved in nicotine addiction include dopaminergic pathways in the nucleus accumbens (14), but serotoninergic and glutaminergic pathways are also involved. New approaches to treating nicotine addiction include cannabinoid CB_1-receptor antagonists, $GABA_B$-receptor agonists, metabotropic glutamante ($mGlu_5$) receptor agonists, dopamine D3-receptor antagonists, serotonin 5-HT_{1A}-receptor antagonists, and agonists of the β2-subunit of nicotinic receptors. Another development is vaccines against nicotine developed by immunizing with a nicotine hapten linked to an immunogenic protein that results in sequestration of nicotine to prevent its entry into the brain (15). These vaccines are now in clinical development.

III. NEW BRONCHODILATORS

Since bronchodilators are the mainstay of current management (16), a logical approach is to improve existing bronchodilators. Several once-daily-inhaled β_2-agonists are now in clinical development, and the once-daily-inhaled anticholinergic tiotropium has recently become available in several countries (17). Long-term studies with tiotropium bromide have demonstrated significant improvement in symptoms and improvement in the quality of life, as well as an unexpected reduction in exacerbations (18). Tiotropium is likely to have additive effects when combined with long-acting β2-agonists making once daily combination bronchodilator inhalers a likely development (19). New classes of bronchodilator, such as potassium channel openers and phosphodiesterase-3 inhibitors, have been difficult to develop as the side effects, particularly vasodilator effects, usually limit the dose.

IV. OVERCOMING CORTICOSTEROID RESISTANCE

As discussed in Chapter 15, corticosteroids do not inhibit the inflammatory process of COPD, with no reduction in cytokines, chemokines, or proteinases even with maximum inhaled doses or high doses of oral corticosteroids (7–9). This is consistent with a failure of inhaled corticosteroids to reduce the progression of the disease (20). There is a small reduction in exacerbations in severe asthma patients, which may imply that some steroid-sensitive component is involved in exacerbations. This might be an effect on mucus hypersecretion or on plasma leakage, both of which increased during exacerbations. This suggests that there may be an active resistance to corticosteroids in COPD patients. As discussed in Chapter 15, this may be due to an inhibitory effect of cigarette smoke on histone deacetylation, which is required for corticosteroids to switch off inflammatory genes (21).

Based on this mechanism, therapeutic strategies that unlock the molecular mechanism of resistance might be possible, as drugs that increase histone deacetylase activity may "resensitize" cells to the effects of corticosteroids. As the effect of cigarette smoke may be mediated through oxidative stress and the generation of peroxynitrite, it is predicted that antioxidants, inhibitors of inducible nitric oxide synthase (iNOS), or peroxynitrite scavengers should reverse the reduction in histone deacetylase 2 (HDAC2) found in asthma and thus restore steroid responsiveness.

A. Theophylline

Theophylline in low concentrations increases the activation of HDACs and increases responsiveness to corticosteroids in vitro (22,23). This effect of theophylline is completely independent of its inhibition of phosphodiesterases (PDE) and adenosine receptor antagonism, which underlie all of the known side effects of theophylline. Theophylline may therefore reverse the reduction in HDAC2 found in COPD macrophages and thus reverse steroid resistance. Clinical trials are now needed to test this hypothesis. The dose of theophylline is limited by side effects, so a search for other drugs like theophylline that activate HDAC may be a novel approach. The molecular mechanisms whereby theophylline activates HDACs are completely unknown, but identification of the molecular target might result in the discovery of alternative HDAC-activating drugs.

B. Combination Inhalers

Combination inhalers of a steroid and a long-acting β_2-agonist appear to be more effective than either drug alone in the management of COPD and it is possible that there is some molecular synergy between these drugs (24,25). There are important interactions between β_2-agonists and corticosteroids

that can account for their beneficial effects in asthma (26), but there are few mechanistic studies in COPD. Transforming growth factor-β (TGF-β) downregulates β_2-receptors in human cell lines by inhibiting gene transcription (27). Transforming growth factor-β inhibits the bronchodilator response to β-agonists in guinea pig trachea in vitro (28), and as there is increased expression of TGF-β in peripheral airways of COPD patients (29), this may downregulate β2-receptor on smooth muscle cells of small airways and impair the bronchodilator response to β2-agonists. Corticosteroids increase the expression of β_2-adrenergic receptors in small airways (30) and thus might restore the reduction if β_2-receptors increase the bronchodilator response to β_2-agonists. β_2-Agonists may also have effects on glucocorticoid receptors, facilitating their nuclear translocation so that the anti-inflammatory effect of corticosteroids is enhanced (31,32). It is possible that this may reduce the resistance to corticosteroids seen in COPD, but further studies are needed to explore this possibility.

