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DISSERTATION

BRONCHIAL CYTOKINES AND GROWTH FACTORS AUGMENT FIBROBLAST-INDUCED AIRWAY REMODELING IN ADULT ASTHMATICS

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring, 2002

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY CHRISTINA CAROL LEWIS ENTITLED BRONCHIAL CYTOKINES AND GROWTH FACTORS AUGMENT FIBROBLAST-INDUCED AIRWAY REMODELING IN ADULT ASTHMATICS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATON

BRONCHIAL CYTOKINES AND GROWTH FACTORS AUGMENT FIBROBLAST-INDUCED AIRWAY REMODELING IN ADULT ASTHMATICS

There is abundant evidence implicating the role of the bronchial fibroblast as a key orchestrating cell type in airway remodeling, which has been described in many asthmatics. However, it is not yet understood whether its role in this phenomenon involves alterations in fibroblast phenotypic expression, increased fibroblast numbers, increased collagen synthesis, decreased collagen breakdown, or a combination thereof. Of primary interest and concern in the presented studies were the potential contributions that specific cytokines and growth factors, elaborated in the asthmatic airway, may have in modulating fibroblast-induced airway remodeling activities. Airway remodeling may lead to a state of fixed, or irreversible, airway obstruction, as well as an enhanced airway hyperresponsiveness, despite appropriate and aggressive anti-inflammatory therapy. Given the progressive nature of airway remodeling, the knowledge to be gained from research of this nature is of utmost importance, as an enhanced understanding of the mechanisms driving this phenomenon may facilitate the development of more accurately targeted treatments. Improved treatment modalities could potentially prevent, or perhaps even reverse, the clinical sequelae of airway remodeling.

The objective of the research studies presented was to explore the role of the bronchial fibroblast in airway remodeling with respect to cell proliferation, collagen

production, receptor expression, and ultimately fibroblast phenotypic expression. The hypothesis was that specific cytokines and growth factors, important in the asthma phenotype, alter bronchial fibroblast-induced airway remodeling activities, thereby contributing to airway remodeling. By studying airway biopsy tissue obtained from well-characterized subjects with and without asthma, many aspects of the disease phenotype that are difficult to account for in alternative experimental models of asthma were not of concern, thus enabling a more focused and precise investigation of fibroblast biology within the context of asthma.

The exposure of bronchial fibroblasts to interleukins 4 and 13, and dexamethasone, each alone and in combination, resulted in enhanced proliferation in mild/moderate asthmatics, as compared to severe asthmatics and normal controls. Stimulation with platelet-derived growth factor isoform BB resulted in significant procollagen production in fibroblasts from severe asthmatics, compared to mild/moderate asthmatics and normal controls, while having no significant effect on proliferation. Conversely, cysteinyl leukotrienes and insulin-like growth factor-I, each alone and in combination, had no effects on fibroblast proliferation or collagen synthesis that differentiated asthmatic populations from each other or from healthy controls. Finally, eosinophil-derived proteins did not affect fibroblast proliferation or procollagen synthesis in any discernable manner that is informative as to the responsiveness of fibroblasts from asthmatics from those of non-asthmatic controls.

The studies presented are suggestive of potentially heterogeneous fibroblast populations as well as asthmatic populations, that are perhaps as of yet uncharacterized. It is conceivable that the asthmatics in these studies, as well as the fibroblasts derived from

their airways, may constitute significant subpopulations in addition to those already defined based on their clinical disease. The existence of such subpopulations may account for the differences in fibroblast responsiveness among the asthmatic populations to the various types of cytokines and growth factors investigated in these studies.

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Chapter 1

Introduction

1.1. Introduction to Asthma

Bronchial asthma is a complex disease of the airways characterized by airway inflammation, airway hyperresponsiveness, and intermittent, reversible airway obstruction. The etiologies of asthma are incompletely understood, but are thought to be multifactorial, with allergenic, environmental, and infectious components. The airway inflammation of asthma is characterized by a cellular infiltrate composed of lymphocytes, eosinophils, macrophages, neutrophils, and mast cells. Each of these cell types is thought to contribute some aspect to the physiologic changes that characterize asthma, although these are still being defined.

1.2. Introduction to Airway Remodeling in Asthma

As a chronic inflammatory disorder of the airways, asthma, like many other chronic inflammatory disorders, is believed to involve tissue injury and subsequent tissue repair processes that accompany its resolution. It has generally been assumed that the abnormalities in airway physiology and the pathologic events associated with asthma are reversible, either spontaneously or as the result of appropriate treatment. However, within the last decade or so, this assumption has undergone reevaluation as many asthmatics have been shown to exhibit irreversible changes in their lung function, despite

appropriate and sometimes even aggressive anti-inflammatory therapy. Furthermore, dramatic alterations in the architecture of the airway wall and parenchyma, which may be irreversible, have been described in the airways of many asthmatics, including abnormal collagen deposition beneath the epithelial basement membrane, hypertrophy and/or hyperplasia of smooth muscle and mucous glands, and increased vascularity. Among these, the hyalinization and thickening of the epithelial basement membrane (subepithelial fibrosis) has received much attention. The mechanisms underlying the development of subepithelial fibrosis are believed to involve the airway fibroblast, as the dysregulation of many of this cell's normal biological functions could achieve such pathologic events.

1.3. Statement of Hypothesis

Since several aspects of fibroblast biology have been shown to be modulated by many of the cytokines and growth factors elaborated in the asthmatic airway, we hypothesized that the bronchial fibroblast phenotype of the asthmatic differs from that of the non-asthmatic, with respect to cell proliferation and collagen synthesis capabilities, which collectively culminate in its contributing to airway remodeling.

Chapter 2

Review of Pertinent Literature

2.1. Asthma

Bronchial asthma is a very complex, often life-long, inflammatory lung disease that affects all portions of the airways (i.e., the large conducting airways, small airways, and the lung parenchyma) (1,2). It is characterized by chronic airway inflammation, nonspecific bronchial hyperresponsiveness, and variable, although often reversible, airway obstruction. The clinical manifestations of asthma include dyspnea, wheezing, chest tightness, and cough, which may vary from mild and almost undetectable to severe and unremitting (status asthmaticus) (3-5). Asthma affects an estimated 17 million Americans and is the ninth leading cause of hospitalization in the U.S. (6). Evidence from many studies indicates that its etiologies are multifactorial, with allergenic, environmental, infectious (7), familial, socioeconomic, and psychosocial contributions (8-17). Asthma is a tremendous disease burden in the U.S., from both an epidemiological and financial standpoint. According to the National Center for Health Statistics, the morbidity and mortality associated with asthma have been increasing for many years. Asthma morbidity has been on the rise since 1960 (11), as indicated by the prevalence of self-reported asthma cases, asthma physician visits, asthma emergency room visits, and asthma hospitalizations. Asthma mortality has been on the rise since 1980. From 1980 to

1987, the number of deaths attributable to asthma (i.e., with asthma as the underlying cause) increased 31% (18). Inevitably, the financial impact resulting from the morbidity and mortality associated with asthma has also increased. Between 1985-1990, the total estimated cost of asthma in the U.S., that includes both the direct medical expenditures related to health care as well as indirect costs associated with missed school/work and mortality, increased from $4.5 billion to $6.2 billion (19). With such trends, efficient treatment methods for asthma are imperative to minimize the national burden associated with it. Yet, many facets of this complex disease are still poorly understood, making treatment of asthma less than optimal. Thus, the progression of research in asthma is critical, as an enhanced understanding of the pathogenesis of asthma may yield significant mechanistic and therapeutic information that could improve treatment methods, and ultimately decrease the burden of the morbidity, mortality, and financial expenditure associated with asthma.

2.2. Descriptive Pathology of Asthma

Numerous studies have been conducted on both autopsy specimens obtained from patients who have died from asthma and bronchial biopsies acquired via fiberoptic bronchoscopy from living patients to investigate the pathologic changes that occur in the lungs with asthma. From such studies, a rather uniform depiction of the pathologic events involved in asthma has emerged, with only a few inconsistencies. Histologic features of asthma, as evidenced by morphometric analyses of these tissue samples, include a prominent inflammatory cell infiltrate present in the airway, a loss of epithelial integrity, and an abnormally thickened basement membrane. The inflammatory cell infiltrate that

has been described is generally composed of lymphocytes, eosinophils, macrophages, neutrophils, and mast cells (1,4,20-33), although details of the contribution of individual cell type(s) is somewhat variable among the studies. In particular, the significance of both the neutrophil and mast cell in airway inflammation of asthmatics remains unclear. While several studies have failed to find an increased presence of neutrophils in asthma (20,22,23,31,33-36), several investigators have described an enhanced neutrophil component in a number of asthma phenotypes, including severe asthma (29), nocturnal asthma (37), and status asthmaticus (38,39). Similarly, while some investigators have reported non-significant differences in mast cell numbers in asthmatic airways (22,32), others have described significant differences in both mast cell numbers (35,36) as well as mast cell activation (4).

In addition to these inflammatory cell changes, as previously mentioned, alterations to some of the structural cells and components of the airway have repeatedly been described as histologic hallmarks of asthma. Extensive bronchial epithelial cell detachment and resultant denudation of the basal epithelial layer in asthmatic airways, as evidenced via morphometric analyses of post-mortem and endobronchial biopsy tissue samples, has been reported in numerous studies (4,20,28,31,35,36,40,41). Whether this epithelial damage represents an artifactual consequence of sampling has long been a source of debate. However, many investigators in the field would argue that the similarities found between the biopsies obtained in living persons by bronchoscopy and the post-mortem tissue specimens, which would theoretically undergo less sampling damage, support the significance of epithelial damage as a histologic feature of asthma.

Other architectural alterations that have been extensively documented in airway sections from individuals with asthma include thickening or hyalinization of the basement membrane, also known as subepithelial fibrosis (see *Descriptive Analysis of Airway Remodeling* below), and thickening of the airway wall (2,4,20,31,33,40,42-48). Subepithelial fibrosis, in particular, has been shown even in milder forms of asthma (20,31,45), suggesting remodeling of the airways is an innate feature of the disease pathology.

In addition to the aforementioned analyses of airway tissue samples, several investigators have examined the bronchoalveolar lavage (BAL) fluid obtained from the airways of living asthmatics to further characterize the constituents of the asthmatic airway. BAL samples have revealed similar inflammatory cell infiltrates, composed predominantly of lymphocytes, mast cells, macrophages and eosinophils (4,20,26,28,29,34,40). Significant numbers of epithelial cells present in the BAL have also been reported by several investigators (4,20,26,28,40), again suggesting that significant bronchial epithelial damage is an important component of asthma. However, similar to the issue of biopsy acquisition, the epithelial shedding that is evidenced via BAL has undergone scrutiny as a potentially greater marker of the sampling procedure than as one of asthma pathogenesis. Furthermore, although BAL sampling is thought to be representative of both the bronchial and alveolar regions of the lung (i.e., both the large and small airways), neither the true origin of the BAL fluid retrieved nor the location of inflammation it represents can be precisely determined (49). Consequently, BAL analyses, as a means by which to study asthma pathogenesis, while useful, have unique limitations.

Along these lines, a rapidly expanding interest and recent focus in asthma research has been the quest to determine if there are significant differences between the large (>2 mm in diameter) and the small (< 2 mm in diameter) airways, with respect to the pathogenesis of asthma. The small airways have been considerably under-investigated in asthma because of the difficulty of *in vivo* sampling. Post-mortem studies, however, have shown that the entire length of the airways is inflamed in asthma (2,25,50). Furthermore, Haley et al. (25) have described regional variations in the inflammatory cell distribution between the small and large airways upon examination of post-mortem tissue specimens obtained from fatal asthmatics. They reported that the inflammatory cell infiltrate of the small airways was characteristically found within the outer region of the airway wall, while that of the large airways was in the inner region of the airway wall. Additionally, Kraft et al. (1) have reported the presence of inflammation in both large and small airways in the lungs of asthmatics, as evidenced by both transbronchial and endobronchial biopsy specimens obtained from living asthmatics. Further studies are needed to differentiate the significance between the large and small airways, and their relative contributions to the pathogenesis of asthma. Several studies have demonstrated the importance of the small peripheral airways in airflow limitation and airway hyper-responsiveness (51-55). Thus, the small airways may have a greater diagnostic and therapeutic importance than is currently appreciated.

While the spectrum of tissue and BAL investigations that have been performed thus far have greatly contributed to our current understanding of the abnormal pathologic events that occur in the asthmatic airway, the cellular and molecular mechanisms underlying those pathologic events are incompletely understood. However, the most

commonly accepted theories that have been extrapolated from studies in the field suggest that many of the pathologic phenomena occurring in asthma arise from interactions between the resident cells of the airways (i.e., both structural and functional cells) and the inflammatory cells that have infiltrated them. Such cellular interactions have been shown to result in the elaboration of a myriad of cytokines, chemokines, and growth factors (see Table 2.1), many of which have repeatedly been shown to alter various aspects of the biology of airway cells (see Chapters 4, 5, 6, & 7).

Table 2.1. Biological Mediators Associated with Asthma Inflammation and/or Remodeling.

Cytokine/ Mediator	Predominant Cellular Source	Predominant Activities
IL-1	Monocytes/ macrophages/ T lymphocyte	Proinflammatory
TNF-α	Monocytes/ macrophages/ B lymphocyte	Proinflammatory (eosinophil survival)
PDGF	Macrophages/ eosinophils	Mitogen for fibroblast, smooth muscle & epithelial cells
TGF-β	Macrophages/ eosinophils/ fibroblasts	Induction of fibroblast collagen deposition
IL-4	T-lymphocytes/ eosinophils/ mast cells	Proinflammatory; production of IgE; eosinophil recruitment
IL-5	T-lymphocytes/ eosinophils/ mast cells	Eosinophil activation, differentiation & survival
IL-13	T-lymphocytes/ basophils	Promotes IgE production & VCAM-1 expression; fibrogenic
MCP-1	Macrophages/ epithelium	Proinflammatory; monocyte chemoattractant
IFN-γ	Lymphocytes	Antifibrogenic; IgE inhibition
LTC_4	Eosinophil	Proinflammatory
IGF-I	Epithelial cells/ macrophages	Mitogen for fibroblasts and smooth muscle cells
MBP, EDN, EPO, ECP	Eosinophil	Cytotoxic & proinflammatory

IL, interleukin; TNF, tumor necrosis factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; MCP, monocyte chemoattractant protein; LTC4, leukotriene C4; IFN, interferon; IGF, insulin-like growth factor; MBP, major basic protein; EDN, eosinophil-derived neurotoxin; EPO, eosinophil peroxidase; ECP, eosinophil cationic protein.

2.3. Functional Consequences of Airway Inflammation

By definition, bronchial asthma is a chronic inflammatory disorder of the airways associated with airway hyperresponsiveness (AHR) to a variety of stimuli (i.e., non-specific hyperresponsiveness). Whether these two characteristics of asthma are causally related or whether they occur as independent events in the clinical manifestation of asthma is not yet known. One possibility is that the AHR observed in asthma may be a consequence of airway inflammation (56) that leads to alterations in airway smooth muscle contractility. Alternatively, AHR may result from alterations in the autonomic regulation of the airways, or even structural changes in the bronchial wall (see *Airway Remodeling* in section 2.4).

The mechanisms by which inflammatory changes in the airways may lead to alterations in airway smooth muscle are unknown. Several investigators have focused on identifying relationships between specific markers of airway inflammation, as assessed by cellular infiltration, and AHR. However, there is discordance in the findings among these investigators. Some groups have shown a strong relationship between the presence of inflammatory cells and enhanced airway responsiveness (22,23,33,34,57,58), whereas others, have failed to establish such a relationship (30,31,59). However, the common observation that asthmatic airways are equally hyperresponsive to a variety of stimuli has challenged the idea that a single inflammatory cell or mediator may be central to the pathogenesis of asthma and AHR.

As previously mentioned, alterations in the neuronal regulation of the airways may lead to AHR. Interestingly, AHR has been reported in lung transplant recipients in the absence of airway inflammation, suggesting that an altered neural control of airway

smooth muscle tone may play a major role in these individuals (48). However, studies in this area are lacking and inconclusive.

Finally, it is feasible that the airway wall thickening that occurs in bronchial asthma due to edema, cellular filtration, vascular engorgement, and other structural changes, may give rise to exaggerated changes in airway caliber for a given degree of airway smooth muscle shortening (60). In this scenario, the prominent structural alterations of the bronchial wall (collectively termed airway remodeling) that often accompany the disease, may lead to a state of " fixed airway obstruction".

2.4. Airway Remodeling

Historically, asthma has been regarded as a completely reversible obstructive disorder. However, in the last decade, asthma has come to be appreciated as having a major irreversible and progressive component to its pathogenesis. Along these lines, a significant number of asthmatics, both children and adults, have shown evidence of residual airway obstruction (48,61-64). Peat et al. (63) found that individuals with asthma had a greater rate of decline in FEV_1 and a lower baseline lung function than did non-asthmatics when observed over an 18-year time period. Similarly, Schacter et al. (62) found that during a 6-year time period, the loss of pulmonary function over time measured in terms of FEV_1 and the forced expiratory flow at 50% of total lung capacity, was consistently greater for asthmatic adults than for non-asthmatic adults. Lange et al. (61) conducted a 15-year follow-up study of pulmonary function in adults with asthma and likewise found that subjects with asthma had substantially greater declines in FEV_1 over time than those without asthma.

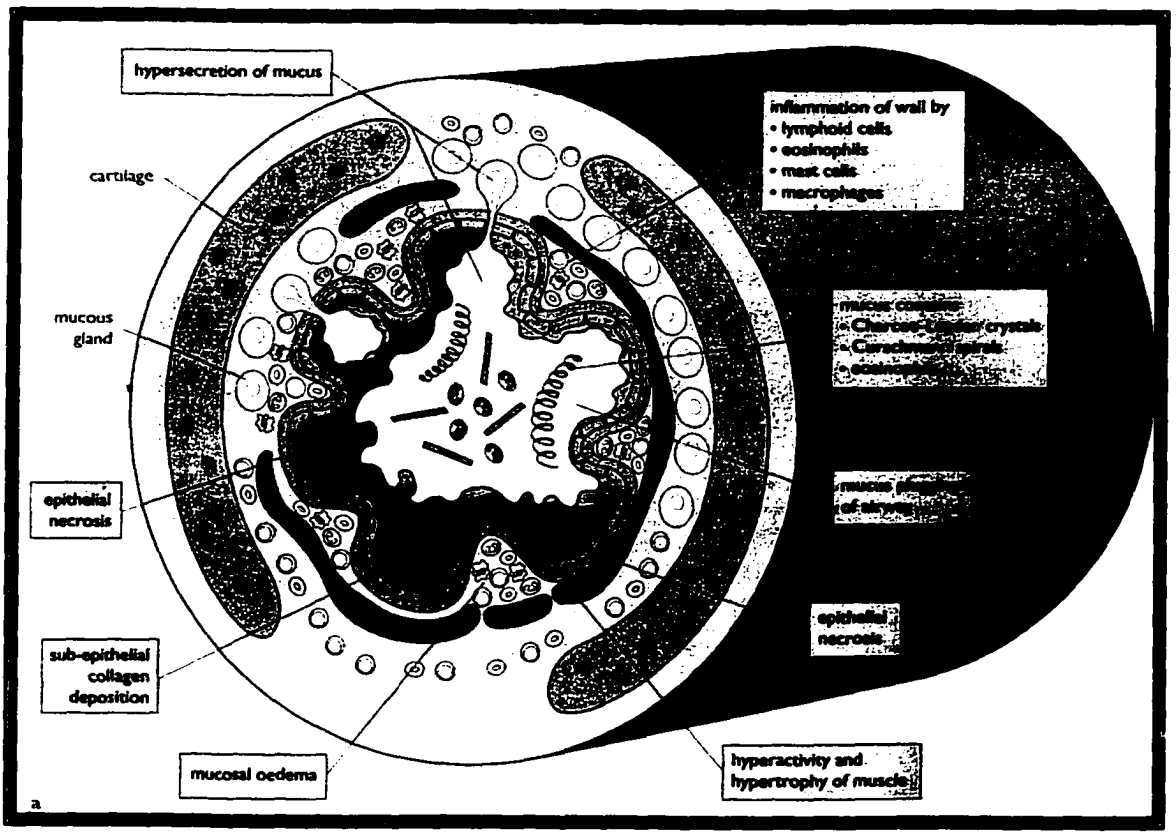
Concomitant to these clinical changes are a spectrum of structural abnormalities and "irreversible" changes of the airways that have been revealed upon evaluation of airway tissues from patients with asthma (46,65,66). These unique pathologic changes, collectively termed airway remodeling, may lead to a state of fixed, or irreversible, airway obstruction, as well as an enhanced AHR despite appropriate and aggressive anti-inflammatory therapy (48,65,67). While the general concept of "remodeling" is often regarded as a healthy response to injury, it is worth noting that in this context, remodeling of the airways is thought of as a deranged wound repair or unchecked inflammation, which are detrimental to the asthmatic airway. Airway remodeling is a dynamic process, likely involving several different events and many cell types, and may potentially be progressive in many asthmatics, yet also potentially preventable and reversible with appropriate therapy (48).