V. MEDIATOR ANTAGONISTS

As discussed in Chapter 11, many mediators are involved in the pathophysiology of COPD, although there is much less information about the mediators of COPD than the mediators of asthma. An approach to new therapies in COPD is to antagonize specific mediators or to prevent their synthesis with enzyme inhibitors. Although this is an easily testable approach, it is unlikely that blocking a single mediator will control the disease, as so many mediators are involved and there is considerable redundancy between these mediators. For example, there are several mediators that may increase neutrophil chemotaxis, including leukotriene B_4 (LTB_4), interleukin (IL) -8 and anaphylotoxins, so that blocking a single mediator may not have a marked effect on neutrophilic inflammation in the lungs.

A. Leukotriene Inhibitors

Leukotriene B_4 is a potent chemoattractant of neutrophils and is increased in the sputum and exhaled breath of patients with COPD (33,34). It is probably derived from alveolar macrophages, as well as neutrophils, and may be synergistic with IL-8. Two subtypes of receptor for LTB_4 have been described; BLT_1 receptors are expressed mainly on granulocytes and monocytes, whereas BLT_2 receptors are expressed on T lymphocytes (35). BLT_1 antagonists, such as LY29311, have now been developed for the treatment of neutrophilic inflammation (36). LY293111 and another antagonist SB225002 inhibit the neutrophil chemotactic activity of sputum from COPD patients, indicating the potential clinical value of such drugs (37,38). Several selective BLT_1 antagonists are now in development. Leukotriene B_4 is synthesized by 5′-lipoxygenase (5-lLO), of which there are several inhibitors, although there

have been problems in clinical development of drugs in this class because of side effects. A recent pilot study in COPD patients with a 5′-lipoxygenase inhibitor BAYxl005 showed only a modest reduction in sputum LTB_4 concentrations but no effect on neutrophil activation markers (39).

Cysteinyl-LTs are important bronchoconstrictors in patients with asthma and are largely derived form mast cells, whereas in COPD cys-LTs do not appear to be increased (34,40). There is, therefore, no rationale for the use of cys-LT receptor antagonists in COPD.

B. Antioxidants

As discussed in Chapter 12, oxidative stress is increased in patients with COPD (41,42), particularly during exacerbations, and reactive oxygen species contribute to its pathophysiology (43). Furthermore, as discussed in Chapter 15, oxidative stress may also lead to steroid resistance through an inhibitory effect on HDAC2 activity. This suggests that antioxidants may be of use in the therapy of COPD. *N*-Acetyl cysteine (NAC) provides cysteine for enhanced production of the antioxidant glutathione (GSH) and has antioxidant effects in vitro and in vivo. A systematic review of studies with oral NAC in COPD suggested a small reduction in exacerbations (44). More effective antioxidants, including stable glutathione compounds, analogs of superoxide dismutase, and selenium-based drugs, are now in development for clinical use (45,46).

C. Nitric Oxide Synthase Inhibitors

Oxidative stress and increased nitric oxide release from activity of iNOS may result in the formation of peroxynitrite; this is a potent radical that nitrates tyrosine residues on proteins and may alter their function. 3-Nitrotyrosine may indicate peroxynitrite formation and is markedly increased in sputum macrophages of patients with COPD (47). Selective inhibitors of iNOS are now in development and one of these, a prodrug of l-N^6-(1-imminoethyl)lysine (L-NIL) gives a profound and long-lasting reduction in the concentrations of nitric oxide in exhaled breath (48). Other drugs, such as ebselen, may act as peroxynitrite scavengers (49).