2.5. Descriptive Analysis of Airway Remodeling

Post-mortem specimens obtained from the lungs of asthmatic individuals who had died in status asthmaticus, as well as bronchial biopsies acquired via fiberoptic bronchoscopy from living individuals with asthma, have revealed striking alterations in the bronchial mucosa and submucosa (see Figure 2.1), including edema, inflammatory cell infiltrates, prominent obstruction of both large and small airways with mucus plugs (2,25,40,42,50), a thickened airway wall (2), hypertrophy and/or hyperplasia of the airway smooth muscle (2,40,68-71), hypertrophy and/or hyperplasia of the mucous glands (2,65,66,72), deposition and/or destruction of elastin (47,65,69), angiogenesis or blood vessel proliferation (73), altered expression of matrix metalloproteinases (74), and

thickening or hyalinization of the epithelial basement membrane (i.e., subepithelial fibrosis) (20,31,33,40,43-48), which is thought to be due primarily to increased collagen deposition just underneath the bronchial epithelium by airway fibroblasts (46,75).

Figure 2.1. **Structural Changes in the Remodeled Asthmatic Airway.**



* Adapted from Stevens, et al., 1995

2.6. Physiologic Significance of Airway Remodeling

It is unclear whether airway remodeling is a separate process in asthma, a consequence of the inflammatory response that is only seen in some asthmatics, or the result of repetitive insults to the asthmatic airway. However, irrespective of its etiology, airway remodeling is generally thought to result in altered airway function in a number of

ways (71), including the enhancement of AHR, as evidenced in methacholine PC_{20} values (48), or by inducing changes in other clinical scores associated with asthma, such as FEV_1. For example, Boulet et al. (76) and Cho et al. (77) demonstrated that bronchial subepithelial fibrosis in asthmatics significantly correlated with the AHR exhibited by these individuals, as indicated by their methacholine PC_{20}. Furthermore, several studies have suggested that such clinical sequelae of airway remodeling may correlate with the severity of the remodeling (57,71,77,78), but this remains unclear (79).

Whether airway remodeling itself is a marker of asthma severity remains unclear. However, some investigators have failed to find any significant correlation between the degree of collagen deposition, or other markers of airway remodeling, and the clinical severity of asthma. Furthermore, airway remodeling has been reported in asthmatics of varying severity as well as in patients with a relatively short clinical history or even a new diagnosis of asthma (79). Nevertheless, mathematical modeling studies have demonstrated that any degree of airway remodeling can exaggerate airway narrowing in the absence of any change in smooth muscle physiology, i.e., for a given degree of muscle shortening (70).

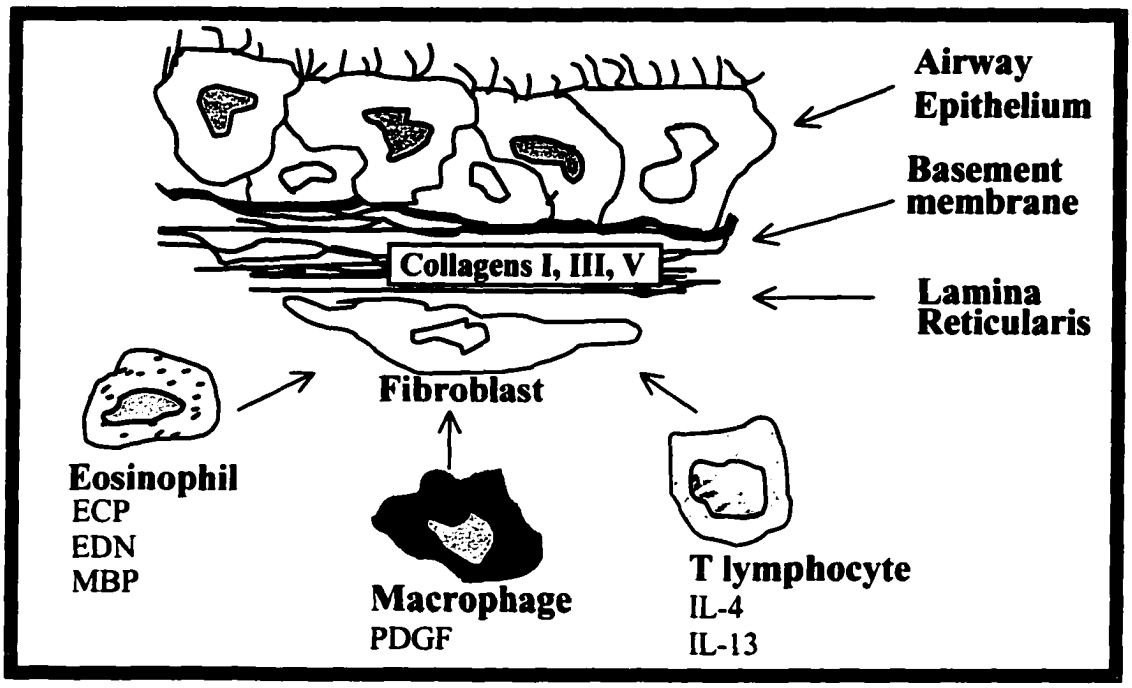
2.7. The Role of the Fibroblast in Airway Remodeling

There are several reasons for implicating the fibroblast as a key orchestrator of the airway remodeling process (see Figure 2.2). First, the fibroblast is known to secrete large quantities of collagen (80), and indeed the subepithelial fibrosis identified in asthmatic airways has been attributed to the abnormal deposition of interstitial collagens (types I, III, V) and fibronectin (31,44,45,75,81). Secondly, it is the reticular layer of the

basement membrane that is primarily thickened in subepithelial fibrosis (31,44,48,59,66,82) and the fibroblast is a major constituent of this layer (45,46,75,83,84). In fact, the prevalence of fibroblasts in this layer appears to be proportional to the severity of remodeling (75,85). Thirdly, cytokines released by the inflammatory and/or epithelial cells of the airways, which are in close proximity to the resident fibroblast, have been shown to modulate various fibroblast functions, including cell proliferation and collagen synthesis (27,44,86-90). Finally, fibroblasts themselves are reportedly capable of producing an array of cytokines and undergoing phenotypic changes that may perpetuate the bronchial inflammatory and/or fibrotic processes (91).

While it is generally agreed upon that the fibroblast is a key contributing cell in the airway remodeling process, it is not yet understood whether its role in this phenomenon involves alterations in fibroblast phenotypic expression, increased fibroblast numbers, increased collagen synthesis, decreased collagen breakdown, or a combination thereof. The relative contributions of each of these mechanisms to airway remodeling are still largely unknown.

Figure 2.2. **Relationship of Fibroblasts and Inflammatory Cells in the Airway.**



2.8. Fibroblast Heterogeneity

Numerous studies support the concept of "tissue-specific fibroblasts." For example, dermal fibroblasts have been shown to differ from pulmonary fibroblasts with respect to morphology, proliferation rates, and synthesis of cytokines and extracellular components (92-94). While this may not seem intuitively unreasonable, as lung and skin have unique functions which likely require specialized populations of fibroblasts, an evolving concept of fibroblast heterogeneity within the same anatomic site has been under investigation. This concept of cellular heterogeneity is not new, as Willmer wrote in 1965 (95): "cells which are morphologically indistinguishable may differ in many biochemical and physiological properties just as men in black coats and white collars may be performing widely different functions in the human community."

Along these lines, several studies involving both normal and disease state fibroblasts indicate that these cells, similar to lymphocytes, are composed of heterogeneous sub-populations with unique phenotypes and functions that have been characterized on the basis of growth, morphology, and metabolic differences. For example, Bordin et al. (96) and Hassell et al. (97) have described functional heterogeneous fibroblast populations derived from healthy gingival tissue with respect to cell morphology, proliferation rates, total protein synthesis, collagen synthesis, and even the types of collagen synthesized. Tipton et al. (98), also studying gingival tissue, found that the basal level of collagenase activity and the production of tissue inhibitor of metalloproteinase (TIMP) varied among fibroblast sub-populations, both between individuals as well as within an individual, and even suggested that such differences may partly explain why some people appear to have a greater susceptibility to fibrotic gingival disorders than do others. Korotzer et al. (99) reported differences among human gingival fibroblast sub-populations to complement-induced cell proliferation. In another model, Breen et al. (100) separated murine lung fibroblasts into distinct sub-populations with differing ratios of type I to type III collagen, a property that influences many important cellular functions, including cell-matrix interactions, proliferation, and cell-cell interactions. Additionally, murine lung fibroblasts have been separated by fluorescence-activated cell sorting on the basis of the thymocyte-1 antigen, the presence or absence of which has been shown to determine morphology, expression of surface markers, antigen presentation to T lymphocytes, ability to synthesize collagen, and cytokine production (101-103). Within the context of asthma, Jordana et al. (104) reported that primary cultures of human adult lung fibroblasts derived from fibrotic lung tissue proliferate

faster *in vitro* than do fibroblasts derived from control lung tissue. Goulet et al. (105) demonstrated that bronchial fibroblasts derived from asthmatics had significantly greater contractile capabilities than did fibroblasts derived from non-asthmatic controls.

Thus, there is abundant evidence that indicates the existence of heterogeneous populations among fibroblasts with respect to a host of important biologic activities, many of which have been implicated in airway remodeling activities (e.g., proliferation, collagen synthesis, collagenolytic activity, and production of TIMP). And, while not all of the aforementioned studies involve pulmonary fibroblasts, the concept of fibroblast heterogeneity as a biological phenomenon that can occur in both healthy and diseased tissue can still be appreciated.

If phenotypically distinct and functionally different sub-populations of fibroblasts exist within the airway tissues of both non-asthmatic and asthmatic individuals, then airway remodeling may not involve cellular injury but instead selection of fibroblast sub-populations with intrinsically greater proliferative and synthetic capabilities. It is conceivable that such fibroblast sub-populations may be positively selected in the inflammatory milieu of the asthmatic lung or under conditions of injury and repair likely to favor the expansion of this phenotype. This seems feasible particularly in light of the fact that various cytokines and inflammatory mediators have been shown to modulate fibroblast proliferation and collagen synthesis. However, whether such unique fibroblast phenotypes require continuous exposure to inflammatory mediators or alternatively the fibroblast subpopulations themselves possess the inherent characteristics such as to explain the abnormalities is unclear. However, needless to say, investigations into the

characterization of fibroblast heterogeneity are critical to our understanding of the pathologic role of the fibroblast in airway remodeling.

2.9. The "Myofibroblast"

The "myofibroblast" phenotype is perhaps the single most well documented fibroblast sub-population known to date. The myofibroblast has structural, chemical, immunological, and functional characteristics that are intermediate between those of the fibroblast and smooth muscle cell (84,93,106,107-110). Immunohistochemically, myofibroblasts are characterized by the presence of actin (a type of microfilamant), vimentin, and/or desmin (intermediate filaments)(106,111). They are specifically distinguished from fibroblasts based on their expression of the α-actin isoform, which is similarly expressed in smooth muscle cells (83,108,110,112-114). It is unclear from which cell type the myofibroblast is truly derived; however, the most likely candidates are the fibroblast (106,113-115), the smooth muscle cell (107,109), and/or the pericyte (i.e., the cell type surrounding small blood vessels) (108,116)

The myofibroblast is known to be an important participant in the normal growth, development, and repair of healthy tissue, as these cells are avid producers of extracellular matrix materials, including collagen, glycosaminoglycans, tenascin, and fibronectin (111,117,118). However, myofibroblast expression is particularly evident during wound repair, where it was first described (119), and during which time its contractile and synthetic activities are essential (111,112,117-120). During normal repair processes, the myofibroblast is only transiently expressed, when additional extracellular matrix materials and contractile activity is needed to facilitate wound closure (83).

However, under pathologic conditions, the myofibroblast can prevail, evolving into an aggressive phenotype, characterized by altered cytokine production (84) and sustained growth and collagen synthesis (43, 83,120-122). In this way, an enhanced expression of myofibroblasts may be representative and/or indicative of major pathology involving tissue fibrosis and remodeling (e.g., idiopathic pulmonary fibrosis, bronchiolitis obliterans and organizing pneumonia, Langerhans cell granulomatosis, hypersensitivity pneumonia, systemic scleroderma, pulmonary hypertension, and nasal polyposis) (83,86,123,124). Along these lines, several investigators have reported increased numbers of myofibroblasts in bronchial biopsies obtained from asthmatics (43,44,75,83,125). The increased number of myofibroblasts and the presence of subepithelial collagen deposition observed in asthmatic airways are thought to reflect the activation of myofibroblasts within the local microenvironment of the airway wall. Interestingly, Gizycki et al. (125) have reported the emergence of myofibroblast populations in endobronchial biopsy specimens from asthmatics following allergen challenge. However, while these studies suggest that the myofibroblast may be a significant fibroblast sub-population underlying airway remodeling activities in asthma, further studies are needed to confirm or refute this concept, as the mechanisms behind the expression of the myofibroblast are incompletely understood, making interpretations about its role difficult and premature. Along these lines, it is worth noting that several studies have indicated that the expression of the myofibroblast phenotype may vary between tissues (116) and can be influenced by numerous environmental factors, such as whether the cells are studied *in situ* or in culture (114,121), and even within a given tissue, whether the cells are activated by hormonal or cytokine treatment or by disease (111). Furthermore, in culture, treatment with

transforming growth factor-β (TGF-β) may induce uniform α-SMA staining of all the cells (83,108), as can interluekin-4 (IL-4) (126) and platelet-derived growth factor (PDGF). Conversely, tumor necrosis factor α (TNF-α), interferon (IFN)-α and IFN-γ decrease the expression of α-SMA in myofibroblasts (108,110).

Chapter 3

Materials and Methods

3.1. Subjects

All study subjects were recruited from the Denver, CO community via newspaper and radio advertisements. According to the July 1, 1997 estimate of the Denver County population demographics, as cited by the Population Estimates Program, Population Division, U.S. Bureau of the Census, the approximate distribution of representative subjects in this area would include 50% female and 36% from minority ethnic groups. The approximate distribution of subjects from ethnic groups would be 21% Hispanic, 12% Black, 2% Asian and Pacific Islander, and 1% Native American. All subjects enrolled in the study were between 18 and 65 years of age, and all female subjects underwent pregnancy testing prior to each bronchoscopy and were required to use an acceptable method of birth control (e.g., birth control pills, diaphragm). A physician or nurse practitioner performed a history and physical examination. Subjects were excluded from the study for any of the following reasons:

1. acute upper and/or lower respiratory infection within 6 weeks of study;
2. use of inhaled or oral steroids, leukotriene receptor antagonists, theophylline, cromolyn, or nedocromil within 6 weeks of the study, and long-acting β_2 adrenergic agonists within 48 hours of study entry;

3. unstable asthma for at least one month (i.e., missed work/school due to asthma or symptoms/signs were not at the usual baseline level);
4. significant non-asthma pulmonary disease, as determined clinically and by the use of spirometery and as diagnosed by the principal investigator Dr. Monica Kraft;
5. pregnancy;
6. a smoking history of greater than 5 pack-years (i.e., 5 years of smoking 1 pack a day) and/or any cigarette use within the last year.

The diagnosis of asthma was made by the principal investigator, Dr. Monica Kraft, in accordance with the criteria of the American Thoracic Society (3). Briefly, subjects were considered to have asthma if they had a clinical history of intermittent chest tightness, wheezing, coughing, and/or shortness of breath, and a documented reversible airway obstruction (12% and/or 200 mL improvement in the forced expiratory volume achieved in one second (FEV_1) in response to inhaled β_2 agonists). In addition to these criteria, all subjects underwent methacholine challenge testing (see *Methacholine Challenge Testing* below), as a measure of bronchial hyperresponsiveness, and pulmonary function tests (spirometry), as an evaluation of airway resistance (127,128). In a methacholine challenge, the bronchial sensitivity of one individual to another is normalized by expressing the degree of bronchoconstriction as a "PC_{20}" value, defined as the provocative concentration of methacholine at which a 20% decrease from the pre-challenge baseline FEV_1 occurs. The lower the PC_{20}, the more sensitive an individual is to methacholine. Subjects were considered to have asthma if they exhibited a methacholine $PC_{20} < 8$ mg/mL and non-asthmatic if they exhibited a methacholine PC_{20} of > 25 mg/mL. Those with methacholine PC_{20} values between 8 and 25 mg/ml were not

included in the study. All asthmatics were maintained on short-acting inhaled β_2 agonists only.

Informed consent was obtained from each subject prior to entry into the study and all aspects of the protocols were explained to each subject by the physician investigator with a technician present as a witness. All research protocols and subject consent forms were approved by the Institutional Review Board at National Jewish Medical and Research Center and by the Colorado State University Human Research Committee.

3.2. Methacholine Challenge Testing

As asthma is a disease characterized by airway hyperresponsiveness, one of the diagnostic criteria used in the assessment of asthma is bronchial reactivity testing. There are several methods currently employed for evaluating bronchial reactivity, most of which yield similar results. However, the most common procedures include histamine and methacholine inhalation, with histamine being the conventional means in Europe and methacholine in North America. Upon inhalation, both histamine and methacholine provoke bronchoconstriction in asthmatics more easily than in non-asthmatics. Methacholine is a derivative of the neurotransmitter acetylcholine, which, upon activation of the cholinergic or muscarinic receptors of the airways, results in bronchial smooth muscle contraction, and subsequent reduction in airway caliber. While the vast majority of normal individuals do not experience any change in lung function following the inhalation of low concentrations of histamine or methacholine, nearly all asthmatics with active disease develop bronchoconstriction with these substances. Furthermore, there is a rough correlation between the sensitivity of the airways to inhaled methacholine and the

severity of asthma, i.e., those with worse asthma tend to be more sensitive.

To determine the presence and/or severity of bronchial hyperresponsiveness and asthma classification for our research protocols, all subjects underwent methacholine challenge testing. The procedures of the methacholine challenge tests were in accordance with the protocols of the National Heart, Blood, and Lung Institute sponsored Asthma Clinical Research Network (ACRN). Inhalation of methacholine was achieved via the dosimeter method (standardized to 0.6 seconds delivery time and 10 seconds standby @ 20.0 psi), in which an electronically controlled valve regulates the amount of methacholine released from a nebulizer to be inhaled during the course of an inspiratory breath. The inspiratory delivery is repeated at 5-minute intervals with increasing concentrations of methacholine (0.078-25 mg/ml; see Table 3.1) and the degree of bronchoconstriction is assessed via alterations in the FEV_1, as measured by the Jaeger Spirometry System (ACRN). The inhalation of methacholine is discontinued when either a 20% or greater reduction in a subject's FEV_1 has occurred or the maximum concentration of methacholine has been administered (25 mg/mL).

Table 3.1. The Sequence of Methacholine Concentrations Administered During a Methacholine Challenge Test.

Solution Sequence	Concentration of Methacholine (mg/ml)
0 (control)	Diluent only
1	0.078
2	0.156
3	0.3125
4	0.625
5	1.25
6	2.5
7	5.0
8	10.0
9	25.0

In accordance with the guidelines of the Asthma Clinical Research Network, subjects were excluded from methacholine challenge testing for any of the following reasons:

1. baseline FEV_1 less than 50% of their predicted values;
2. upper respiratory infection within six weeks (since mild respiratory infections can increase bronchial reactivity for a prolonged period);
3. acute asthma exacerbation requiring the use of oral steroids (e.g., prednisone or a similar anti-inflammatory agent) within six weeks; or
4. use of medications and/or substances that may interfere with accurate measurements of a subject's bronchial reactivity (see Table 3.2).

Table 3.2. Examples of Medications and Substances to Discontinue Prior to Methacholine Testing.

Before test	8 hr Before test	24 hr Before test	48 hr Before test
Alupent	Colas	Choledyl	Salmeterol
Berotec	(Pepsi, Coke)	Palaron	Terfenadine
Brethaire	Coffee	Phylocontin	Tamazepam
Bricanyl	Mello-Yellow	Quibron	Acetominophen
Bronchometer	Mountain Dew	Slo-Bid	with Codeine
Maxair	Tea	Somophyllin	Chlorpheniramine
Proventil	Chocolate	Theo-Dur	Oxymetazoline
Theolair	Alcohol	Theo 24	
Tornolate	Anacin	Uniphyl	
Ventolin	Esgic		
Atrovent	Darvon compound		
Combid	Excedrin		
	Fioricet		
	Fiorinol		
	NoDoz		
	Norgesic		
	Vivarin		

With appropriate precautions, methacholine challenge testing is considered to be a safe procedure, even in severe asthmatics. Routine precautions that ensure the safety of testing subjects, include:

1. the administration of an inhaled bronchodilator to reverse the effects of methacholine if the challenge was terminated because of a 20% reduction in a subject's FEV_1 ; testing subjects are not discharged from the clinic until their FEV_1 has returned to at least 90% of their baseline level;
2. a physician on-site who is readily available to evaluate a subject and supervise treatment in the very unlikely event that there is a severe reaction to methacholine inhalation; and

3. a clearly marked "Emergency Drug Box" is kept accessible in the same room where the methacholine challenge testing is performed so that reactions, should they occur, can be treated promptly. The box includes a metered-dose inhaler bronchodilator (albuterol); iptratropium bromide; ampules of pre-measured bronchodilator and saline, which can be administered via the same nebulizer as the methacholine; injectable epinephrine or terbutaline; and injectable atropine, the specific antidote for methacholine overdose.

3.3. Bronchoscopy & Endobronchial Biopsy

Bronchoscopy with endobronchial biopsy was performed using a fiberoptic bronchoscope (Pentax-FB-15X, Pentax-FB-18P; Orangeburg, NY), as previously described (128). On the day of the procedure, subjects arrived in the laboratory approximately one hour before the bronchoscopy to undergo spirometry. Spirometry was performed using standard ATS procedure (127), both before and after the administration of 0.18 mg of albuterol from a metered dose inhaler. For safety precautions, all research subjects had to demonstrate a post-bronchodilator FEV_1 of > 50% of their predicted value prior to undergoing bronchoscopy. Upon meeting these spirometric requirements, an intravenous catheter was placed in an antecubital vein and prebronchoscopy medications were administered in preparation for the bronchoscopy (see Table 3.3). Lidocaine (4%) was atomized to the oropharynx and nasopharynx to anesthetize the upper airways, and lidocaine (1%) was directly applied to the laryngeal area, trachea, carina, the right and left main stem bronchi, as well as the orifice of the right lower or left lower lobe bronchi through the bronchoscope. The total amount of lidocaine used for local anesthesia for an individual is calculated by using 9 mg/kg conversion (or a maximum of 600 mg if the 9

mg/kg is >600). This standard of administration has been employed because the resultant serum lidocaine levels (< 2.5 mg/L) have been shown to have no clinical toxicity associated with them (129).

Table 3.3. Medications Administered Prior to Bronchoscopy.

Medication	Administered Dose	Target & Administration	Activity
Lidocaine (4%)	5-10 ml (200-400 mg)	Atomized to oropharynx & nasopharynx	Anesthetize upper airway
Lidocaine (2% viscous)	2-4 ml (40-80 mg)	Distributed around mouth or nares where bronchoscope is introduced	Lubrication; not absorbed systemically
Lidocaine (1%)	5-10 ml (100-200 mg)	Vocal cords, trachea, mainstem bronchus & segment location of biopsies	Anesthetize upper & lower Airway
Atropine	0.4 mg	Intravenous	Dry secretions; Vasovagal inhibition
Versed	0.5-2 mg	Intravenous	Sedation
Fentanyl	50-100 μg	Intravenous	Analgesia & Cough Suppression

During the bronchoscopy, endobronchial biopsies were obtained from the second to fourth generation airways. The site of biopsy was randomized to either the right or left lower lobe. Four to five endobronchial biopsy specimens were taken from the tertiary carinae of the right or left lower lobes under direct visualization, using "alligator" type forceps (Olympus, FB-15C). Upon retrieval, biopsies were immediately placed in regular

growth medium (Dulbecco's Modified Eagle's Medium) (see *Cell Culture* below) and held on ice for the duration of the procedure.

Before, after, and throughout the bronchoscopy procedure, supplemental oxygen (4 L/min) was administered via a nasal cannula, and heart rate, blood pressure, and arterial oxygen saturation were monitored continuously. All subjects remained in the laboratory for approximately 1-2 hours for postbronchoscopy observation. Vital signs (blood pressure, heart rate, electrocardiogram, and oxygen saturation) were monitored every 15 minutes for 60 minutes and then every 30 minutes until discharged. Spirometry was performed after the bronchoscopy, and each subject was discharged from the laboratory when his/her FEV_1 was 90% of the prebronchoscopy, postbronchodilator value. If the FEV_1 was < 90% of the pre-bronchoscopy post-bronchodilator value, an inhaled β_2 agonist was administered, and spirometry was repeated after 15 minutes. Each subject was required to have an escort to take him/her home.

Immediately following the bronchoscopy, the biopsy specimens were washed several times with regular growth medium to remove residual mucus and blood, and to prepare the tissue for culturing.

3.4. Cell Culture

Fibroblasts were cultured from the endobronchial biopsies, as previously described (105). Each biopsy (1-2 mm x 1-2 mm) was cut with a surgical blade (size 22) into several smaller pieces (approximately 5-6) and placed into wells (2 pieces/well) of a flat-bottomed, 24-well tissue culture plate (Becton Dickinson, Franklin Lakes, NJ), containing 500 µl of Dulbecco's Modified Eagle Medium (DMEM; containing 1.000

mg/L D-glucose, L-glutamine, pyridoxine hydrochloride, 110 mg/L sodium pyruvate, and 3.7 g sodium bicarbonate, pH ~7.4)(Sigma, St. Louis, MO), supplemented with fetal bovine serum (10%) (GibcoBRL, Grand Island, NY), streptomycin (10,000 μg/ml) (Sigma), penicillin (10,000 U/ml)(Sigma), and gentamicin (100 μg/ml)(Sigma). The biopsies were incubated at 37°C/5% CO_2 (Napco 6500; Precision Scientific;Winchester, VA), and the medium was changed every 2-3 days. The tissue was cultured until the fibroblast growth generated from the biopsies established >50% confluency within the well (approximately 8-20 days). Upon establishing >50% confluency, the fibroblasts were trypsinized (1X Trypsin/EDTA; 0.5 g porcine trypsin, 0.2 g EDTA, 4 Na/L HBSS; Sigma) for 2-5 min to enable cell detachment, after which time the trypsin was deactivated with an equal volume of fetal bovine serum. Freshly trypsinized fibroblasts were centrifuged (Beckman GS-6R; rotor size GH 3.8) @ 1100 rpm for 10 min to obtain a cell pellet. Fibroblast cell pellets were resuspended in 2-4 ml of DMEM, supplemented with 10% FBS, and cell yield and viability were assessed using a hemacytometer and the trypan blue exclusion method, respectively. Quantified fibroblasts were either seeded for experimentation (see *[^{3}H]-thymidine Incorporation* and *Collagen Production* sections below) or plated in tissue culture dishes (60 x 15 mm, polystyrene; Beckman, Fullerton, CA) for a second passage. For repassage, 4 x 10^4 fibroblasts were seeded in a culture dish, containing 2-3 ml of DMEM, supplemented with 10% FBS, incubated at 37°C/5% CO_2, and media changed every 2-3 days. The subsequent fibroblast passages were cultured to near confluency (80-90%)(approximately 5-7 days), at which time the fibroblasts were again trypsinized, quantified, and plated for experimentation and repassaged.

Surplus fibroblasts remaining after experimentation and repassaging demands were resuspended in pure fetal bovine serum, supplemented with 10% dimethyl sulfoxide (DMSO; Sigma) and cryopreserved in liquid nitrogen for future studies.

3.5. Immunohistochemistry

Within each fibroblast passage, an aliquot of 4×10^4 cells was seeded for growth in a standard culture dish (60 x 15 mm, polystyrene) containing cover slips (18 mm glass circles; VWR Scientific, Media, PA) (4 cover slips/ dish) for the purposes of immunohistochemistry. Fibroblasts growing on cover slips were cultured in parallel and under identical culture conditions as the repassaged cells. Upon establishing >50% confluency, the medium was discarded and the cover slips with adherent cells were fixed in methanol/acetone fixative (60:40) and mounted onto slides (1 cover slip per slide) for immunohistochemical staining. Fibroblasts were stained with mouse anti-human antibodies against vimentin (1:25) (DAKO, Carpinteria, CA), fibroblast antigen Ab-1 (1:20)(Calbiochem, San Diego, CA), and α-smooth muscle actin (1:200)(DAKO) to confirm fibroblast and myofibroblast origin.

3.6. [^{3}H]-thymidine Incorporation

As an index of cell proliferation, measurements of DNA synthesis were determined via the [^{3}H]-thymidine incorporation assay, as previously described (122,130,131). Freshly trypsinized and quantified fibroblasts suspended in DMEM supplemented with 10% FBS were plated at a density of 10^4 cells/well in flat-bottomed, 96-well microplates and assayed in triplicate. Seeded fibroblasts were incubated for 24 h

to permit cell adherence to the culture dish. After 24 h, adhered fibroblasts were rinsed with PBS, and replaced with DMEM and serum-starved for an additional 24 h incubation to establish cell quiescence. After this time, the cells were incubated in the presence of the mediator of choice and [^{3}H]-thymidine (5 μCi/ml) (specific activity 6.7 Ci/mmol) (ICN Biomedicals; Irvine, CA) for an additional 24 hours. At the end of this period, the medium was removed, cells rinsed with PBS, and lysed with 200 μl of 0.2 M NaOH (Sigma). Cellular DNA was harvested (FilterMate Harvester, 96-well; Packard Instruments, Meriden, CT) onto glass fiber filter paper (easy tab-C; Packard), and scintillation cocktail (Microscint 20; Packard) was added to the filter papers. The glass fiber filters were then analyzed with a scintillation counter (TopCount Microplate Scintillation & Luminescence Counter; Packard Instruments) to determine the [^{3}H]-thymidine incorporation, as an index of fibroblast proliferation. For all subjects, results were expressed mean [^{3}H]-thymidine incorporation (counts per minute; cpm) ± SEM of all three replicate wells at each passage. Each cell population was passed serially through four passages.

3.7. Quantification of Procollagen I

Fibroblast production of procollagen I was quantified in fibroblast culture supernatant using a sandwich enzyme-linked immunoabsorbent assay (ELISA). For these purposes, fibroblasts were cultured and prepared as described for DNA synthesis except that 5 x 10^4 cells/well were seeded in 24-well plates containing 500 μl of DMEM supplemented with FBS (10%) and incubated for 24 h to permit cell attachment. At the end of this period, the medium was removed, the adherent cells gently rinsed with PBS,

and 500 µl of fresh DMEM (without FBS) were added for an additional 24 h incubation to induce quiescence. After this time, the mediator(s) of choice and ascorbic acid (50 µg/ml)(Sigma) were added to each well and fibroblasts incubated for an additional 24 h. After 24 h, fibroblast culture supernatants were removed, diluted 1:100 in EIA buffer (1 L contained 0.38 g EDTA, 0.1 g NaN_3, 100 ml PO_4 buffer, 23.4 g NaCl, 1 g BSA from Sigma Fraction V; pH ~7.4), and subsequently used to determine procollagen type I production using a standard sandwich ELISA.

Briefly, microtiter plates (96-well; NUNC, Roskilde, Denmark) were coated (100 µl into each well) with mouse anti-human procollagen I (Takara PC-8-7 through Panvera) and diluted in PBS (pH 7.4) to a concentration of 1.0 µg/ml. This medium was allowed to sit 16 to 24 h at room temperature, at which time 225 µl of a saturation buffer (0.1 M potassium phosphate, 1.5 mM sodium azide, 0.4 M sodium chloride, 0.1 mM EDTA, and 0.3% bovine serum albumin) was added to each well. After blocking, ELISA plates were washed (PO_4/Tween buffer) and samples (100 µl) were added to wells in triplicate and incubated for 3 h at 37°C. At this time, plates were again washed and 100 µl of detection antibody (Takara MK101) were added to each well. After an overnight incubation at 4°C, the plates were washed and TMB substrate added. Plates developed for 10-30 min and then development was halted by the addition of 100 µl of 1M phosphoric acid. Plates were read at 450 nm and standard curves were generated with 4-parameter function on Delta Soft software. The assay is sensitive over the range of (0.2 to 100 ng/ml).

3.8. Data Analyses

Only cells from the first through fourth passages were used for data collection, given the potential concern for changes in cell function, including enhanced senescence, that may occur after several subsequent passages (122). The main outcome variables, though somewhat variable between projects, were fibroblast proliferation and procollagen I production at baseline (unstimulated control) and after exposure to mediators. Data were expressed as both absolute values and as fold inductions (i.e., a given condition expressed as a ratio of its unstimulated control). Data from multiple passages (first through fourth) within a subject were averaged together to yield a single data point per condition. The data for each outcome variable was analyzed for between-group comparisons using a simple one-way ANOVA. However, the distributions of all data were determined via the Shapiro-Wilk goodness-of-fit test for normality prior to performing statistical analyses. Further explanation and details of the statistical analyses employed for the individual projects are provided in subsequent chapters.

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Chapter 4

Interleukin (IL)-4, IL-13 and Dexamethasone Augment Fibroblast Proliferation in Asthma

ABSTRACT

Background: IL-4 and IL-13 have been shown to be critical for expression of the asthma phenotype in a murine model, and may modulate human fibroblast function.

Objective: We hypothesized that IL-4 and IL-13 would increase airway fibroblast proliferation and reduce the ability of dexamethasone to decrease this proliferation.

Methods: Six subjects with severe asthma, five subjects with mild asthma, and five healthy subjects underwent bronchoscopy with endobronchial biopsy. Biopsy specimens were placed in Dulbecco's Modified Eagle Medium and cultured, and only fibroblasts from the first and second passages were evaluated. Cells were incubated with IL-4 (50 ng/mL), IL-13 (10 ng/mL), and the combination for 48 hours in the presence and absence of dexamethasone, 10^{-7} mol/L, and 10^{-8} mol/L. Fibroblasts were also incubated with IFN-γ at 50 ng/mL to assess the response of a T_H1 cytokine on proliferation.

Results: Fibroblast proliferation, determined by [^{3}H]-thymidine incorporation, was significantly increased by IL-4 in subjects with mild asthma and healthy subjects ($p=0.003$), IL-13 ($p=0.011$), and the combination ($p=0.004$). Dexamethasone also increased proliferation in the group with mild asthma as compared with the group with

severe asthma and the healthy group (10^{-7} mol/L, p=0.02; 10^{-8} mol/L, p=0.02). IFN-γ did not significantly alter airway fibroblast proliferation.

Conclusion: IL-4, IL-13, and dexamethasone all significantly increased fibroblast proliferation in mild asthma.

ABBREVIATIONS:

IL-4	Interleukin-4
IL-13	Interleukin-13
FBS	Fetal bovine serum
DMEM	Dulbecco's Modified Eagle's Medium
IFN-γ	Interferon-gamma
T_H2	T-helper-2
T_H1	T-helper-1

INTRODUCTION

Airway remodeling in asthma has received attention as a significant alteration of the airways due to derangements in wound repair after injury. Analysis of airway biopsy specimens from patients with asthma has revealed evidence of significant changes in airway structure in addition to increased airway inflammation as compared to controls. Specifically, one such change is subepithelial basement membrane thickening, which has been shown in some studies to correlate with bronchial hyper-responsiveness (1). This morphologic change is believed to be due to increased collagen deposition, particularly types I, III, and V, produced by airway fibroblasts adjacent to the collagen layer (2).

These fibroblasts, which also include myofibroblasts, produce collagen and have been shown to produce specific cytokines that may attract inflammatory cells to the airway and perpetuate inflammatory and fibrotic processes (3,4). These processes, although still being defined, are believed to be detrimental to the host in that they may lead to fixed airflow limitation (5-7).

The cytokines IL-4 and IL-13 are part of the T_H2 subset of cytokines, which are involved in the regulation of B cells and immunoglobulin isotype switching. IL-13, unlike IL-4, acts primarily in the monocyte population to regulate surface antigen expression, antibody-dependent cellular cytotoxicity, and cytokine synthesis (8-10). It has been shown to reduce glucocorticoid binding affinity in monocytes but not in T lymphocytes (11). We have previously shown that in nocturnal asthma, IL-4 and IL-13 in combination reduced steroid responsiveness and glucocorticoid receptor β expression of alveolar macrophages at 4 am (12). In addition, both cytokines have been shown to bind to a human lung fibroblast cell line and induce proliferation (13,14). It is not known whether these receptors are present in fibroblasts from patients with asthma or whether these cytokines modulate fibroblast function in this disease process.

Corticosteroids are first-line therapy in patients with asthma more severe than mild intermittent (15). However, it is unclear whether corticosteroids affect the remodeling process. In regard to fibroblast function, corticosteroids may either reduce or increase fibroblast proliferation and also may exhibit differential effects on other aspects of fibroblast function (16). Some investigators have shown a decrease in proliferation by corticosteroids (17,18), whereas others have shown an increase (19-22). The same issue may be true for collagen production and contractile properties of airway fibroblasts (23).

For instance, Skold et al. (23) have shown that glucocorticoids augment contraction of collagen gels by inhibition of prostaglandin E_2. In vivo, it is unclear whether inhaled corticosteroids reduce subepithelial collagen deposition significantly because the data are conflicting (24,25). We hypothesized that IL-4 and IL-13 would increase airway fibroblast proliferation in patients with asthma and reduce the ability of dexamethasone to decrease this proliferation.

METHODS

Subjects

Six subjects with asthma that was considered severe by American Thoracic Society criteria, five subjects with mild asthma by American Thoracic Society criteria, and five healthy subjects were recruited from the general Denver community. All subjects with asthma met diagnostic criteria for asthma (26), exhibiting a methacholine PC_{20} of less than 8 mg/mL, and were receiving on inhaled β_2-agonists only. The healthy group had no history of asthma and no bronchial hyper-responsiveness (methacholine $PC_{20} > 25$ mg/mL). Exclusion criteria included use of cromolyn, nedocromil, inhaled or oral corticosteroids, leukotriene modifiers, and/or theophylline within the previous 6 weeks; immunotherapy within the previous 3 months; history of cigarette use; and significant nonasthma pulmonary disease or other medical illnesses. Informed consent was obtained from all patients for this institutional review board-approved protocol.

Protocol

Subjects underwent bronchoscopy with endobronchial biopsy as previously described (27). The site of biopsy was randomized to either the right or left lower lobe, and 5 to 6 endobronchial biopsy specimens were taken from the tertiary carinae of the right or left lower lobes under direct visualization. Prior to the bronchoscopy, spirometry was performed before and after administration of 0.18 mg of albuterol from a metered dose inhaler and 0.4 mg atropine intravenously. Xylocaine (4%) was used to anesthetize the upper airway and Xylocaine (1%) was applied to the laryngeal area, trachea, and orifice of the right lower or left lower lobe bronchi via the bronchoscope. Supplemental oxygen was administered throughout the procedure along with monitoring of heart rate and oxygen saturation. Subjects' vital signs were monitored in the laboratory after the procedure, and subjects were discharged from the laboratory when their FEV_1 was 90% of the prebronchoscopy, postbronchodilator value.

Fibroblast Culture

Biopsy specimens were cut into several smaller pieces (~5-6 per biopsy) and placed into wells (2 pieces/well) of a 24-well tissue culture plate containing Dulbecco's Modified Eagle Medium (DMEM), supplemented with FBS (10%), streptomycin (100 μg/mL), penicillin (10,000 U/mL), and gentamicin (100 μg/mL). This medium does not support growth of other cells, such as epithelial and endothelial cells. The tissue pieces were incubated at 37°C/5% CO_2 and cultured until cell growth was established at greater than 50% confluency (~8-20 days), with the media changed every 2-3 days. Upon reaching greater than 50% confluency, the tissue pieces were removed, and the cells were

trypsinized, counted, tested for viability, stained for identification, and plated in 96-well plates for proliferation experiments and in 15 x 60-mm culture dishes for subsequent cell passages (Becton-Dickinson, Franklin Lakes, NJ). All cells were stained with monoclonal mouse anti-human antibodies against fibroblast antigen (Ab-1; Calbiochem, San Diego, CA), vimentin (DAKO, Carpinteria, CA), and α-smooth muscle actin (DAKO), to confirm fibroblast, myofibroblast, and mesenchymal cell specificity, respectively. First-passage cells generated directly from the biopsy tissue were stained on cytospin slides, whereas second-passage fibroblasts were grown directly onto 18-mm coverslips (Fisher Scientific, Pittsburgh, PA) that were placed in the culture dishes upon repassage. Only cells from the first (200-400K) and second (700K-1 million) passages were used for experiments, given the potential concern for changes in cell function, including enhanced senescence, that may occur after several passages (28).

Measurement of Fibroblast Proliferation

For the cytokine and dexamethasone modulation studies, the cells were seeded at a density of 10^4 cells/well in 96-well culture plates in DMEM with 0.5% FBS. A proliferation dose-response study was performed in two subjects with mild asthma and a human lung fibroblast cell line (Clonetics, Walkersville, MD). The concentrations of IL-4 used were 1, 10, 20, 30, 40, 50 and 100 ng/mL. The concentrations of IL-13 used were 10, 20, 30, 40, 50 and 100 ng/ml. After 48 hours, [^{3}H]-thymidine (1 mCi/mL) was added to the cells and incubated for an additional 24 hours. The cells were harvested onto glass fiber filter papers, and [^{3}H]-thymidine incorporation was measured. There was no

significant proliferation dose responses for IL-4 and IL-13, although all concentrations increased proliferation above unstimulated control.

To determine the effects of a T_H1 cytokine on airway fibroblast proliferation, we performed a proliferation dose-response study in two subjects with mild asthma and a human lung fibroblast cell line (Clonetics, Walkersville, MD), using interferon-gamma (IFN-γ). The concentrations of IFN-γ evaluated were 100, 200, 300, 400, 500, 600, and 1000 U/ml. Because there was no significant dose response with either subject or the cell line, fibroblasts from the remaining subjects of the mild asthma group and the healthy group were incubated with IFN-γ at 300 U (50 ng/mL). After 48 hours, [^{3}H]-thymidine (1 mCi/mL) was added to the cells and incubated for an additional 24 hours. The cells were harvested onto glass fiber filter papers, and [^{3}H]-thymidine incorporation was measured.