D. Chemokine Inhibitors

As discussed in Chapter 11, several chemokines are involved in neutrophil chemotaxis, these being mainly chemokines of the CXC family, and chemokine antagonists are of potential therapeutic benefit in COPD (50). Interleukin-8 levels are markedly elevated in the sputum of patients with COPD and are correlated with disease severity (51). Blocking antibodies to IL-8 reduce the chemotactic response of neutrophils to sputum from COPD patients (33,38). A human monoclonal antibody to IL-8 is now in

clinical trials for COPD, but other CXC chemokines are also involved in COPD so that this may not be very effective. Interleukin-8 activates neutrophils via a specific low-affinity G-protein-coupled receptor (CXCR1) coupled to activation and degranulation and via a high-affinity receptor (CXCR2), shared with other members of the CXC family, which is important in chemotaxis. Other CXC chemokines, such as growth-related oncoprotein-α (GRO-α), are also elevated in COPD (52) and therefore a CXCR2 antagonist is likely to be more useful than a CXCRl antagonist, particularly as CXCR2 is also expressed on monocytes. Indeed, inhibition of monocyte chemotaxis may prevent the marked increase in macrophages found in the lungs of patients with COPD. Small molecule inhibitors of CXCR2, such as SB225002, have now been developed and are entering clinical trials (53).

CC-Chemokines are also involved in COPD. There is increased expression of monocyte chemotactic protein-1 (MCP-1) and its receptor CCR2 in macrophages and epithelial cells from COPD patients and this may play a role in recruitment of blood monocytes to the lungs of COPD patients (54). This suggests that CCR2 antagonists may be of use and small molecule inhibitors are now in clinical development.

Chemokine receptors are also important for the recruitment of $CD8^+$ T cells, which predominate in COPD airways and lungs and might contribute to the development of emphysema, as discussed in Chapter 9. $CD8^+$ cells show increased expression of CXCR3 and there is upregulation of CXCR3 ligands, such as CXCL10 (IP-10), in peripheral airways of COPD patients (55). This suggests that CXCR3 antagonists might also be useful.

E. Tumor Necrosis Factor-α Inhibitors

Tumor necrosis factor-α (TNF-α) and soluble TNF receptor concentrations are raised in the sputum of COPD patients (51,56). Tumor necrosis factor-α augments inflammation and induces IL-8 and other chemokines in airway cells via activation of the transcription factor nuclear factor-κB (NF-κB). As discussed in Chapter 17, the severe wasting in some patients with advanced COPD might be due to skeletal muscle apoptosis, resulting from increased circulating TNF-α. COPD patients with cachexia have increased release of TNF-α from circulating leukocytes (57). Humanized monoclonal TNF antibody (infliximab) and soluble TNF receptors (etanercept) that are effective in other chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease, should also be effective in COPD, particularly in patients who have systemic symptoms (58). Trials of anti-TNF therapies in patients with systemic features of COPD are currently underway. Tumor necrosis factor-α converting enzyme (TACE), which is required for the release of soluble TNF-α, may be a more attractive target, as it is possible to discover small molecule TACE inhibitors, some of which are also matrix metalloproteinase (MMP) inhibitors (59). General anti-inflammatory

drugs such as PDE inhibitors, NF-κB, and p38 mitogen-activated protein (MAP) kinase inhibitors also potently inhibit TNF-α expression.

VI. OTHER ANTI-INFLAMMATORY DRUGS

A. Phosphodiesterase-4 Inhibitors

Phosphodiesterase-4 (PDE4) is the predominant PDE expressed in neutrophils, $CD8^+$ cells, and macrophages, suggesting that PDE4 inhibitors would be effective in controlling inflammation in COPD (60). Selective PDE4 inhibitors, such as cilomilast and roflumilast, are active in animal models of neutrophil inflammation. Cilomilast had promising beneficial clinical effects in a 6-week study in patients with moderate to severe COPD (61) and has some anti-inflammatory effects measurable in airway biopsies (62). Roflumilast appears to be better tolerated than cilomilast at doses that significantly inhibit TNF-α release from peripheral blood monocytes. Phosphodiesterase-4 inhibitors have been limited by side effects, particularly nausea and other gastrointestinal effects, but it might be possible to develop more selective inhibitors in the future, which are less likely to be dose-limited by adverse effects.

Several steps may be possible to overcome the limitation of side effects. It now seems likely that vomiting is due to inhibition of a particular subtype of PDE4. At least four human PDE4 genes have been identified and each has several splice variants (63). This raises the possibility that subtype-selective inhibitors may be developed that may preserve the anti-inflammatory effect, while having less propensity to side effects. PDE4D appears to be of particular importance in nausea and vomiting and is expressed in the chemosensitive trigger zone in the brain stem (64), and in mice, deletion of the gene for PDE4D prevents a behavioral equivalent of emesis (65). This isoenzyme appears to be less important in anti-inflammatory effects, and targeted gene disruption studies in mice indicate that PDE4B is more important than PDE4D in inflammatory cells (66). PDE4B-selective inhibitors may therefore have a greater therapeutic ratio and theoretically might be effective anti-inflammatory drugs. Cilomilast is selective for PDE4D and therefore has a propensity to cause emesis, whereas roflumilast, which is nonselective for PDE4 isoenzymes, has a more favorable therapeutic ratio. Several potent PDE4 inhibitors with a more favorable therapeutic ratio are now in clinical development for COPD. Another approach is to give the PDE4 inhibitor by inhalation and some PDE4 inhibitors have a low oral bioavailability and are retained in the lung, so appear to be suitable for inhaled delivery (67), but no clinical studies of inhaled PDE4 inhibitors have yet been reported.

B. Nuclear Factor-κB Inhibitors

Nuclear factor-κB regulates the expression of IL-8 and other chemokines, TNF-α and other inflammatory cytokines, and some MMPs including the

critical MMP-9. Nuclear factor-κB is activated in macrophages and epithelial cells of COPD patients, particularly during exacerbations (68,69). There are several possible approaches to inhibition of NF-κB, including gene transfer of the inhibitor of NF-κB (IκB), inhibitors of IκB kinases (IKK), NF-κB-inducing kinase (NIK), and IκB ubiquitin ligase, which regulate the activity of NF-κB, and the development of drugs that inhibit the degradation of IκB (70). The most promising approach may be the inhibition of IKK-2 by small molecule inhibitors, several of which are now in development (71). A small molecule IKK-2 inhibitor suppresses the release of inflammatory cytokines and chemokines from alveolar macrophages (72) and might be effective in COPD, as alveolar macrophages are resistant to the anti-inflammatory actions of corticosteroids (10). One concern about long-term inhibition of NF-κB is that effective inhibitors may result in immune suppression and impair host defenses, as mice that lack NF-κB genes succumb to septicemia. However, there are alternative pathways of NF-κB activation via kinases other than IKK that might be more important in inflammatory disease (73,74).

C. p38 MAP Kinase Inhibitors

Mitogen-activated protein kinases play a key role in chronic inflammation, and several complex enzyme cascades have now been defined (75). One of these, the p38 MAP kinase pathway is activated by cellular stress and regulates the expression of inflammatory cytokines, including IL-8, TNF-α, and MMPs (76). Small molecule inhibitors of p38 MAP kinase, such as SB 203580, SB 239063, and RWJ 67657, have been developed and these drugs have a broad range of anti-inflammatory effects (76,77). SB 239063 reduces neutrophil infiltration after inhaled endotoxin and the concentrations of IL-6 and MMP-9 in bronchoalveolar lavage fluid of rats, indicating its potential as an anti-inflammatory agent in COPD (78). It is likely that such a broad-spectrum anti-inflammatory drug will have some toxicity, but inhalation may be a feasible therapeutic approach. Several p38 inhibitors are now in clinical development and some are in Phase II trials for other inflammatory indications.

D. Phosphoinositide 3-Kinase Inhibitors

Phosphoinositide 3-kinases (PI-3Ks) are a family of enzymes that lead to the generation of lipid second messengers that regulate a number of cellular events. A particular isoform, PI-3Kγ, is involved in neutrophil recruitment and activation. Knock-out of the PI-3Kγ gene results in inhibition of neutrophil migration and activation, as well as impaired T-lymphocyte and macrophage function (79). This suggests that selective PI-3Kγ inhibitors may have relevant anti-inflammatory activity in COPD and small molecule inhibitors of PI-3Kγ and PI-3Kδ are in development (80).

E. Peroxisome Proliferator-Activated Receptor Activators

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated nuclear hormone receptors belonging to the steroid receptor superfamily, and the three recognized subtypes PPAR-α, -γ, and -δ are widely expressed. There is evidence that activation of PPAR-α and -δ may have anti-inflammatory and immunomodulatory effects (81). For example, PPARγ agonists, such as troglitazone, inhibit the release of inflammatory cytokines from monocytes and induce apoptosis of T lymphocytes, suggesting that they may have anti-inflammatory effects in COPD (82,83).