Incubation with Cytokines and Dexamethasone

Given the lack of a proliferation dose response with IL-4 and IL-13, the concentrations of cytokines chosen were those used by Doucet et al. (14) in their study of IL-4 and IL-13 effects on a human lung fibroblasts cell line. IL-4 at 50 ng/mL and IL-13 at 10 ng/mL and the combination were added to each well in triplicate (R&D, Minneapolis, MN). The fibroblasts were also incubated with the cytokines in the conditions described above in the presence of dexamethasone to achieve concentrations of 10^{-7} mol/L and 10^{-8} mol/L and dexamethasone alone. These concentrations were chosen as they represent the physiologic range of dexamethasone relative to cortisol given their difference in potency. After 48 hours, [^{3}H]-thymidine (1 mCi/mL) was added

to the cells and incubated for an additional 24 hours. The cells were harvested onto glass fiber filter papers and [^{3}H]-thymidine incorporation was measured. Results are expressed as percentage of unstimulated control.

Statistical Analysis

The main outcome variable was fibroblast proliferation at baseline (unstimulated control) and after exposure to IL-4, IL-13, dexamethasone, and the combination of cytokines and dexamethasone. The data were analyzed with a mixed-effects model with fixed effects for group, cytokine, and dexamethasone concentrations; the interactions between dexamethasone, cytokines and groups; plus a random subject effect (29-31). If an interaction was significant, pair-wise comparisons of least squares means were made within groups and within times. This statistical analysis, which is a repeated-measures design, initially provides *p* values when all three groups are compared with one another. Contrasts are then performed to evaluate differences between pairs, and adjustments are made for multiple comparisons. This method was chosen given that repeated measures under different conditions were performed within each group, and a mixed-effects model allows evaluation of all the conditions simultaneously. When *p* values were significant, contrasts were performed to evaluate significance between groups. Within-group comparisons were also performed with means within one group, in which a specific condition is compared with an unstimulated control for that group.

All tests were 2 sided and conducted at the 5% significance level. Data are expressed as mean ± SEM because the data were normally distributed, as determined by the Shapiro-Wilk test (32).

RESULTS

Subjects

Subject characteristics are shown in Table 4.1.

Table 4.1. Subject Characteristics.

	Healthy Group	Mild Asthma	Severe Asthma	P Value
Sex (M/F)	3/2	0/5	4/2	–
Age (y)	32.4 ± 3.4	32.2 ± 3.7	39.8 ± 4.7	0.13
Medicines	None	β_2-agonists	β_2-agonists	–
FEV_1 (L)	3.8 ± 0.4	2.7 ± 0.2	2.4 ± 0.2	0.009
FEV_1(% Predicted)	99.8 ± 4.8	80.8 ± 1.6	65.6 ± 2.6	0.0001

Proliferation

Immunostaining with vimentin (DAKO), Ab-1 (Calbiochem), and α-smooth muscle actin (DAKO) confirmed the fibroblast phenotype where cells were 100% vimentin, more than 95% Ab-1 positive, and less than 5% α-smooth muscle actin positive. The baseline proliferation without stimulation or exposure to cytokines and dexamethasone was greatest in the healthy group, but not significantly so ($p=0.09$, Table 4.2). To take into account differences in baseline proliferation and evaluate proliferation after exposure to cytokines and dexamethasone, we present the data as a percentage of unstimulated control.

Table 4.2. Results of Proliferation of Airway Fibroblasts Incubated with IL-4, IL-13, IFN-γ, and Dexamethasone.

	Healthy Group (n=5)	Mild Asthma (n=5)	Severe Asthma (n=6)	Statistical Condition	*P* value*
Baseline Proliferation†	3384 ± 1136	1386 ± 451	705 ± 177	H > M and S	0.09
Effects of Cytokines & Dex ‡:					
IL-4 (50 ng/mL)	136 ± 10	191 ± 19	77 ± 5	M > H > S	0.003
IL-13 (10 ng/mL)	124 ± 12	177 ± 15	11 ± 12	M > S and H	0.011
IL-4 and IL-13	130 ± 17	196 ± 23	82 ± 10	M > S and H	0.004
IFN-γ (300 U/mL or 50 ng/mL)	89 ± 1	126 ± 33	ND		0.43
Dex 10^{-7} mol/L	100 ± 10	192 ± 31	67 ± 22	M > H, M > S	0.02/0.09
Dex 10^{-8} mol/L	100 ± 10	177 ± 22	58 ± 50	M > S and H	0.02
IL-4, IL-13, & Dex 10^{-7} mol/L	115 ± 13	243 ± 53	103 ± 4	M > S and H	0.07
IL-4, IL-13, & Dex 10^{-8} mol/L	120 ± 17	246 ± 45	190 ± 66	M > S and H	0.10

H, Healthy; *M*, Mild asthma; *S*, severe asthma; *Dex*, dexamethasone; *ND*, not done.
*The depicted *P* value for the designated statistical condition in which the results of the 3 groups were compared by mixed-effects model (see Methods).
†Baseline proliferation is expressed as mean ± SEM in counts per minute of incorporated [3H]-thymidine during 48 hours of incubation without cytokines or dexamethasone.
‡Results for effects of cytokines and dexamethasone are expressed as mean ± SEM of percentage increase from unstimulated control fibroblast cultures

Analysis of proliferation between the three groups revealed that IL-4 and IL-13, each alone and in combination, significantly increased proliferation in the mild asthma group as compared with the severe asthma and healthy groups. Specifically, proliferation after exposure to IL-4 was significantly greater in the mild asthma group as compared with the severe asthma and the healthy groups (p=0.003) (Table 4.2, Figure 4.1). Proliferation after exposure to IL-13 was also significantly greater in the mild asthma group (p=0.011)(Table 4.2, Figure 4.1). The combination of IL-4 and IL-13 also

increased fibroblast proliferation significantly in the mild asthma group as compared to the severe asthma and healthy groups (p=0.004)(Table 4.2, Figure 4.1).

When each group was analyzed separately, within-group comparisons revealed a significant effect by IL-4, IL-13, and the combination of IL-4 and IL-13 in both the mild asthma group (p=0.002 when compared with unstimulated control) and healthy group (p=0.03), but not in the severe asthma group (p=0.81).

Analysis of proliferation between the three groups with the mixed-effects model also revealed a significant group by steroid interaction at p=0.009. Specifically, dexamethasone at 10^{-7} and 10^{-8} mol/L each significantly increased proliferation in the mild asthma group as compared to the severe asthma and healthy group. Proliferation after exposure to dexamethasone at 10^{-7} mol/L was significantly higher in the mild asthma group than in the healthy group (p=0.02). However, proliferation in the mild asthma group was greater than in the severe asthma group, but not significantly so (p=0.09)(Table 4.2). Dexamethasone at 10^{-8} mol/L increased fibroblast proliferation in the mild asthma group as compared with both the severe asthma and healthy groups (p=0.02)(Table 4.2, Figure 4.2).

When each group was analyzed separately, within-group comparisons revealed that dexamethasone at both concentrations increased proliferation significantly in the mild asthma group (p=0.0008 for both concentrations as compared to unstimulated, control), but not in the healthy group (p=0.71). Dexamethasone at both concentrations decreased proliferation in the severe asthma group, and this effect trended toward significance (p=0.06).

The combination of dexamethasone and IL-4/13 also increased proliferation primarily in the mild asthma group, but the differences between the three groups trended toward significance: IL-4/13 + dexamethasone 10^{-7} mol/L, p=0.07 (Table 4.2, Figure 4.2); IL-4/13 + dexamethasone 10^{-8} mol/L, p=0.10 (Table 4.2, Figure 4.2). The same trends were observed when IL-4 and IL-13 were used separately with dexamethasone: IL-4 + dexamethasone 10^{-7} mol/L, p=0.12; IL-4 + dexamethasone + 10^{-8} mol/L, p=0.07; IL-13 + dexamethasone 10^{-7} mol/L, p=0.09; IL-13 + dexamethasone + 10^{-8} mol/L, p=0.18.

Exposure of airway fibroblasts with IFN-γ did not significantly increase proliferation in either the mild asthma or healthy groups (p=0.43, Table 4.2).

Figure 4.1. **Airway Fibroblast Proliferation in Response to IL-4 & IL-13.**

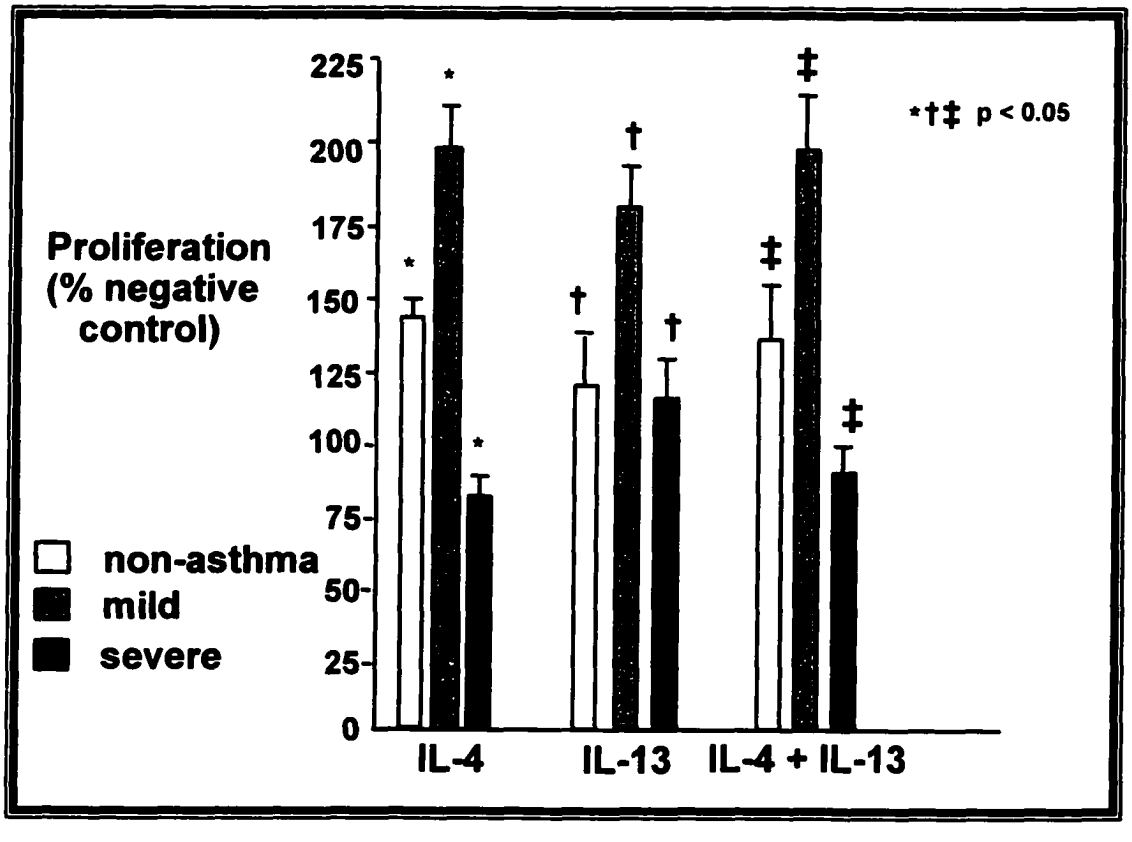
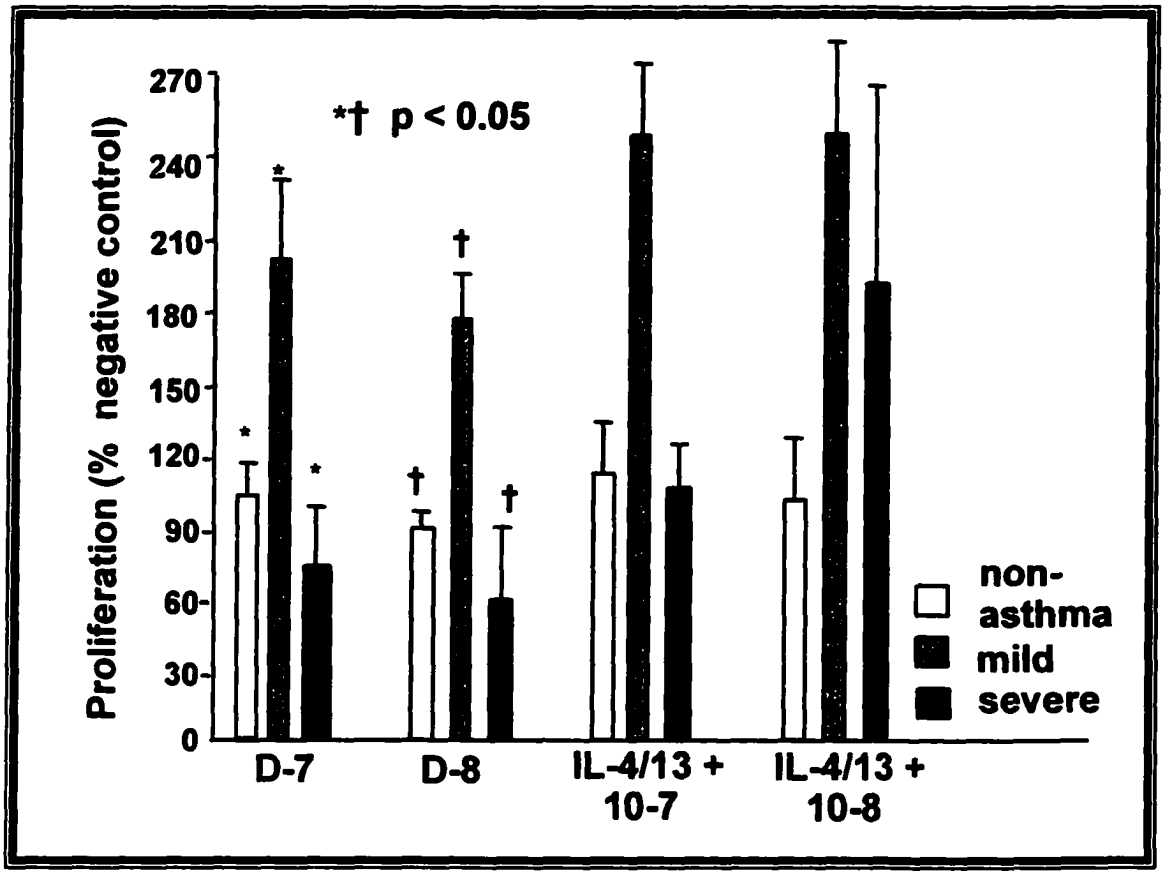


Figure 4.2. Airway Fibroblast Proliferation in Response to IL-4, IL-13, & Dexamethasone.



DISCUSSION

We have shown that in mild asthma, dexamethasone augmented fibroblast proliferation and did not diminish the ability of the cytokines IL-4 and IL-13 to increase proliferation. The augmentation of proliferation did not occur to the same extent in the severe asthma and the healthy group. IFN-γ, a T_H1 cytokine, had no affect on fibroblast proliferation.

The effect of corticosteroids on fibroblast function is controversial. Warshamana et al. (22) have shown in a human lung fibroblast cell line and early-passage rat

fibroblasts that dexamethasone increased fibroblast proliferation. The proliferative effect of corticosteroids was blocked by a polyclonal antibody to the AB isoform of platelet-derived growth factor (PDGF). Dexamethasone also upregulated PDGF receptor α mRNA and protein. The authors concluded that dexamethasone-induced fibroblast proliferation is mediated in part by PDGF-AA, another isoform of PDGF that binds to the PDGF receptor alpha. Dube et al. (28), in fibroblasts obtained from a group of subjects with mild asthma and a group of healthy subjects, determined that dexamethasone also increased proliferation in airway fibroblasts over control. The proliferation observed in the mild asthma group was 73.9% above control, as compared with that in our subjects with mild asthma, who demonstrated proliferation at 91% above control. The subjects studied by Dube et al. had mild asthma by description, but pulmonary function was not reported. In addition, they also showed that PDGF-BB, another isoform of PDGF, increased proliferation in asthmatic fibroblasts 25.3% above control, without any significant change in the fibroblasts from healthy control subjects. In our study, corticosteroids did not affect fibroblast proliferation significantly in the healthy control subjects, trended toward decreasing proliferation in the severe asthma group, and increased proliferation in the mild asthma group. Therefore, it is not clear whether corticosteroids significantly attenuate the remodeling process in asthma, if we are to use fibroblast proliferation as a surrogate measurement. In fact, these data suggest that corticosteroids may enhance proliferation and wound repair as part of the remodeling process, particularly in mild asthma. Clearly, there are other consequences of remodeling that include smooth muscle hypertrophy/hyperplasia, collagen expression, and mucous gland hyperplasia, which were not assessed in this study. In addition, we are using an *in*

vitro model, which may not precisely mimic asthma *in vivo*. However, the fibroblasts were obtained from well-characterized subjects with and without asthma, and only the first and second passages were evaluated.

Each of the cytokines was able to significantly augment proliferation of airway fibroblasts in the mild asthma and healthy groups, with the greatest effect in the mild asthma group. There was no significant effect of either cytokine or the combination on fibroblasts from the severe asthma group. A shared receptor for IL-4 and IL-13 has been demonstrated in a lung fibroblast cell line and thus may provide a mechanism by which both cytokines can augment proliferation without acting synergistically when the two are combined (13,14). Doucet et al. (14) evaluated three different types of fibroblasts (ICIG7, CCL202 and FPA) and found that they all express functional, but different IL-4/IL-13 receptors. The CCL202 and FPA fibroblasts also expressed the IL-13Rα_2 and IL-2Rγ subunits, suggesting the presence of a heterotrimeric receptor (IL-4Rα/IL-13Rα/IL-2Rγ) able to bind IL-4 and IL-13. The presence of these receptors may have implications beyond fibroblast proliferation because IL-4 and IL-13 each have been shown to increase production of the proinflammatory cytokines IL-6 and monocyte chemoattractant protein 1 by airway fibroblasts (13,14). The lack of effect of IFN-γ, a T_H1 cytokine, on airway fibroblast proliferation suggests that the fibroblasts may exhibit differential responses to T_H1 versus T_H2 cytokines. These observations are preliminary, and further study of this differential response is required for validation.

It is of interest that the greatest augmentation of fibroblast proliferation by cytokines and dexamethasone occurred in the subjects with mild asthma. It is possible that the fibroblasts from subjects with severe asthma, exposed to presumed persistent

inflammation and elevated cytokine levels *in vivo*, were unable to respond further to cytokine exposure *in vitro*. Perhaps the receptor number or function has changed in these patients, resulting in reduced responses. Similarly, the receptors may be intact and functional in the healthy control subjects; thus, their fibroblasts are responsive to cytokines *in vitro*. It is also possible that receptor number or function changed *in vitro*, resulting in reduced or increased effects. Although the healthy group's fibroblasts responded to cytokine stimulation, they did not respond to dexamethasone. Thus, there may be a mechanism unique to mild asthma that allows enhanced response to these particular cytokines and to corticosteroids. Further study of cytokine receptors and the modulation of fibroblast production by corticosteroids are needed to elucidate the mechanisms driving these observations.

In summary, we have shown that dexamethasone, IL-4 and IL-13, and the combination of corticosteroid and cytokines augment airway fibroblast proliferation in patients with mild asthma, as compared with subjects with severe asthma and healthy control subjects. Because corticosteroids are recommended as first-line therapy for asthma more severe than mild intermittent, further study regarding steroid and cytokine modulation of fibroblast function is required to determine whether patients experience long-term benefits with the available therapies for asthma.

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Chapter 5

Platelet-Derived Growth Factor Induces a Synthetic Fibroblast Phenotype in Asthma

ABSTRACT

Asthma is characterized by airway inflammation, with subsequent injury and repair. Among the numerous growth factors thought to be active in tissue inflammation and repair processes, platelet-derived growth factor (PDGF) may have a significant role in airway remodeling, as it is a powerful inductor of many fibroblast-induced activities, including proliferation and collagen synthesis. We hypothesized that platelet-derived growth factor is a significant contributor to fibroblast-induced remodeling activities in asthmatics by enhancing airway fibroblast proliferation and procollagen production. Ten subjects with mild to moderate asthma, five with severe asthma, and six non-asthmatic normal controls underwent bronchoscopy with endobronchial biopsy. Biopsies were placed in Dulbecco's Modified Eagle Medium and cultured. Fibroblasts were incubated with PDGF isoforms -AA, -BB, and -AB (1, 5, 10, 100 ng/ml), and [^{3}H]-thymidine incorporation determined. Data were expressed as ratios to the negative control or baseline proliferation. Compared to baseline values, our results indicate that none of the PDGF isoforms significantly enhanced fibroblast proliferation in asthmatics as compared to normal controls. However, PDGF-BB significantly enhanced fibroblast procollagen

production in severe asthmatics, as compared to mild/moderate asthmatics and normal controls. Finally, baseline expression of PDGFR-β was significantly enhanced in severe asthmatics, as compared to mild/moderate asthmatics and normal controls.