F. Adhesion Molecule Blockers

Recruitment of neutrophils, monocytes, and cytotoxic T cells into the lungs and respiratory tract is dependent on adhesion molecules expressed by these cells and on endothelial cells in the pulmonary and bronchial circulations. Several adhesion molecules can now be inhibited pharmacologically. For example, E-selectin on endothelial cells interacts with sialyl-Lewis^x on neutrophils. A mimic of sialyl-Lewis^x, bimosiamose, blocks selectins and inhibits granulocyte adhesion, with preferential effects on neutrophils (84). However, there are concerns about this therapeutic approach for a chronic disease, as an impaired neutrophilic response may increase the susceptibility to infection. The expression of Mac-1 (CDllb/CD18) is increased on neutrophils of patients with COPD, suggesting that targeting this adhesion molecule, which is also expressed on monocytes and macrophages, might be beneficial (85).

G. Interleukin-10

Interleukin-10 (IL-10) is a cytokine with a wide spectrum of anti-inflammatory actions. It inhibits the secretion of TNF-α and IL-8 from macrophages and tips the balance in favor of antiproteases by decreasing the expression of MMP while increasing the expression of endogenous tissue inhibitors of MMPs (TIMP). Interleukin-10 concentrations are reduced in induced sputum from patients with COPD, so that this may be a mechanism for increasing lung inflammation (86). Interleukin-10 is currently in clinical trials for other chronic inflammatory diseases (inflammatory bowel disease, rheumatoid arthritis, and psoriasis), including patients with steroid resistance, but IL-10 may cause hematological side effects (87). Interleukin-10 may have therapeutic potential in COPD, especially if a selective activator of IL-10 receptors or unique signal transduction pathways can be developed in the future.

H. Resveratrol

Resveratrol is a phenolic component of red wine that has anti-inflammatory and antioxidant properties. It has a marked inhibitory effect on cytokine release from alveolar macrophages from COPD patients that show little

or no response to corticosteroids (88). The molecular mechanism or this action is currently unknown, but identification of the cellular target for resveratrol may lead to the development of a novel class of anti-inflammatory compounds. Resveratrol itself has a very low oral bioavailability; so, related drugs or a suitable inhaled formulation will need to be developed.

VII. DRUGS ACTING ON STRUCTURAL CELLS

A. Mucoregulators

Mucus hypersecretion is commonly seen in cigarette smokers, but is not necessarily associated with airflow limitation. In individuals with COPD, mucus hypersecretion is associated with more rapid decline in FEV_1 and increased frequency of exacerbations (89). Reducing mucus hypersecretion may therefore have therapeutic benefit, although suppression of the normal airway mucus secretion may be detrimental. Mucolytic drugs have been used for many years to reduce mucus viscosity but these drugs do not appear to have any clinical value. As discussed in Chapter 5, there are many inflammatory products and neural mechanisms that regulate mucus secretion and therefore there are several targets for the development of mucoregulators.

Several novel approaches to inhibiting mucus hypersecretion are currently being explored (90). Mucus hypersecretion appears to be largely driven in COPD by the neutrophil inflammatory response, so that effective anti-inflammatory treatments would be expected to reduce mucus hypersecretion (90).

Epidermal growth factor (EGF) plays a critical role in airway mucus secretion from goblet cells and submucosal glands and appears to mediate the mucus secretory response to several secretagogs, including oxidative stress, cigarette smoke, and inflammatory cytokines (91). Epidermal growth factor may also be responsible for the mucus hyperplasia seen in chronic bronchitis. Small molecule inhibitors of EGF receptor kinase, such as gefitinib, have now been developed for clinical use.

Another novel approach involves inhibition of calcium-activated chloride channels (CACC), which are important in mucus secretion from goblet cells. Activation of human hCLCAl induces mucus secretion and mucus gene expression and may therefore be a target for inhibition. Small molecule inhibitors of CACC, such as niflumic acid and MSI 1956, have been developed (92). Other approaches include inhibition of the neural mechanisms driving mucus secretion, including tachykinin receptor antagonists and potassium channel openers (93).