ABBREVIATIONS:

PDGF	Platelet-derived growth factor
PDGFR-α	Platelet-derived growth factor receptor alpha
PDGFR-β	Platelet-derived growth factor receptor beta
DMEM	Dulbecco's Modified Eagle's Medium

INTRODUCTION

Significant structural alterations of the bronchial wall (collectively termed airway remodeling) have been repeatedly described in the airway tissues of many asthmatics, leading to the concerning complication of a state of fixed or irreversible airway obstruction (1-3). Among these abnormalities, hyalinization and thickening of the subepithelial basement membrane has been reported (4-6). This morphologic change is believed to be due to increased collagen deposition, particularly types I, III, and V, by the airway fibroblasts adjacent to the collagen layer (7,8). These fibroblasts have been shown to produce specific cytokines that may attract inflammatory cells to the airway, and perpetuate inflammatory and fibrotic processes (9).

Among the numerous growth factors that are active in airway inflammation and tissue repair, platelet-derived growth factor (PDGF) may have a prominent role in altering fibroblast activities linked to airway remodeling. PDGF is a powerful inductor of

fibroblast chemotaxis (10,11), proliferation (12-16), and production of extracellular materials, particularly collagen (14,17). Furthermore, most inflammatory cell types involved in airway inflammation are capable of synthesizing and secreting PDGF, particularly activated macrophages (13,14,17,18), and eosinophils (19), two cell types that have often been linked to asthma (20). Additional evidence of PDGF's potential role in airway remodeling comes from studies that have examined PDGF in the context of airway fibrosis. Several investigators have reported an enhanced expression of PDGF in localized areas of fibrotic lung in patients with idiopathic pulmonary fibrosis (IPF) (17,21,22). Martinet et al. (23) even correlated the spontaneous release of PDGF by alveolar macrophages and the development of fibrosis in IPF.

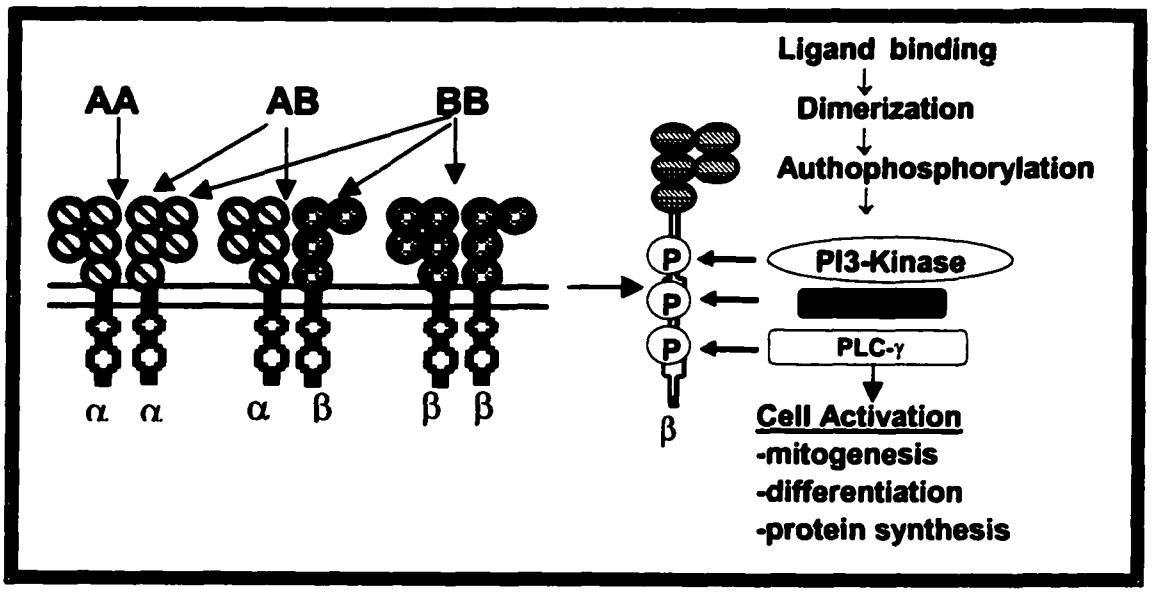
PDGF exists as a homo- or heterodimer consisting of two polypeptide chains, denoted A & B, which can be dimerized via sulfhydryl bridges to form three bioactive isoforms (PDGF-AA, -BB, -AB) (24,25).. The three isoforms bind differentially to cell surface PDGF-α and PDGF-β receptor subunits, which differ in their biologic and functional activities. (26-31). The α and β receptor subunits are dimerized into functional homo- or heterodimer PDGF receptors upon binding of individual PDGF chains to the receptor subunits (32). Binding studies have shown that the α-subunit of the receptor (PDGFR-α) can bind to either the PDGF-A chain or the PDGF-B chain, whereas the β-subunit (PDGFR-β) can bind to only the PDGF-B chain. Thus, PDGF-BB can bind to any one of the three receptor subunit combinations (αα, αβ, ββ); PDGF-AA can bind only to an αα receptor; and PDGF-AB can bind either to an αα or αβ receptor (see Figure 5.1) (27,33,34). Consequently, the biological activities of the PDGF isoforms on a target cell are ultimately dependent upon the expression of receptor subtypes on that cell, as these

will dimerize into the functional receptors accordingly. The functional PDGF receptor is a transmembrane polypeptide (~180 kDa) and a member of the tyrosine kinase family of receptors, which are capable of transducing their signals via autophosphorylation of their own intracellular tyrosine-containing regions of receptor (25,30,31,35-37). Upon phosphorylation, these intra-cellular tyrosine residues physically associate themselves with various intracellular substrates (e.g., MAPK, PLCγ-1, PI3-kinase, GTPase activating proteins, JAK-STAT), which subsequently participate in various signal transduction mechanisms (e.g., stimulation of protein serine/threonine phosphorylation, Ca^{2+} mobilization, cytoplasmic pH changes, expression of certain genes, and rearrangement of actin filaments) that lead to the various PDGF-induced cellular responses, including mitogenesis and protein synthesis (15, 25).

While all of the PDGF isoforms and receptor types have been previously identified on human fibroblasts (28,38), the expression and balance of the various components of this sophisticated system are virtually uncharacterized in the human lung. Consequently, it is also unclear as to whether inflammatory and wound repair mechanisms within the context of asthma and airway remodeling might induce alterations in the PDGF system, thereby perpetuating remodeling activities.

Given the prominent role of PDGF in inflammation and repair, including its ability to activate fibroblasts in a number of ways, we hypothesized that PDGF is a significant contributor to fibroblast-induced remodeling activities in asthmatics. More specifically, we hypothesized that fibroblasts derived from asthmatics exhibit a greater responsiveness to PDGF stimulation than do fibroblasts from healthy controls, which, in part, is due to an altered expression of PDGF receptors.

Figure 5.1. PDGF Dimeric Molecules Bind to Cell Surface Receptor Subunits α and β with Differing Affinity to Elicit Various Biologic Responses.



MATERIALS & METHODS

Subjects

A group of subjects consisting of five severe asthmatics, 10 mild asthmatics (severity determined by American Thoracic Society standards), and six normal, healthy individuals without asthma (non-asthmatic) were recruited from the general Denver community. All asthmatic subjects met diagnostic criteria for asthma (39), exhibiting a methacholine PC_{20} of less than 8 mg/ml, and were maintained on inhaled β_2 agonists only. Exclusion criteria included use of cromolyn, nedocrimil, inhaled or oral corticosteroids, leukotriene modifiers and/or theophylline within 6 weeks prior to the study; an upper respiratory tract infection within 6 weeks prior to the study; immunotherapy within 3 months prior to the study; cigarette use; and, significant non asthma pulmonary disease or other medical illnesses. Informed consent was obtained

from all subjects participating in the study, in accordance with the standards of the institutional review board.

Protocol

Subjects underwent bronchoscopy with endobronchial biopsy as previously described (40). The site of biopsy was randomized to either the right or left lower lobe, and 5 to 6 endobronchial biopsy specimens were taken from the tertiary carinae of the right or left lower lobes under direct visualization. Prior to bronchoscopy, spirometry was performed before and after the administration of 0.18 mg of albuterol from a metered dose inhaler and 0.4 mg atropine, introduced intravenously. Xylocaine (4%) was used to anesthetize the upper airway, and Xylocaine (1%) was applied to the laryngeal area, trachea, and orifice of the right lower or left lower lobe bronchi via the bronchoscope. Supplemental oxygen was administered throughout the procedure along with monitoring of heart rate and oxygen saturation. Subjects' vital signs were monitored in our laboratory after the procedure, and subjects were discharged once their FEV_1 was 90% of their pre-bronchoscopy, post-bronchodilator value.

Fibroblast Culture

Fibroblasts were cultured from endobronchial biopsy tissue as previously described (41). Briefly, biopsy specimens were rinsed and cut into several small pieces (approximately 5-6 per biopsy) and placed into wells (2 pieces/well) of a 24-well tissue culture plate, containing Dulbecco's Modified Eagle's Medium (DMEM), supplemented with fetal bovine serum (10%), streptomycin (100 μg/ml), penicillin (10,000 U/ml), and

gentamicin (100 μg/ml). The tissue pieces were incubated at 37°C/5% CO_2 and cultured until cell growth established >50% confluency (approximately 8-20 days), with the media changed every 2-3 days. Upon reaching >50% confluency, the tissue pieces were removed, and the cells were trypsinized, counted, tested for viability, stained for identification, and placed into 96- and 24-well plates for proliferation and collagen experiments, respectively. Additionally, cells were seeded in 15 X 60-mm culture dishes for subsequent cell passages (Becton Dickinson, Franklin Lakes, NJ). All cells were stained with monoclonal mouse anti-human antibodies against fibroblast antigen (Ab-1; Calbiochem, San Diego, CA), vimentin (DAKO, Carpinteria, CA) and α-smooth muscle actin (DAKO) to confirm fibroblast, myofibroblast, and mesenchymal cell specificity, respectively. First passage cells generated directly from the biospy tissue were stained on cytospin slides while second passage fibroblasts were grown directly onto 18 mm coverslips (Fisher Scientific, Pittsburgh, PA) that were placed in the culture dishes upon repassage. Only cells from the first through fourth passages were used for experiments, given the potential concern for changes in cell function including enhanced senescence that may occur after several passages (42).

Fibroblast Proliferation

To determine cell proliferation, [^{3}H]-thymidine incorporation was measured. Fibroblasts were seeded in 96-well culture plates at a density of 10^4 cells/ well in 200 μl of DMEM supplemented with FBS (10%) and incubated for 24 h to allow cell attachment. The adherent cells were then rinsed with PBS and fresh DMEM supplemented with 0.5% FBS was added to the wells for an additional 24 h incubation to

establish quiescence. After this time, recombinant human PDGF isoforms AA, BB, and AB (R&D Systems; Minneapolis, MN) (1, 5, 10, and 20 ng/ml) and [^{3}H]-thymidine (5 μCi/ml; ICN) were added to each well in triplicate and incubated for an additional 24 h. Following a 24 h incubation with PDGF and [^{3}H]-thymidine, the cell media was removed, and the cells lysed with 0.2 M NaOH. Cellular DNA was harvested onto glass fiber filter papers and the ^{3}H counted via a scintillation counter. Results are expressed as mean counts per minute (cpm) ± SEM.

Quantification of Procollagen I Production

For this assay, cells were seeded at a density of 5 x 10^4 cells/well in 24-well culture plates containing 500 μl of DMEM supplemented with FBS (10%) and incubated for 24 h to permit cell attachment. The cells were then rinsed with PBS and fresh DMEM (without FBS) was added to the wells for an additional 24 h incubation to establish quiescence. After this time, recombinant human PDGF isoforms AA, BB, and AB (R&D Systems; Minneapolis, MN) (1, 5, 10, and 20 ng/ml) and ascorbic acid (50 μg/ml) were added to each well in triplicate and incubated for an additional 24 h. After 24 h, fibroblast culture supernatants were removed, diluted 1:100 in EIA buffer and analyzed for procollagen type I using a standard sandwich ELISA.

Briefly, microtiter plates (96-well; NUNC, Roskilde, Denmark) were coated (100 μl into each well) with mouse anti-human procollagen I (Takara PC-8-7 through Panvera) and diluted in PBS (pH 7.4) to a concentration of 1.0 μg/ml. This medium was allowed to sit 16 to 24 h at room temperature, at which time 225 μl of a saturation buffer (0.1 M potassium phosphate, 1.5 mM sodium azide, 0.4 M sodium chloride, 0.1 mM EDTA, and

0.3% bovine serum albumin) was added to each well. After blocking, ELISA plates were washed (PO_4/Tween buffer) and samples (100 µl) were added to wells in triplicate and incubated for 3 h at 37°C. At this time, plates were again washed and 100 µl of detection antibody (Takara MK101) were added to each well. After an overnight incubation at 4°C, the plates were washed and TMB substrate added. Plates developed for 10-30 min and then development was halted by the addition of 100 µl of 1M phosphoric acid. Plates were read at 450 nm and standard curves were generated with 4-parameter function on Delta Soft software. The assay is sensitive over the range of 0.2 to 100 ng/ml.

Immunofluorescence

Fibroblast expression of PDGF receptors α and β was demonstrated via immunofluoresence cell staining. For immunofluorescence experiments, fibroblasts were grown on 4-well chamber slides (10^4 cells/well), containing DMEM, supplemented with fetal bovine serum (10%), streptomycin (100 µg/ml), penicillin (10,000 U/ml), and gentamicin (100 µg/ml). Freshly seeded fibroblasts were incubated at 37°C/5% CO_2 for 24 h to allow cell attachment. The adherent cells were then rinsed with PBS and fresh DMEM, with no FBS, was added to the chambers for an additional 48 h incubation to establish cell quiescence. After this time, the medium was removed, the adherent cells rinsed with PBS, subsequently fixed with methanol/acetone (60:40), and stored in –20 °C until a later time. For cell staining, slides were first incubated with 10% normal blocking serum diluted in PBS at room temperature for 20 min to suppress non-specific binding of IgG. The slides were then washed with PBS, and incubated with rabbit polyclonal anti-human PDGFR-β (1:20) and PDGFR-α (1:10)(Santa Cruz Biotechnology) antibodies

overnight at 4°C in a humidified box with lid. Subsequently, the slides were rinsed with PBS and incubated with swine anti-rabbit, FITC-conjugated immunoglobulins (1:25, DAKO) for 1 h in the dark at room temperature. After this time, slides were again rinsed with PBS, and 1 drop of fluorescent mounting medium (DAKO) was added to the center of the slide. The fibroblast PDGF receptors were examined under the fluorescent microscope and photographed.

Obtaining Cell Lysate

Whole fibroblast cell lysate samples were obtained in triplicate from fibroblast monolayer cultures (15 x 60-mm culture dishes). Briefly, the medium was removed and the adherent cells were washed with PBS. The adherent fibroblasts were then gently detached from the tissue culture dish via a cell scraper, and 500 µl of PBS containing 50 mM EDTA. A cell pellet was obtained from the cell solution by low speed centrifugation (4500 rpm in microfuge for 4.5 min at 4°C). After microcentrifugation, the supernatant was removed and discarded. Subsequently, 100 µl of ice-cold RIPA lysis buffer (containing 1X PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM EDTA), with freshly added protease inhibitors (PMSF, 10 µl/ml; sodium orthovandate, 10 µl/ml; and Sigma protease inhibitor cocktail for mammalian tissues, containing 104 mM AEBSF, 0.08 mM Aprotonin, 2.1 mM Leupeptin, 3.6 Mm Bestatin, 1.5 mM Pepstatin A, 1.4 mM E-64), was added to the cell pellet. The solution was gently mixed with a pipette and allowed to incubate on ice for 15 min to complete cell membrane disruption, after which time samples were stored in –70 °C freezer, until a later time. Subsequently, the cell lysate samples were thawed gently by keeping on ice. The cell

lysates were further homogenized by passing through a 21-gauge needle. The cell lysate was then spun in microcentrifuge at 10,000xg for 12 minutes at 4°C. After microcentrifugation, the supernatant fluid (total cell lysate) was transferred to a fresh centrifuge tube and the resultant pellet discarded. The cell lysates were kept on ice at all times.

Quantification of Total Cell Protein

Total fibroblast protein in individual cell lysate samples was colorometrically detected and quantified using the bicinchoninic acid (BCA) assay (Pierce; Rockford, IL), according to the manufacturer's protocol. For this purpose, the cell lysates were diluted (1:10) and assayed in RIPA lysis buffer with freshly added protease inhibitors (see above description). Plates were read at 570 nm and standard curves were generated with linear fit on Delta Soft software. The assay is sensitive over the range of 15.6 to 2000 μg/ml.

Quantification of PDGF Receptors α and β

Baseline PDGF receptor protein expression was quantified in fibroblast cell lysates using sandwich ELISAs specific to each of the receptors, α and β. Briefly, microtiter plates (96-well; NUNC, Roskilde, Denmark) were coated (100 μl into each well) with monoclonal mouse anti-human PDGFR-α or -β antibodies (R & D Systems, Minneapolis, MS) diluted in PBS (pH 7.4) to a concentration of 2.0 μg/ml. This medium was allowed to sit 16 to 24 h at room temperature, at which time 225 μl of a saturation buffer (0.1 M potassium phosphate, 1.5 mM sodium azide, 0.4 M sodium chloride, 0.1 mM EDTA, and 0.3% bovine serum albumin) were added to each well. After blocking,

ELISA plates were washed (PO_4/Tween buffer) and samples (100 µl) were added to wells in triplicate and incubated overnight at 4°C. After this time, plates were again washed and 100 µl of the biotinylated polyclonal antibody conjugate were added to each well and the plates incubated for an additional 2 h at room temperature on a shaker. After 2 h, the plates were washed and 100 µl of Avidin-HRP conjugate were added to each well and the plates incubated for 2 h at room temperature on a shaker. Finally, the plates were washed and TMB substrate added. Plates developed for 5-10 min and then development was halted by the addition of 100 µl of 1M phosphoric acid. Plates were read at 450 nm and standard curves were generated with 4-parameter function on Delta Soft software. The sensitivity of the PDGFR-α assay is 0.39 to 50 ng/ml, and that of the PDGFR-β assay is 0.039 to 5 ng/ml. PDGF receptor protein levels were normalized to the total cell protein present in a sample, as obtained via the BCA assay, as described above. Thus, PDGF receptor protein data were expressed as ng of receptor protein/ 100 µg of total cell protein.

Statistical Analyses

The outcome variables were fibroblast proliferation and procollagen I production at baseline (unstimulated control) and after exposure to PDGF-AA, -BB, and –AB, as well as baseline PDGF receptor expression (α and β). Data were expressed as both absolute values and as fold inductions (i.e., a given condition expressed as a % of its unstimulated control). Data from multiple passages (first through fourth) within a subject were averaged together to yield a single data point per condition. The data for each outcome variable was analyzed for between-group comparisons using a simple one-way

ANOVA. The effect(s) of PDGF on the outcome variables was compared between all groups (i.e., severe asthma, mild/moderate asthma, and normal controls) as well as between a pooled asthmatic population (i.e., mild/moderate and severe asthmatics together) and normal controls.

RESULTS

Subjects

Subject characteristics are shown in Table 5.1.

Table 5.1. Subject Characteristics.

	Non-Asthmatic Controls	Mild/Moderate Asthmatics	Severe Asthmatics	P Value
Sex (M/F)	2/4	5/5	4/1	–
Age (y)	26.4 ± 3.7	34.6 ± 2.63	37 ± 3.7	0.13
Medicines	None	β_2-agonists	β_2-agonists	–
FEV_1 (L)	3.67 ± 0.35	3.22 ± 0.25	2.6 ± 0.35	0.12
FEV_1(% Predicted)	102 ± 5.79	89.8 ± 4.10	64.7 ± 5.79	0.0008

Data are presented as means ± standard error of the mean.

Proliferation

Immunostaining with vimentin, Ab-1, and α-smooth muscle actin confirmed the fibroblast phenotype, with 100% of the cells positive for vimentin, >95% positive for Ab-1 expression, and <5% positive for α-smooth muscle actin. The results of fibroblast proliferation with PDGF stimulation are shown in Tables 5.2 and 5.3 (see Appendix for graphical representation). As shown in Table 5.2 and Figure 5.1, baseline fibroblast proliferation without stimulation or exposure to PDGF was numerically greatest in the non-asthmatic group, although the differences between the groups were not statistically

significant when analyzed by either the severity of asthma (severes: 6965 ± 2215 cpm, mild/moderates: 6864 ± 1566 cpm, normal controls: 9101 ± 2022 cpm; p=0.68) or when asthmatic populations were pooled (asthma: 6977 ± 1245 cpm, normal controls: 9101 ± 1968 cpm; p=0.37). However, to account for these differences in baseline proliferation and evaluate true proliferative changes that may have occurred upon exposure to PDGF, the data are also presented as fold inductions, or % of negative, unstimulated control.