B. Fibrosis Inhibition

Transforming growth factor-β1 (TGF-β1) is highly expressed in airway epithelium and macrophages of small airways in patients with COPD

(29,94). It is a potent inducer of fibrosis, partly via the release of the potent fibrogenic mediator connective tissue growth factor, and may be important in inducing the fibrosis and narrowing of peripheral airways (obstructive bronchiolitis) in COPD. Transforming growth factor-β1 also activates MMP-9, which then further activates TGF-β1, thus providing a link between small airway fibrosis and emphysema in COPD. MMP-9 may mediate proteolysis of TGF-β-binding protein (LTBP1), and this may be a mechanism for physiological release of TGF-β1 (95). Inhibition of TGF-β1 signaling may therefore be a useful therapeutic strategy in COPD. Small molecule antagonists which inhibit TGF-β receptor kinase or TGF-β-activated pathways are now in development (96,97), although the long-term safety of such drugs might be a problem, particularly as TGF-β affects tissue repair and is a potent anti-inflammatory mediator.

Proteinase-activated receptor-2 (PAR-2) expression is widespread in the airways, and expression is increased in the central airways of smokers and nonsmokers (98). Proteinase-activated receptor-2 may be involved in MMP-9 release from airway epithelial cells and proliferation of fibroblasts (99,100). However, a potential drawback for strategies to antagonize PAR-2 is that activation of epithelial PAR-2 causes bronchoprotection in the airways (101).

C. Antiproteases

As discussed in Chapters 13 and 14, there is compelling evidence for an imbalance between proteases that digest elastin (and other structural proteins) and antiproteases that protect against this. This suggests that either inhibiting these proteolytic enzymes or increasing endogenous antiproteases may be beneficial and theoretically should prevent the progression of airflow obstruction in COPD. Considerable progress has been made in identifying the enzymes involved in elastolytic activity in emphysema and in characterizing the endogenous antiproteases that counteract this activity (102). The fact that there are so many proteinases implicated in COPD might mean that blocking a single enzyme may not have a major effect.

One approach is to give endogenous antiproteases (α_1-antitrypsin, secretory leukoprotease inhibitor, elafin, tissue inhibitors of MMP) either in recombinant form or by viral vector gene delivery (103,104). These approaches are unlikely to be cost effective, as large amounts of protein have to be delivered and gene therapy is unlikely to provide sufficient protein.

A more promising approach is to develop small molecule inhibitors of proteases, particularly those that have elastolytic activity (105). Small molecule inhibitors, such as ONO-5046 and FR901277, have been developed which have high potency (106). These drugs inhibit neutrophil elastase-induced lung injury in experimental animals, whether given by inhalation or systemically and also inhibit the other serine proteinases released from

neutrophils, cathepsin G, and proteinase-3. Small molecule inhibitors of neutrophil elastase are in clinical development, but there is concern that neutrophil elastase may not play a critical role in emphysema and that other proteinases are more important in elastolysis. Inhibitors of elastolytic cysteine proteinases, such as cathepsins K, S, and L, which are released from macrophages are also in development (107). Matrix metalloproteinases with elastolytic activity (such as MMP-9) may also be a target for drug development, although nonselective MMP inhibitors, such as marimastat, appear to have considerable musculoskeletal side effects (108). It is possible that side effects could be reduced by increasing selectivity for specific MMPs or by targeting delivery to the lung parenchyma. MMP-9 is markedly overexpressed by alveolar macrophages from patients with COPD and is the major elastolytic enzyme released by these cells (109); so, a selective MMP-9 inhibitor might be useful in the treatment of emphysema.

D. Lung Regeneration

As a major mechanism of airway obstruction in COPD is loss of elastic recoil due to proteolytic destruction of lung parenchyma, it seems unlikely that this could be reversible by drug therapy, although it might be possible to reduce the rate of progression by preventing the inflammatory and enzymatic disease process. Retinoic acid increases the number of alveoli in developing rats and, remarkably, reverses the histological and physiological changes induced by elastase treatment of adult rats (110,111). However, this is not observed in other species (112). Retinoic acid activates retinoic acid receptors, which act as transcription factors to regulate the expression of many genes involved in growth and differentiation. The molecular mechanisms involved and whether this can be extrapolated to humans is not yet known. Several retinoic acid receptor subtype agonists have now been developed that may have a greater selectivity for this effect and therefore a lower risk of side effects. The receptor mediating the effect on alveoli appears to be the RAR-γ receptor (111). A short-term trial of all-*trans*-retinoic acid in patients with emphysema did not show any improvement in clinical parameters (113), but a longer study is currently underway. This approach is unlikely to be successful as adult human lung, unlike rat lung, has less potential for repair after surgical resection for example.