Analyses of the absolute values of [^{3}H]-thymidine detected in the proliferation experiments, expressed in cpms, revealed that, with the exceptions of PDGF-BB and -AB at their highest concentrations (20 ng/ml), there were no significant between-group differences in fibroblast proliferation when stimulated with any of the PDGF isoforms. At 20 ng/ml, both PDGF-BB and -AB exposure resulted in greater proliferation numbers in the non-asthmatic controls (p=0.007 and 0.056, respectively), as compared to both mild/moderate asthmatics and severe asthmatics. However, when the proliferation numbers resulting from exposure to these conditions (BB and AB at 20 ng/ml) were expressed as a % relative to their unstimulated or negative control, the data failed to demonstrate the same significant between-group responses (p=0.57 for BB20 and p=0.81 for AB20). Thus, the greater proliferation numbers observed for these conditions in the normal controls are likely only reflections of the greater baseline proliferation values inherent to the cells of this group, rather than a real PDGF-induced response that is preferential to the group. This inference is further supported by the previously stated observation that fibroblasts derived from the non-asthmatic group exhibited greater baseline proliferation values than did the asthmatic groups, although this difference was not statistically significant.

When the asthmatic populations were pooled and compared to the normal controls, analyses still revealed significant between-group differences in response to BB and AB (20 ng/ml; p=0.002 and p=0.05, respectively) that again could not be supported when the data was expressed as % of negative, unstimulated control (p=0.29 and p=0.84). Additionally, when the asthmatic populations were pooled, analyses demonstrated a significantly higher proliferation response in the normal controls when stimulated with the lowest concentration of BB (1 ng/ml), and the data expressed as a % of its negative control (asthmatics, 1.18 ± 0.08; normal controls, 1.47 ± 0.12; p=0.05). However, this enhanced proliferative response in the normal control group as compared to the asthmatic group is not consistently expressed at higher concentrations of PDGF-BB, nor is it evident when the data are expressed as ratios to the negative control. Thus, our data do not indicate that any isoforms of PDGF truly affect fibroblast proliferation differentially between asthmatics and normal controls, or even between asthmatic groups of different disease severity.

Figure 5.2. **Baseline Fibroblast Proliferation Among Subject Groups.**

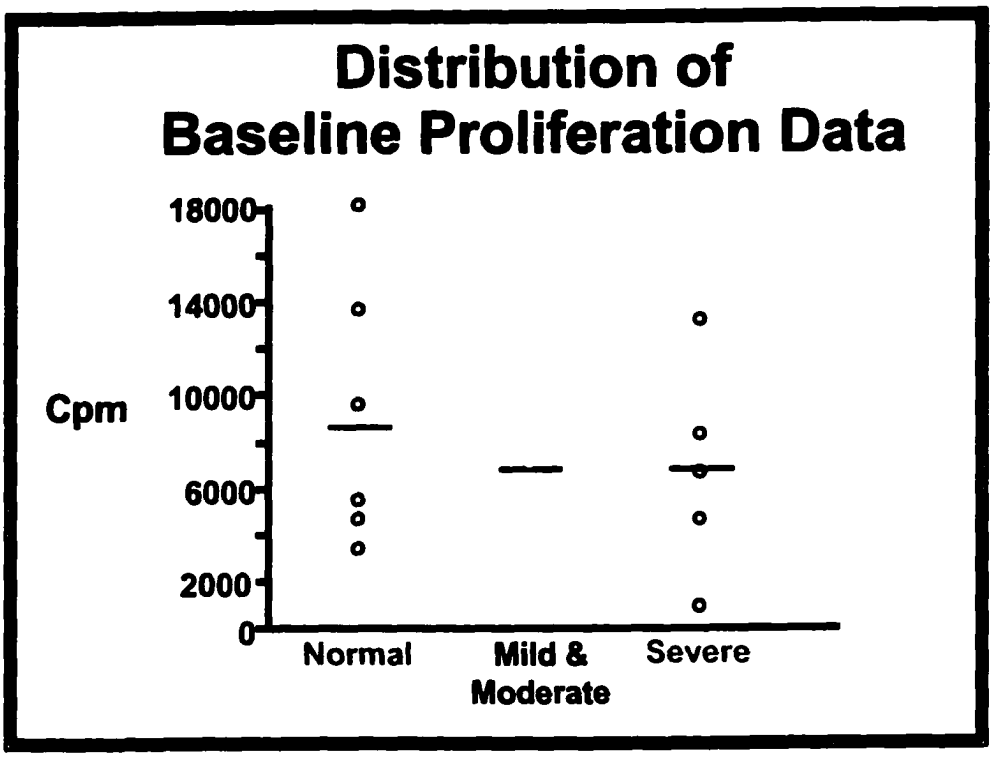


Table 5.2. Results of Fibroblast Proliferation with PDGF Stimulation Analyzed by Severity of Asthma.

	Normal Controls (n=6)	Mild/Moderate Asthma (n=10)	Severe Asthma (n=5)	*P* value
Baseline Proliferation (cpm)	9101 ± 2022	6984 ± 1566	6965 ± 2215	0.68
Effects of PDGF:				
AA (1 ng/mL)	9734 ± 1971 †	7286 ± 1526	8023 ± 2159	0.62
	1.10 ± 0.06 ‡	1.09 ± 0.05	1.10 ± 0.06	0.98
AA (5 ng/mL)	14711 ± 2805	12559 ± 2173	13971 ± 3073	0.82
	1.71 ± 0.30	2.33 ± 0.23	2.10 ± 0.33	0.28
AA (10 ng/mL)	19403 ± 3256	15408 ± 2522	16720 ± 3567	0.63
	2.41 ± 0.49	2.98 ± 0.37	2.43 ± 0.53	0.58
AA (20 ng/mL)	25752 ± 3616	17466 ± 2801	19886 ± 3961	0.22
	3.44 ± 0.59	3.35 ± 0.46	3.17 ± 0.65	0.95
BB (1 ng/mL)	12522 ± 2451	8020 ± 1899	8346 ± 2685	0.34
	1.47 ± 0.01	1.18 ± 0.10	1.18 ± 0.14	0.17
BB (5 ng/mL)	18584 ± 3196	16122 ± 2475	16440 ± 3501	0.82
	2.42 ± 0.55	3.11 ± 0.43	2.67 ± 0.60	0.59
BB (10 ng/mL)	25298 ± 3362	20881 ± 2604	20621 ± 3683	0.54
	3.78 ± 0.93	4.69 ± 0.72	3.46 ± 1.02	0.56
BB (20 ng/mL)	41109 ± 3810	23616 ± 2951	27885 ± 4173	**0.007**
	6.62 ± 1.14	5.34 ± 0.88	4.94 ± 1.25	0.57
AB (1 ng/mL)	9849 ± 2266	9673 ± 1755	9025 ± 2482	0.97
	1.26 ± 0.11	1.46 ± 0.08	1.30 ± 0.12	0.31
AB (5 ng/mL)	20565 ± 3275	18200 ± 2537	19253 ± 3588	0.85
	2.84 ± 0.60	3.62 ± 0.46	3.17 ± 0.65	0.58
AB (10 ng/mL)	27487 ± 3365	22648 ± 2606	23934 ± 3686	0.53
	3.70 ± 0.86	4.88 ± 0.67	4.24 ± 0.94	0.56
AB (20 ng/mL)	36943 ± 4182	23642 ± 3239	25055 ± 4581	**0.056**
	5.27 ± 1.00	5.31 ± 0.78	4.46 ± 1.10	0.81

All Data are Expressed as means ± SEM. † Data shown are absolute values; ‡ Data shown are fold inductions, i.e, condition as a % of unstimulated control. All P values are for between group comparisions.

Table 5.3. Results of Fibroblast Proliferation with PDGF Stimulation, Analyzed with Pooled Asthmatic Populations.

	Normal Controls (n=6)	Asthmatics* (n=15)	*P* value
Baseline Proliferation (cpm)	9101 ± 1968	6977 ± 1245	0.37
Effects of PDGF:			
AA (1 ng/mL)	9734 ± 1922 †	7531 ± 1216	0.35
	1.10 ± 0.06 ‡	1.09 ± 0.04	0.87
AA (5 ng/mL)	14711 ± 2741	13030 ± 1734	0.61
	1.71 ± 0.29	2.25 ± 0.19	0.13
AA (10 ng/mL)	19403 ± 3177	15846 ± 2009	0.36
	2.41 ± 0.48	2.79 ± 0.30	0.51
AA (20 ng/mL)	25752 ± 3543	18272 ± 2241	0.09
	3.44 ± 0.58	3.29 ± 0.36	0.83
BB (1 ng/mL)	12521 ± 2387	8129 ± 1509	0.14
	1.47 ± 0.12	1.18 ± 0.08	**0.057**
BB (5 ng/mL)	18584 ± 3111	16228 ± 1967	0.53
	2.42 ± 0.54	2.96 ± 0.34	0.40
BB (10 ng/mL)	25298 ± 3273	20795 ± 2070	0.26
	3.78 ± 0.93	4.28 ± 0.59	0.65
BB (20 ng/mL)	41109 ± 3779	25039 ± 2390	**0.0019**
	6.62 ± 1.11	5.21 ± 0.70	0.30
AB (1 ng/mL)	9849 ± 2208	9457 ± 1397	0.88
	1.26 ± 0.11	1.41 ± 0.07	0.26
AB (5 ng/mL)	20565 ± 3193	18551 ± 2020	0.60
	2.84 ± 0.59	3.47 ± 0.37	0.37
AB (10 ng/mL)	27487 ± 3283	23077 ± 2076	0.27
	3.70 ± 0.84	4.67 ± 0.53	0.35
AB (20 ng/mL)	36943 ± 4077	24113 ± 2579	**0.0155**
	5.27 ± 0.99	5.03 ± 0.62	0.84

** Includes mild, moderate, and severe asthmatics. All Data are Expressed as means ± SEM. † Data shown are absolute values; ‡ Data shown are fold inductions, i.e, condition as a % of unstimulated control. All P values are for between group comparisions.*

Procollagen Production

The results of fibroblast procollagen I production with PDGF stimulation are shown in Tables 5.4, 5.5, and Figures 5.5, 5.6, and 5.7 (see Appendix for additional graphical representations). Interestingly, as shown in Table 5.2 and Figure 5.5, the baseline fibroblast production of procollagen I without stimulation or exposure to PDGF was greatest in the severe asthmatic group, and this difference between the groups trended toward significance (severes, 3489 ± 705 ng/ml; mild/moderates, 1482 ± 499 ng/ml; normal controls, 2430 ± 644 ng/ml; p=0.08). However, this effect was blunted when the asthmatic populations were pooled (asthma: 2151 ± 452 ng/ml; normal controls 2429 ± 714 ng/ml; p=0.75). While these differences in baseline procollagen production are not statistically significant, the data are shown as both absolute values and as well as fold inductions, or % of negative, unstimulated control, just as with the proliferation data previously mentioned, to account for true PDGF-induced alterations in procollagen production.

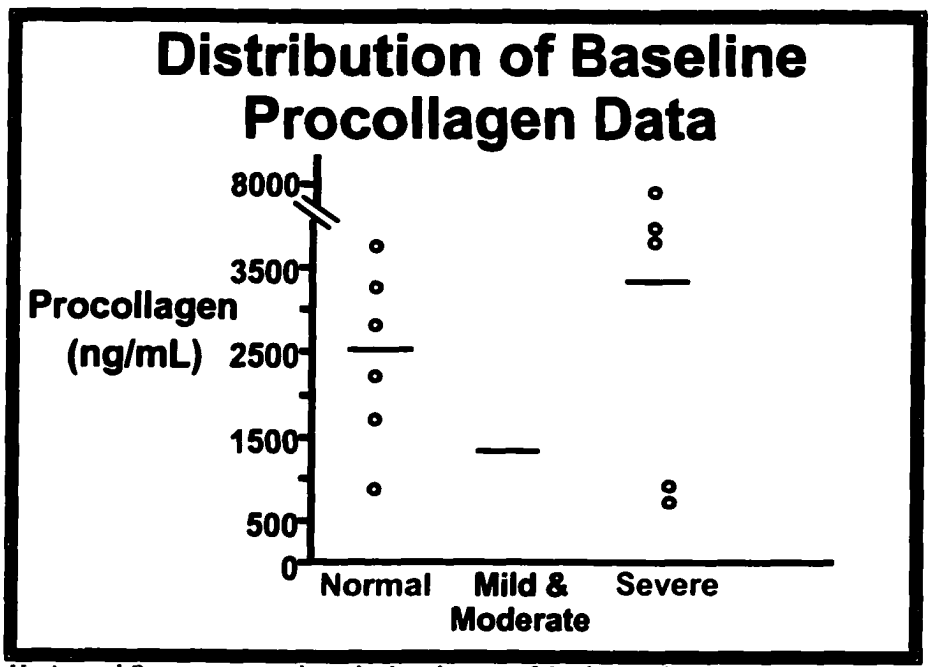
Analyses of the absolute values of procollagen I measured, expressed in ng/mL, revealed that the fibroblasts of severe asthmatics produced a significantly greater amount of procollagen in response to PDGF-BB (20 ng/ml) than did either the mild/moderate asthmatics or the normal controls (severes, 6173 ±1245; mild/moderates, 1781 ± 880; normal controls 2398 ± 1136; p=0.028). Interestingly, the significance of this observation was maintained when the data were expressed as a ratio to the non-stimulated, negative control (Figure 5.7)(severes, 1.63 ± 0.1; mild/moderates, 1.21 ± 0.1; normal controls, 1.04 ± 0.12; p=0.0133).

Further analyses of the procollagen data in this way (when expressed as % of unstimulated control) indicated that the fibroblasts from severed asthmatics also produced more procollagen than did the mild/moderate asthmatics or normal controls at both 5 ng/mL (severes, 1.51 ± 0.1; mild/moderates, 1.15 ± 0.1; normal controls, 0.96 ± 0.12; p=0.0025), and 10 ng/mL (severes, 1.72 ± 0.17; mild/moderates, 1.14 ± 0.12; normal controls, 1.08 ± 0.15; p=0.0018) of PDGF-BB (Figure 5.7). Additionally, as shown in Figure 5.6, stimulation with PDGF-AA (20 ng/mL), also resulted in a greater procollagen production in the fibroblasts of severe asthmatics, as compared to both the mild/moderate asthmatics and the normal controls (severes, 1.43 ± 0.08; mild/moderates, 1.14 ± 0.6; normal controls, 1.09 ± 0.07; p=0.005). There were no significant between-group differences in procollagen production with PDGF-AB stimulation (Figure 5.8). However, it is of great interest that, even when accounting for the differences in baseline procollagen production, the fibroblasts of severe asthmatics produced 40-70% more collagen than did the normal controls with PDGF-BB (5, 10, 20 ng/ML) and PDGF-AB (20 ng/mL) stimulation.

Finally, when the asthmatic populations were pooled and compared to the normal controls, analyses still revealed significant between-group differences in response to PDGF-BB at 5 ng/mL (p=0.018) and at 20 ng/mL (p=0.05), with the fibroblasts of asthmatics producing significantly more procollagen than those of the normal controls. Thus, our data indicate that the PDGF isoform BB significantly increased fibroblast production of procollagen in asthmatics, as compared to normal controls, and that this effect appears to be dependent upon disease severity.

production of procollagen in asthmatics, as compared to normal controls, and that this effect appears to be dependent upon disease severity.

Figure 5.3. **Baseline Fibroblast Procollagen I Production Among Subject Groups.**



Horizontal Bars represent the calculated mean of the data within a group.

Table 5.4. Results of Fibroblast Procollagen Production with PDGF Stimulation, Analyzed by Severity of Asthma.

	Normal Controls (n=6)	Mild/Moderate Asthma (n=10)	Severe Asthma (n=5)	*P* value
BaselineProcollagen (ng/mL)	2430 ± 644	1482 ± 499	3489 ± 705	0.09
Effects of PDGF:				
AA (1 ng/mL)	2893 ± 658 †	1567 ± 570	3315 ± 721	0.15
	1.06 ± 0.11 ‡	1.07 ± 0.09	1.01 ± 0.11	0.91
AA (5 ng/mL)	2627 ± 1004	1634 ± 820	4575 ± 1100	0.13
	1.11 ± 0.09	1.12 ± 0.08	1.17 ± 0.10	0.92
AA (10 ng/mL)	2401 ± 733	1912 ± 598	4212 ± 803	0.09
	1.12 ± 0.10	1.26 ± 0.08	1.21 ± 0.11	0.51
AA (20 ng/mL)	3204 ± 5250	1945 ± 4067	15243 ± 5751	0.18
	1.09 ± 0.07	1.14 ± 0.06	1.43 ± 0.08	**0.005**
BB (1 ng/mL)	2606 ± 4968	1692 ± 4057	13818 ± 5443	0.20
	0.93 ± 0.52	1.09 ± 0.46	2.35 ± 0.61	0.19
BB (5 ng/mL)	2499 ± 1274	1774 ± 1040	6008 ± 1396	0.07
	0.96 ± 0.09	1.15 ± 0.08	1.51 ± 0.10	**0.0025**
BB (10 ng/mL)	2428 ± 1639	1806 ± 1338	7184 ± 1796	0.07
	1.08 ± 0.15	1.14 ± 0.12	1.72 ± 0.17	**0.018**
BB (20 ng/mL)	2398 ± 1136	1781 ± 880	6173 ± 1245	**0.0288**
	1.04 ± 0.12	1.21 ± 0.10	1.63 ± 0.14	**0.0133**
AB (1 ng/mL)	2090 ± 633	1631 ± 517	3219 ± 693	0.21
	0.99 ± 0.06	1.03 ± 0.06	0.89 ± 0.08	0.36
AB (5 ng/mL)	2455 ± 1693	1648 ± 1311	6447 ± 1854	0.13
	1.20 ± 0.09	1.11 ± 0.07	1.27 ± 0.11	0.48
AB (10 ng/mL)	2465 ± 1941	1713 ± 1503	7119 ± 2126	0.13
	1.06 ± 0.11	1.12 ± 0.09	1.38 ± 0.12	0.15
AB (20 ng/mL)	2693 ± 884	1692 ± 685	4235 ± 968	0.13
	1.15 ± 0.10	1.27 ± 0.09	1.4 ± 0.12	0.31

All Data are Expressed as means ± SEM. † Data shown are absolute values; ‡ Data shown are fold inductions, i.e, condition as a % of unstimulated control. All P values are for between group comparisions.

Table 5.5. Results of Fibroblast Procollagen Production with PDGF Stimulation Analyzed with Pooled Asthmatic Populations.

	Normal Controls (n=6)	Asthmatics* (n=15)	*P* value
Baseline Procollagen (ng/mL)	2429 ± 714	2151 ± 452	0.75
Effects of PDGF:			
AA (1 ng/mL)	2893 ± 707 † 1.06 ± 0.11 ‡	2239 ± 480 1.05 ± 0.07	0.45 0.91
AA (5 ng/mL)	2627 ± 1099 1.11 ± 0.09	2684 ± 720 1.14 ± 0.06	0.97 0.84
AA (10 ng/mL)	2401 ± 815 1.12 ± 0.09	2733 ± 534 1.25 ± 0.06	0.74 0.27
AA (20 ng/mL)	3204 ± 5593 1.09 ± 0.07	6378 ± 3538 1.24 ± 0.05	0.64 0.08
BB (1 ng/mL)	2606 ± 5262 0.93 ± 0.54	6023 ± 3445 1.54 ± 0.38	0.59 0.37
BB (5 ng/mL)	2499 ± 1437 0.96 ± 0.10	3286 ± 941 1.28 ± 0.07	0.65 **0.0188**
BB (10 ng/mL)	2428 ± 1843 1.04 ± 0.16	3727 ± 1207 1.37 ± 0.11	0.56 0.11
BB (20 ng/mL)	2398 ± 1337 1.04 ± 0.13	3245 ± 846 1.35 ± 0.09	0.60 **0.0590**
AB (1 ng/mL)	2090 ± 673 0.99 ± 0.07	2198 ± 441 0.98 ± 0.05	0.89 0.89
AB (5 ng/mL)	2455 ± 1841 1.20 ± 0.09	3247 ± 1164 1.16 ± 0.06	0.72 0.77
AB (10 ng/mL)	2465 ± 2103 1.06 ± 0.11	3515 ± 1330 1.20 ± 0.08	0.68 0.28
AB (20 ng/mL)	2693 ± 964 1.15 ± 0.10	2540 ± 610 1.31 ± 0.07	0.89 0.20

** Includes mild, moderate, and severe asthmatics. All Data are Expressed as means ± SEM. † Data shown are absolute values; ‡ Data shown are fold inductions, i.e, condition as a % of unstimulated control. All P values are for between group comparisions.*

Figure 5.4. **Fibroblast Procollagen I Production with PDGF-AA Stimulation by Severity of Asthma.**

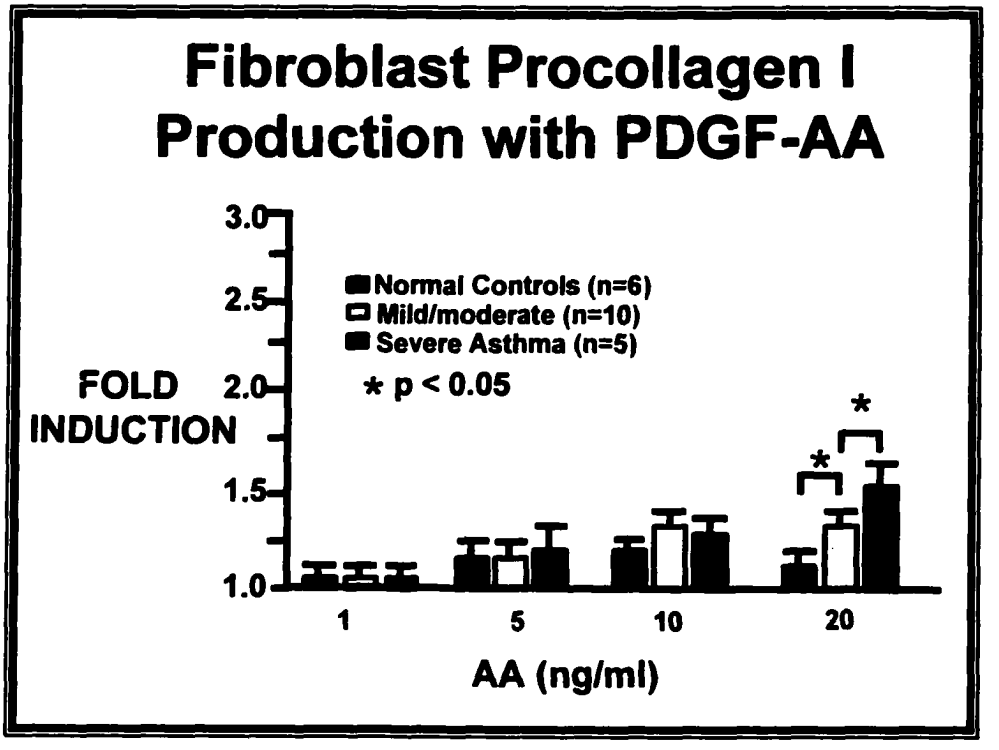


Figure 5.5. **Fibroblast Procollagen I Production with PDGF-BB Stimulation by Severity of Asthma.**

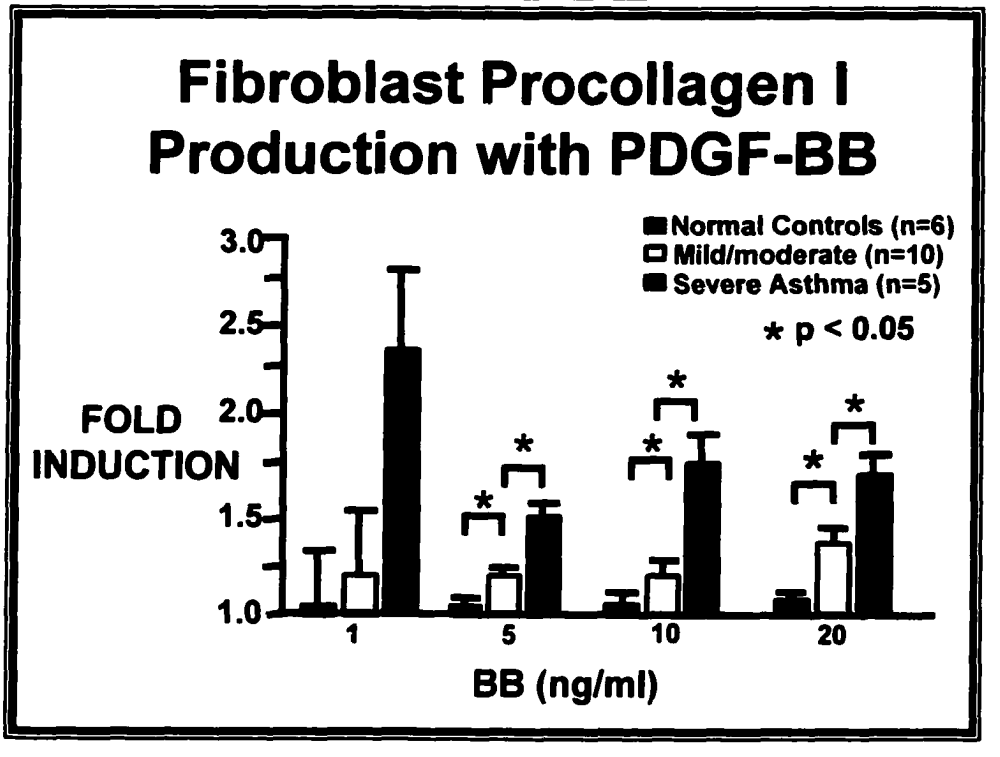
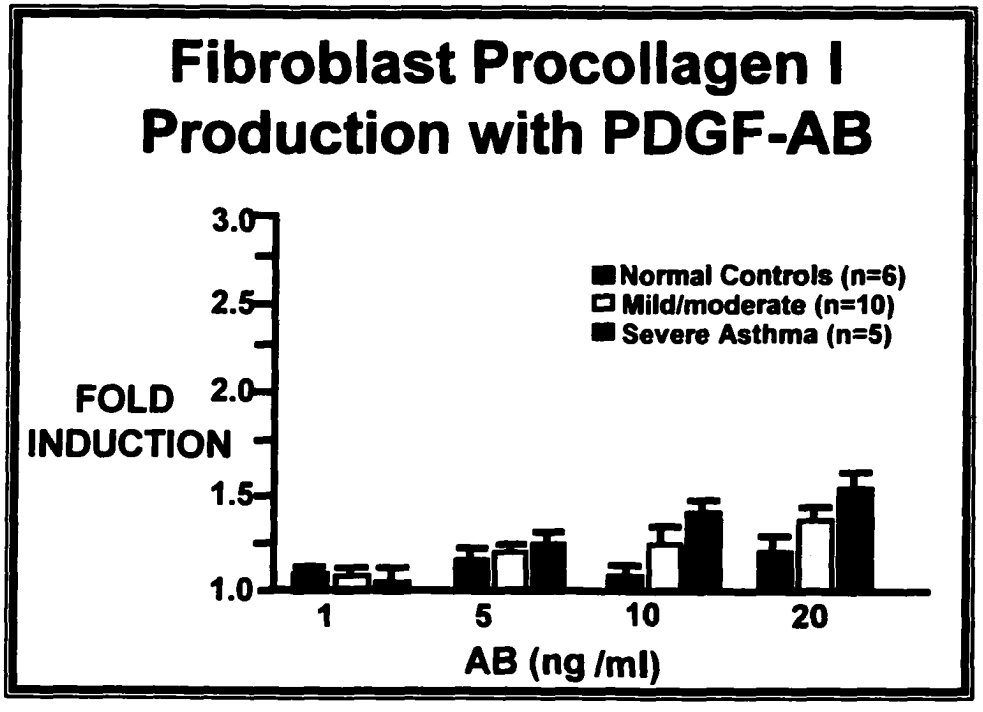


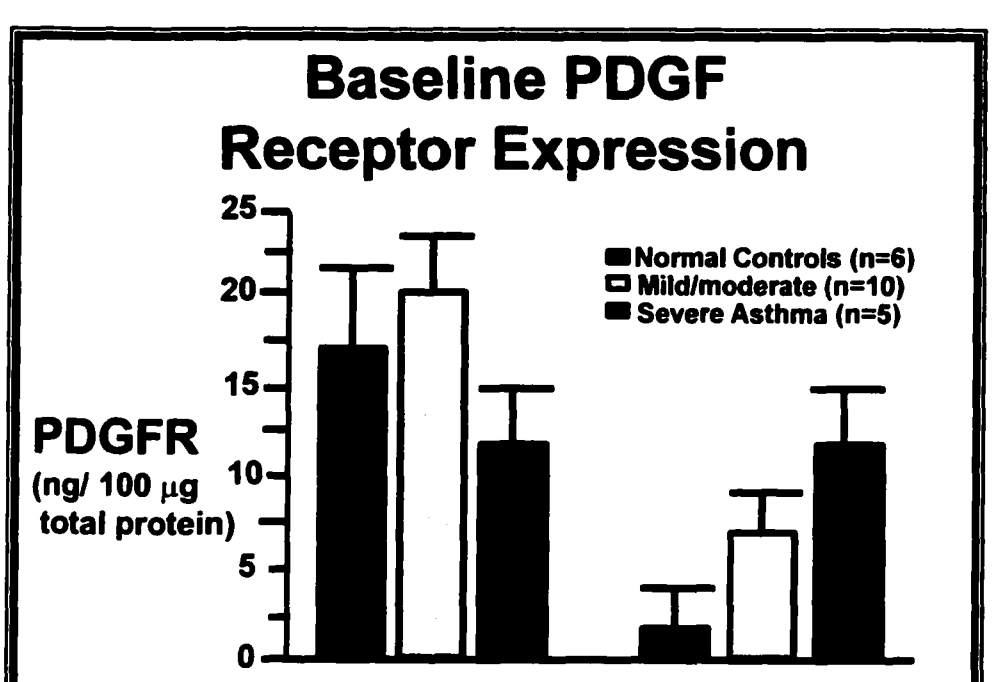
Figure 5.6. **Fibroblast Procollagen I Production with PDGF-AB Stimulation by Severity of Asthma.**



PDGF Receptor Expression

The baseline expression of PDGF receptors α and β are shown in Figure 5.9. Data were normalized to total cell protein and expressed as ng/per 100 μg of total cell protein. There were no significant differences in baseline expression of PDGFR-α between the groups (severe, 11.52 ± 4.91; mild/moderates, 20.27 ± 3.47; normal controls, 17.17± 4.48; p=0.37), or when the asthmatic populations were pooled (asthmatics, 17.36 ± 2.91; normal controls, 17.17 ± 4.61; p=0.97). However, there was a trend of significance in baseline PDGFR-β expression when analyses were performed both between the groups (severe, 11.81 ± 3.48; mild/moderates, 7.05 ± 2.46; normal controls, 1.37 ± 3.17; p=0.11), and when the asthmatic populations were pooled (asthmatics, 8.64 ± 2.02; normal controls, 1.37 ± 3.19; p=0.06).

Figure 5.7. Fibroblast Baseline PDGF Receptor Expression.



DISCUSSION

We have shown that, with the exception of BB (1 ng/mL), none of the PDGF isoforms (AA, BB, nor AB) induced proliferation of fibroblasts differently in asthmatics, as compared to normal controls, when the data were normalized to accomodate the differences in baseline fibroblast proliferation observed between these groups. At its lowest experimental concentration (1 ng/mL), PDGF-BB exposure increased proliferation in the fibroblasts of normal controls, as compared to asthmatics (severe and mild/moderate asthmatic populations pooled). However, this effect was not maintained with higher concentrations of PDGF-BB stimulation, and the level of significance was borderline ($p=0.057$).

Conversely, we have shown significant alterations in fibroblast procollagen production with PDGF-BB stimulation, with the fibroblasts from severe asthmatics

exhibiting a significantly greater production at 5,10, and 20 ng/mL, as compared to mild/moderate asthmatics and normal controls, as well as with AA (20 ng/mL). Although the baseline procollagen production was higher in the severe asthmatics than the other groups (p=0.0890), the data were normalized by expressing the procollagen produced in response to a given condition, as a ratio to its negative, or unstimulated control. In doing so, the procollagen production from fibroblasts of severe asthmatics still maintained significantly higher levels in response to PDGF-BB stimulation at 3 of the 4 concentrations, as did that of PDGF-AA.

Immunofluorescence cell staining revealed the presence of both PDGF-α and -β receptor subunits on the bronchial fibroblasts of both asthmatics and normal controls, with a more predominant expression of PDGF-α as a generalization (data not shown). The intensity of the fluorescence was not quantified, but instead was meant to serve as a basic characterization of the presence of the receptors of interest in our model. For quantification of receptor expression, we chose instead to utilize sandwich ELISAs, specific for each of the receptor subunits. Our data demonstrated that there were no differences in the baseline expression of PDGFR-α between asthmatics (either by group or pooled) and normal controls. However, interestingly, we found trends toward significance in the baseline expression of the PDGFR-β in fibroblasts of severe asthmatics, as compared to mild/moderate asthmatics and normal controls (p=0.11). When the asthmatic populations were pooled, the trend became even closer to significance (p=0.06).

Our proliferation, procollagen, and receptor data are in support of one another because according to the currently accepted model proposed by Seifert et al. (27), greater

amounts of PDGFR-β, and equal amounts of PDGFR-α, would result in an enhanced responsiveness to only PDGF-BB, as this is the PDGF isoform that is able to utilize the functional receptor combination of ββ. PDGF-AB could bind to α β receptor combinations, although the affinity for such binding has been shown to be lower than that of the ββ for PDGF-BB (27). Furthermore, because the biological activities of the PDGF isoforms on a target cell are ultimately dependent upon the expression of receptor subtypes on that cell (i.e., the spatial influence of receptor subunits coming together to dimerize), an enhanced expression of PDGFR-β subunit would undoubtedly influence the effectiveness of PDGF-BB stimulation.

We speculate that the inflammatory milieu of the asthmatic airway drives the over-expression of the PDGFR-β subunit in fibroblasts of asthmatics, and that this effect persists in culture. Furthermore, we believe that this over-expression of PDGFR-βdrives the expression of the synthetic fibroblast phenotype observed in our studies, having altered capabilities in collagen production. Our immunohistochemical analyses, which reveal a fibroblast population with <5% α-smooth muscle actin positivity, rule out the expression of the myofibroblast phenotype as the source of enhanced collagen production observed in the fibroblasts of asthmatics. The existence of a "synthetic" fibroblast phenotype in asthmatics is of great interest and significance as a potential mechanism underlying the fibroblast-induced activities that are thought to contribute to airway remodeling in asthmatic airways.

Our data are in agreement with those of several investigators who have observed the increased expressions of PDGFR-β subunits in conjunction with various conditions of inflammation and wound repair (43-47). Additionally, our data agree with Aubert et al.

(18), who reported an enhanced expression of PDGFR-β in asthmatics when analyzing total lung RNA samples, although this was in comparison to subjects with COPD.

More peripherally, the findings of Oates et al. (48) have revealed the ability of TGF-β treatment of human periodontal ligament cells to induce an increased number of β receptor subunits displayed by the cells. This is of interest because TGF-β is a potent fibrogenic mediator in many systems, and its ability to enhance PDGFR-β expression under pro-fibrotic conditions may suggest a connection between the two. However, this is purely speculative, and the aforementioned study did not address collagen synthesis.

Finally, Hagood et al. (49) revealed that heterogeneous populations of rat lung fibroblasts, classified on the basis of surface expression of the Thy-1 glycoprotein, expressed significantly different amounts of PDGFR-α, both at the protein and RNA levels. In their studies, they found that these differences in receptor expression could account for differential proliferative responses to PDGF-AA, with those cells expressing decreased levels of PDGFR-α lacking a mitogenic response to AA. These findings demonstrate an important and relevant functional difference between these fibroblast subsets and support the concept of fibroblast heterogeneity with respect to PDGFR subunit expression.

There is much work to be done in this area, particularly within the context of asthma and airway remodeling, where inflammatory and wound repair mechanisms likely involving PDGF at some level are activated, and the knowledge of PDGF's involvement is largely unexplored.

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Chapter 6

The Role of the Cysteinyl Leukotrienes and the Insulin-like Growth Factor Axis in the Fibroblast

ABSTRACT

Significant eosinophil influx with evidence of activation and degranulation has been described in the airways of many asthmatics. Additionally, increased numbers of eosinophils and eosinophil products have been described in areas of fibrosis. Upon activation, eosinophils are capable of producing large amounts of cysteinyl leukotrienes. Leukotrienes have long been recognized in asthma for their ability to induce bronchial constriction and increase vascular permeability, but a role in remodeling has not been determined. We hypothesized that the eosinophil directly potentiates the airway remodeling process by altering the proliferative and synthetic capabilities of the fibroblast, and that this effect is mediated through its enhanced production of cysteinyl leukotrienes and subsequent modulation of the IGF axis in the fibroblast. For our studies, 10 subjects with mild to moderate asthma, 5 with severe asthma, and 8 normal controls underwent bronchoscopy with endobronchial biopsy. Biopsies were placed in Dulbecco's Modified Eagle Medium and cultured. Fibroblasts were incubated with LTC4 (10^{-6}, 10^{-8}, 10^{-10}, 10^{-12} M), in the presence and absence of IGF-I (100 ng/ml) and [^{3}H]-thymidine incorporation determined. Procollagen I production was determined via a sandwich

ELISA. Data were expressed as ratios to the negative control or baseline proliferation. Compared to baseline values, our results indicate that LTC_4 and IGF-I, alone and in combination, did not significantly enhance fibroblast proliferation or fibroblast procollagen production in asthmatics as compared to normal controls. However, fibroblast IGF-BP3 production was significantly greater in normal controls, as compared to the mild/moderate and severe asthmatic groups, in response to some combinations of cysteinyl leukotrienes and IGF-I.

ABBREVIATIONS:

IGF-I	Insulin-like growth factor I
IGFBP3	Insulin-like growth factor binding protein 3
LTC_4	Leukotriene C_4
LTD_4	Leukotriene D_4
LTE_4	Leukotriene E_4
DMEM	Dulbecco's Modified Eagle's Medium

INTRODUCTION

As a consequence of chronic inflammation, asthma is invariably associated with airway tissue remodeling as the lungs continually attempt to heal (1), thus often leading to prominent structural alterations of the bronchial wall (collectively termed airway remodeling) that may lead to fixed airway obstruction. Although airway remodeling likely results from several events, proliferative and synthetic abnormalities of airway fibroblasts have been strongly implicated in this process. However, the link between

airway inflammation and airway remodeling has yet to be clarified. While the role(s) that each of the inflammatory cell types may contribute to asthma and airway remodeling has not been elucidated, the role of the eosinophil as a participant in these processes has been repeatedly demonstrated (2-7). Eosinophilia of the blood (3,4), bronchial tissues (3,8,9), and sputum (10,11) has historically been recognized as one of the classical hallmarks for many, although not all (12), asthmatics. Furthermore, the extent of peripheral blood eosinophilia has been shown to be inversely correlated to both the decrease in FEV_1 (7,13) observed in bronchial asthma and to the clinical severity of bronchial hyperresponsiveness (3,7,10,14-16). Thus, in this context, the eosinophil is believed to be a significant contributor to the pathogenesis of asthma, via its ability to act as a proinflammatory and epithelial-damaging effector cell (5,6,17-20). However, very little is understood about its role, if any, in the repair and/or remodeling process that is often observed in the asthmatic airway. Yet, eosinophils are capable of releasing an array of products upon activation, including several cytokines, unique granule proteins (5,20-23), prostaglandins, and leukotrienes (24,25). Among these, the leukotrienes (LTs) have been implicated in many of the inflammatory symptoms associated with asthma (26-29). Leukotrienes (LTs) are metabolites of arachidonic acid, and include LTA_4, LTB_4, LTC_4, LTD_4, and LTE_4. Elevated levels of LTC_4 have been described in the bronchoalveolar lavage (BAL) fluid of patients with asthma, both during spontaneous asthma exacerbations (30) and after antigen challenge (31). Classically, the LTs in asthma have been recognized for their ability to stimulate the contraction of bronchial and vascular smooth muscle, increase vascular permeability, and induce metabolic activation of leukocytes (26-28).

Outside the context of asthma, previous investigations have found LTC_4 to be mitogenic in human airway epithelial cells (32), epidermal keratinocytes (33), glomerular epithelial cells (34), arterial smooth muscle cells (35), and even fibroblasts (36). These findings may provide a link between the eosinophil and the fibroblast, and the role that the interaction between these two cells has in airway remodeling activities. While the physiological mechanism(s) by which cysteinyl leukotrienes promote proliferation in these cells has yet to be elucidated. it is possible that these mediators, when present in abnormally high concentrations, can modulate the homeostasis of the normally occurring growth mechanism in the fibroblast known as the insulin-like growth factor (IGF) axis.