Another approach to repairing damaged lung in emphysema is the use of stem cells to seed the lung (114). Type 2 pneumocytes and Clara cells might be suitable for alveolar repair and this is currently an active area of research.

E. Pulmonary Vascular Drugs

Pulmonary vascular remodeling as a result of chronic hypoxia is important in some patients with severe disease and leads to right heart failure and

a worse prognosis, as discussed in Chapter 19. Long-term oxygen is the most specific therapy for this complication and reduces mortality. However, long-term delivery of oxygen is expensive; so, there is a search for other drugs that prevent hypoxic vasoconstriction or that selective vasodilate the pulmonary circulation. Although there are so entirely selective pulmonary vasodilators, there is increasing evidence that endothelin antagonists (ET_A-receptor antagonists) may reduce pulmonary hypertension (115). Several endothelin antagonists are in clinical development and Bosentan is now used in the therapy of pulmonary hypertension. Stable prostacyclin analogs are also effective in the therapy of pulmonary hypertension and may be given by inhalation (iloprost), orally (beraprost sodium), or subcutaneous injection (treprostinil) (116). However, endothelin antagonists and prostacyclin analogs have not yet been tested in the treatment of secondary pulmonary hypertension in COPD patients (117).

VIII. DRUG DELIVERY

Bronchodilators are currently given as metered dose inhalers or dry powder inhalers that have been optimized to deliver drugs to the respiratory tract in asthma. However, in emphysema, the inflammatory and destructive process is localized to the lung parenchyma and, in chronic obstructive bronchitis, the predominant irreversible changes are in small airways. This implies that if a drug is to be delivered by inhalation then it should have a lower mass median diameter, so that there is preferential deposition in the lung periphery. Furthermore, patients with severe COPD have a markedly reduced inspiratory capacity and find it difficult to inhale drugs. Hence, it may be more appropriate to give therapy parenterally, as it will reach the lung parenchyma and terminal airways via the pulmonary circulation, but parenteral administration may increase the risk of systemic side effects. Targeted delivery of drugs to particular cell types is another approach to limit toxicity. For example, alveolar macrophages may be targeted by molecules that are packaged to be phagocytosed by these cells. Another important concept is the idea of disease activation of drugs; for example, in COPD, active drugs that are released from inactive prodrugs by elastases might be considered. This would concentrate the active drug at the site of disease activity and reduce systemic exposure.

IX. FUTURE DIRECTIONS

New drugs for the treatment of COPD are greatly needed. While preventing and quitting smoking is the obvious preferred approach, this has proved to be very difficult in the majority of patients. It is important to identify the genetic factors that determine why only a minority of heavy smokers

develop COPD (118), and identification of genes that predispose to the development of COPD may provide novel therapeutic targets, as discussed in Chapter 16. However, it will be difficult to demonstrate the efficacy of novel treatments on the rate of decline in lung function, as this requires large studies over 3 years. Hence, there is a need to develop novel outcome measures and surrogate biomarkers, such as analysis of sputum parameters (cells, mediators, enzymes) or exhaled condensates (lipid mediators, reactive oxygen species) (119). The use of imaging techniques, such as high-resolution computerized tomography (CT), to measure disease progression is another promising approach, as scanning resolution increases (120). It may also be important to more accurately define the presence of emphysema vs. small airway obstruction using CT scans, as some drugs may be more useful for preventing emphysema, whereas others may be more effective against the small airway inflammatory-fibrotic process. More research on the basic cellular and molecular mechanisms of COPD is urgently needed to aid the logical development of new therapies for this common and important disease, for which no effective preventative treatments currently exist.

Of the drugs currently in development, PDE4 inhibitors, p38 MAP kinase inhibitors, and CXCR2 antagonists show particular promise as anti-inflammatory therapies over the next 5–10 years. It is likely that effective anti-inflammatory therapies would not only reduce exacerbations, but also improve symptoms and health status. In the long term, these drugs should slow the decline in lung function and prevent the considerable morbidity imposed by this common disease.

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about the book . . .

Emphasizing the urgent need to clearly understand the underlying cellular and molecular mechanisms involved in COPD, this reference provides an up-to-date perspective on the inflammatory cells, mediators, and molecular pathology of COPD.

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about the editor . . .

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