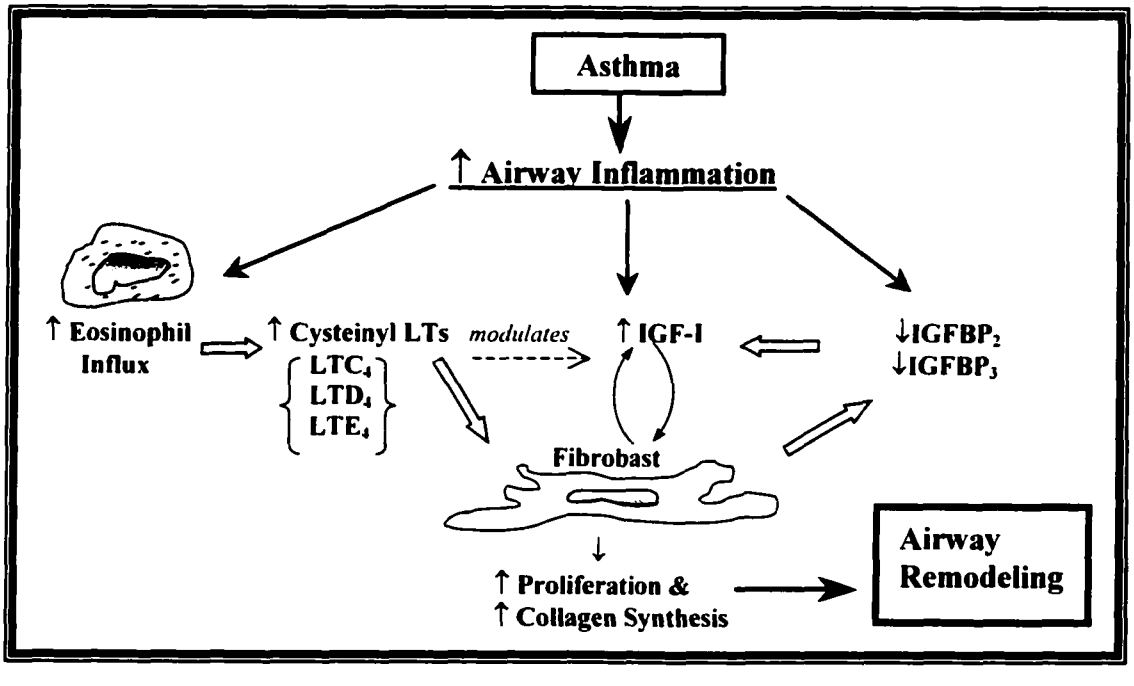
The IGF axis is a fundamental and ubiquitously expressed pathway that is crucial to the regulation of *in vitro* and *in vivo* growth and proliferation in a wide variety of cell types, including the fibroblast (37,38). The components of this system include the IGFs (IGFs -I and IGF-II), their receptors. the IGF binding proteins (IGFBPs), and the IGFBP proteases, with a change in any one of these elements resulting in alterations of the growth potential of the surrounding cells and tissue. More specifically, the IGFs are potent mitogens whose actions are determined by their availability to interact with their respective receptors. The IGFBPs comprise a family of six proteins that bind to the IGFs with high affinity and specificity, thereby regulating their bioavailability, and therefore IGF-dependent action (39). Finally, the cleavage of IGFBPs by specific proteases modulates levels of both free IGFs and IGFBPs and therefore their actions.

The expression of the various components of the IGF axis is tissue specific and tightly regulated. An emerging theme of recent studies is that many hormones, growth factors, and mitogenic peptides influence cell proliferation, in part, by altering the

expression of IGF, IGFBP, and IGF receptors regulating IGFBPs (40,41). Interestingly Yateman et al. (42) have demonstrated that the cytokines TGF-β and TNF-α resulted in changes in fibroblast proliferation via modulation of the fibroblast's production of IGFBP3. Additionally, Cohen et al. (43) and Rajah et al. (44) have shown that LTD_4 facilitates airway smooth muscle cell proliferation via modulation of the IGF axis in this cell. However, to our knowledge, there have been no studies conducted on the modulation of the IGF axis in fibroblasts derived from the airways of asthmatics.

As schematically depicted in Figure 6.1, we hypothesized that the eosinophil directly potentiates the airway remodeling process by altering the proliferative and synthetic capabilities of the fibroblast, and that this effect is mediated through its enhanced production of cysteinyl leukotrienes and subsequent modulation of the IGF axis in the fibroblast.

Figure 6.1. Cysteinyl Leukotriene-Mediated Enhancement of the IGF Axis in the Fibroblast.



MATERIALS & METHODS

Subjects

A group of subjects consisting of five severe asthmatics, 10 mild asthmatics (severity determined by American Thoracic Society standards), and six normal, healthy individuals without asthma (non-asthmatic) were recruited from the general Denver community. All asthmatic subjects met diagnostic criteria for asthma (45), exhibiting a methacholine PC_{20} of less than 8 mg/ml, and were maintained on inhaled β_2 agonists only. Exclusion criteria included use of cromolyn, nedocrimil, inhaled or oral corticosteroids, leukotriene modifiers and/or theophylline within 6 weeks prior to the study; an upper respiratory tract infection within 6 weeks prior to the study; immunotherapy within 3 months prior to the study; cigarette use; and, significant non asthma pulmonary disease or other medical illnesses. Informed consent was obtained from all subjects participating in the study, in accordance with the standards of the institutional review board.

Protocol

Subjects underwent bronchoscopy with endobronchial biopsy as previously described (46). The site of biopsy was randomized to either the right or left lower lobe, and 5 to 6 endobronchial biopsy specimens were taken from the tertiary carinae of the right or left lower lobes under direct visualization. Prior to bronchoscopy, spirometry was performed before and after the administration of 0.18 mg of albuterol from a metered dose inhaler and 0.4 mg atropine, introduced intravenously. Xylocaine (4%) was used to anesthetize the upper airway, and Xylocaine (1%) was applied to the laryngeal area,

trachea, and orifice of the right lower or left lower lobe bronchi via the bronchoscope. Supplemental oxygen was administered throughout the procedure along with monitoring of heart rate and oxygen saturation. Subjects' vital signs were monitored in our laboratory after the procedure, and subjects were discharged once their FEV_1 was 90% of their pre-bronchoscopy, post-bronchodilator value.

Fibroblast Culture

Fibroblasts were cultured from endobronchial biopsy tissue as previously described (47). Briefly, biopsy specimens were rinsed and cut into several small pieces (approximately 5-6 per biopsy) and placed into wells (2 pieces/well) of a 24-well tissue culture plate, containing Dulbecco's Modified Eagle's Medium (DMEM), supplemented with fetal bovine serum (10%), streptomycin (100 μg/ml), penicillin (10,000 U/ml), and gentamicin (100 μg/ml). The tissue pieces were incubated at 37°C/5% CO_2 and cultured until cell growth established >50% confluency (approximately 8-20 days), with the media changed every 2-3 days. Upon reaching >50% confluency, the tissue pieces were removed, and the cells were trypsinized, counted, tested for viability, stained for identification, and placed into 96- and 24-well plates for proliferation and collagen experiments, respectively. Additionally, cells were seeded in 15 X 60-mm culture dishes for subsequent cell passages (Becton Dickinson, Franklin Lakes, NJ). All cells were stained with monoclonal mouse anti-human antibodies against fibroblast antigen (Ab-1; Calbiochem, San Diego, CA), vimentin (DAKO, Carpinteria, CA) and α-smooth muscle actin (DAKO) to confirm fibroblast, myofibroblast, and mesenchymal cell specificity, respectively. First passage cells generated directly from the biospy tissue were stained on

cytospin slides while second passage fibroblasts were grown directly onto 18 mm coverslips (Fisher Scientific, Pittsburgh, PA) that were placed in the culture dishes upon repassage. Only cells from the first through fourth passages were used for experiments, given the potential concern for changes in cell function including enhanced senescence that may occur after several passages (48).

Fibroblast Proliferation

To determine cell proliferation, [^{3}H]-thymidine incorporation was measured. Fibroblasts were seeded in 96-well culture plates at a density of 10^4 cells/ well in 200 µl of DMEM supplemented with FBS (10%) and incubated for 24 h to allow cell attachment. The adherent cells were then rinsed with PBS and fresh DMEM supplemented with 0.5% FBS was added to the wells for an additional 24 h incubation to establish quiescence. After this time, recombinant human IGF-I (R&D Systems: Minneapolis, MN) (100 ng/ml), LTC_4 (Caymen Chemical; MI)(10^{-6}, 10^{-8}, 10^{-10}, and 10^{-12} M) and [^{3}H]-thymidine (5 µCi/ml; ICN) were added to each well in triplicate and incubated for an additional 24 h. Following a 24 h incubation with the mediators and [^{3}H]-thymidine, the cell media was removed, and the cells lysed with 0.2 M NaOH. Cellular DNA was harvested onto glass fiber filter papers and the ^{3}H counted via a scintillation counter. Results are expressed as mean counts per minute (cpm) ± SEM.

Quantification of Procollagen I Production

For this assay, cells were seeded at a density of 5 x 10^4 cells/well in 24-well culture plates containing 500 µl of DMEM supplemented with FBS (10%) and incubated

for 24 h to permit cell attachment. The cells were then rinsed with PBS and fresh DMEM (without FBS) was added to the wells for an additional 24 h incubation to establish quiescence. After this time, recombinant human IGF-I (R&D Systems; Minneapolis, MN) (100 ng/ml), LTC_4 (Caymen Chemical; MI)(10^{-6}, 10^{-8}, 10^{-10}, and 10^{-12} M), and ascorbic acid (50 μg/ml) were added to each well in triplicate and incubated for an additional 24 h. After 24 h, fibroblast culture supernatants were removed, diluted 1:100 in EIA buffer and analyzed for procollagen type I using a standard sandwich ELISA.

Briefly, microtiter plates (96-well; NUNC, Roskilde, Denmark) were coated (100 μl into each well) with mouse anti-human procollagen I (Takara PC-8-7 through Panvera) and diluted in PBS (pH 7.4) to a concentration of 1.0 μg/ml. This medium was allowed to sit 16 to 24 h at room temperature, at which time 225 μl of a saturation buffer (0.1 M potassium phosphate, 1.5 mM sodium azide, 0.4 M sodium chloride, 0.1 mM EDTA, and 0.3% bovine serum albumin) was added to each well. After blocking, ELISA plates were washed (PO_4/Tween buffer) and samples (100 μl) were added to wells in triplicate and incubated for 3 h at 37°C. At this time, plates were again washed and 100 μl of detection antibody (Takara MK101) were added to each well. After an overnight incubation at 4°C, the plates were washed and TMB substrate added. Plates developed for 10-30 min and then development was halted by the addition of 100 μl of 1M phosphoric acid. Plates were read at 450 nm and standard curves were generated with 4-parameter function on Delta Soft software. The assay is sensitive over the range of 0.2 to 100 ng/ml.

Quantification of Insulin-like Growth Factor Binding Protein-3 (IGFBP3)

Fibroblast production of IGFBP3 was quantified in fibroblast culture supernatant using a sandwich enzyme-linked immunoabsorbent assay (ELISA). For these purposes, fibroblasts were cultured and prepared as described for DNA synthesis To quantify fibroblast production of IGFBP3, fibroblast supernatants were quantified using a sandwich ELISA. Fibroblasts were seeded at a density of 50,000 cells/well in 24-well culture plates containing 500 μl of DMEM supplemented with FBS (10%) and incubated for 24 h to permit cell attachment. After this time, the supernatant was discarded and the cells rinsed with PBS and fresh DMEM (without FBS) was added to the wells for an additional 24 h incubation to induce quiescence. After this time, recombinant human PDGF isoforms AA, BB, and AB (1, 5, 10, and 20 ng/ml) (R&D Systems; Minneapolis, MN) and ascorbic acid (50 μg/ml)(Sigma) were added to each well in triplicate and incubated for an additional 24 h. After 24 h, fibroblast culture supernatants were removed and used to determine procollagen type I production using a standard sandwich ELISA.

Briefly, microtiter plates (96-well; NUNC, Roskilde, Denmark) were coated (100 μl into each well) with mouse anti-human procollagen I (Takara PC-8-7 through Panvera) diluted in PBS (pH 7.4) to a concentration of 1.0 μg/ml. This medium was allowed to sit 16 to 24 h at room temperature, at which time 225 μl of a saturation buffer (0.1 M potassium phosphate, 1.5 mM sodium azide, 0.4 M sodium chloride, 0.1 mM EDTA, and 0.3% bovine serum albumin) was added to each well. After blocking, ELISA plates were washed (PO_4/Tween buffer) and samples (100 μl) added to wells in triplicate and incubated for 3 h at 37°C. At this time, plates were again washed and 100 μl of detection

antibody (Takara MK101) was added to each well. After an overnight incubation at 4°C, the plates were washed and TMB substrate added. Plates developed for 10-30 min and then development was halted by the addition of 100 µl of 1M phosphoric acid. Plates were read at 450 nm and standard curves generated with 4-parameter function on Delta Soft software. The assay is sensitive over the range of (0.2 to 100 ng/ml).

Statistical Analyses

The outcome variables were fibroblast proliferation, procollagen I production, and $IGFBP_3$ production at baseline (unstimulated control) and after exposure to LTC_4 and IGF-I. Data were expressed as both absolute values and as fold inductions (i.e., a given condition expressed as a ratio to its unstimulated control). Data from multiple passages (first through fourth) within a subject were averaged together to yield a single data point per condition. The data for each outcome variable was analyzed for between-group comparisons using a simple one-way ANOVA. The effect(s) of LTC_4 and IGF-I on the outcome variables was compared between all groups (i.e., severe asthma, mild/moderate asthma, and normal controls) as well as between a pooled asthmatic population (i.e., mild/moderate and severe asthmatics together) and normal controls.

RESULTS

Subjects

Subject characteristics are shown in Table 6.1.

Table 6.1. Subject Characteristics.

	Non-Asthmatic Controls	Mild/Moderate Asthmatics	Severe Asthmatics	P Value
Sex (M/F)	4/4	5/5	4/1	–
Age (y)	30.4 ± 3.5	34.6 ± 2.63	37 ± 3.7	0.48
Medicines	None	β_2-agonists	β_2-agonists	–
FEV_1 (L)	3.8 ± 0.26	3.22 ± 0.25	2.6 ± 0.35	0.03
FEV_1(% Predicted)	102.5 ± 4.24	89.8 ± 4.10	64.7 ± 5.79	0.0001

Data are presented as means ± standard error of the mean.

Proliferation

Immunostaining with vimentin, Ab-1, and α-smooth muscle actin confirmed the fibroblast phenotype, with 100% of the cells positive for vimentin, >95% positive for Ab-1 expression, and <5% positive for α-smooth muscle actin. The results of fibroblast proliferation with LTC_4 and IGF-I stimulation are shown in Tables 6.2 and 6.3 (see Appendix for graphic representations). As shown in Table 6.2, baseline fibroblast proliferation without stimulation or exposure to LTC_4 and IGF-I was greatest in the non-asthmatic group, although the differences between the groups were not statistically significant when analyzed by either the severity of asthma (severe: 5568 ± 2027 cpm, mild/moderates: 6558 ± 1308 cpm, normal controls: 8018 ± 1602 cpm; p=0.62), or when asthmatic populations were pooled (asthma: 6267 ± 1079 cpm, normal controls: 8018 ± 1573 cpm; p=0.37). However, to account for these differences in baseline proliferation and to evaluate true proliferative changes that may have occurred upon exposure to LTC_4 and IGF-I, the data are also presented as fold inductions, or as ratios to the negative, unstimulated control.

Analyses of the absolute values of [^{3}H]-thymidine detected in the proliferation experiments, expressed in cpms, revealed that none of our chosen combinations or concentrations of LTC_4 or IGF-I resulted in significant between-group differences in fibroblast proliferation. Furthermore, when the proliferation numbers were expressed as ratios to the unstimulated or negative control, the data still failed to demonstrate significant between-group differences in fibroblast proliferation. Additionally, analyses of pooled asthmatic populations revealed no significant between-group differences in proliferation in response to LTC_4 or IGF-I (alone or in combination). This held true for both the absolute numbers and when the data were expressed as a ratio to its negative control. Thus, our data do not indicate that any of our chosen concentrations of LTC_4 or IGF-I (alone or in combination) affects fibroblast proliferation differentially between asthmatics and normal controls, or even between asthmatic groups of different disease severity.

Table 6.2. Results of Fibroblast Proliferation with LTC_4/IGF Stimulation Analyzed by Severity of Asthma.

	Normal Controls (n=8)	Mild/Moderate Asthma (n=10)	Severe Asthma (n=5)	*P* value
Baseline Proliferation (cpm)	8018 ± 1602	6558 ± 1308	5568 ± 2027	0.62
Effects of LTC₄/IGF:				
LTC_4 (10^{-6} M)	8355 ± 1666 †	7093 ± 1360	6670 ± 2107	0.78
	1.07 ± 0.09 ‡	1.22 ± 0.08	1.13 ± 0.11	0.41
LTC_4 (10^{-8} M)	7307 ± 1469	6126 ± 1199	5459 ± 1858	0.71
	0.95 ± 0.02	0.94 ± 0.01	0.98 ± 0.02	0.27
LTC_4 (10^{-10} M)	7240 ± 1389	6378 ± 1134	5571 ± 1756	0.75
	0.97 ± 0.03	0.95 ± 0.03	0.98 ± 0.04	0.76
LTC_4 (10^{-12} M)	6951 ± 1443	6368 ± 1178	5751 ± 1825	0.87
	0.91 ± 0.03	0.97 ± 0.03	1.00 ± 0.04	0.18
IGF-I (100 ng/mL)	11164 ± 2017	9063 ± 1647	9479 ± 2552	0.72
	1.52 ± 0.09	1.56 ± 0.08	1.69 ± 0.12	0.52
LTC_4 (10^{-6} M) + IGF-I (100 ng/mL)	14136 ± 2217	10872± 1810	10766 ± 2804	0.48
	2.04 ± 0.22	2.14 ± 0.20	1.96 ± 0.28	0.86
LTC_4 (10^{-8} M) + IGF-I (100 ng/mL)	11352 ± 1795	8904 ± 1465	9124 ± 2271	0.56
	1.69 ± 0.12	1.60 ± 0.11	1.71 ± 0.15	0.77
LTC_4 (10^{-10} M) + IGF-I (100 ng/mL)	13162 ± 1978	9722 ± 1615	11162 ± 2505	0.42
	1.87 ± 0.14	1.78 ± 0.13	2.13 ± 0.18	0.31
LTC_4 (10^{-12} M) + IGF-I (100 ng/mL)	12918 ± 1923	10027 ± 1640	11218 ± 2433	0.53
	1.82 ± 0.15	1.73 ± 0.14	2.14 ± 0.19	0.24

All Data are Expressed as means ± SEM. † Data shown are absolute values; ‡ Data shown are fold inductions, i.e, condition as a ratio of unstimulated control. All P values are for between group comparisions.

Table 6.3. Results of Fibroblast Proliferation with LTC_4/IGF Stimulation, Analyzed with Pooled Asthmatic Populations.

	Normal Controls (n=8)	Asthmatics* (n=15)	*P* value
Baseline Proliferation (cpm)	8018 ± 1573	6267 ± 1079	0.37
Effects of LTC$_4$/IGF:			
LTC_4 (10^{-6} M)	8355 ± 1630 †	6968 ± 1118	0.49
	1.07 ± 0.08 ‡	1.19 ± 0.06	0.26
LTC_4 (10^{-8} M)	7307 ± 1439	5930 ± 987	0.44
	0.95 ± 0.02	0.96 ± 0.01	0.69
LTC_4 (10^{-10} M)	7240 ± 1363	6141 ± 935	0.51
	0.97 ± 0.03	0.96 ± 0.02	0.84
LTC_4 (10^{-12} M)	6951 ± 1414	6186 ± 970	0.66
	0.91 ± 0.03	0.98 ± 0.02	0.08
IGF-I (100 ng/ml)	11164 ± 1974	9185 ± 1354	0.42
	1.52 ± 0.09	1.61 ± 0.07	0.46
LTC_4 (10^{-6} M) + IGF-I (100 ng/ml)	14136 ± 2168	10841 ± 1487	0.22
	2.04 ± 0.22	2.08 ± 0.16	0.88
LTC_4 (10^{-8} M) + IGF-I (100 ng/ml)	11352 ± 1756	8969 ± 1205	0.27
	1.69 ± 0.12	1.63 ± 0.08	0.68
LTC_4 (10^{-10} M) + IGF-I (100 ng/ml)	13162 ± 1945	10145 ± 1334	0.21
	1.87 ± 0.15	1.90 ± 0.11	0.89
LTC_4 (10^{-12} M) + IGF-I (100 ng/ml)	12918 ± 1886	10400 ± 1334	0.29
	1.82 ± 0.16	1.86 ± 0.12	0.83

* *Includes mild, moderate, and severe asthmatics. All Data are Expressed as means ± SEM.* † *Data shown are absolute values;* ‡ *Data shown are fold inductions, i.e, condition as a ratio to unstimulated control. All P values are for between group comparisions.*

Procollagen Production

The results of fibroblast procollagen I production with LTC_4 and IGF-I stimulation are shown in Tables 6.4 and 6.5 (see Appendix for graphical representations).

Interestingly, as shown in Table 6.4, baseline fibroblast production of procollagen I without stimulation or exposure to LTC_4 and/or IGF-I was greatest numerically in the severe asthmatic group, although this difference was not significant (severe, 3948 ± 1124 ng/ml; mild/moderates, 1838 ± 838 ng/ml; normal controls, 3126 ± 1026 ng/ml; p=0.32). However, this effect was blunted when the asthmatic populations were pooled (asthma: 2592 ± 695 ng/ml; normal controls 3126 ± 1026 ng/ml; p=0.68). For convenience, the data are shown as both absolute values and as well as fold inductions, or ratios to the negative, unstimulated control, just as with the proliferation data previously mentioned, to account for true alterations in procollagen production that may result from exposure to LTC_4 and/or IGF-I.

Analyses of the absolute values of procollagen I measured, expressed in ng/ml, revealed that none of the conditions (LTC_4 or IGF-I alone or in combination) resulted in significant between-group differences in fibroblast procollagen production. Furthermore, when the procollagen numbers were expressed as ratios to the unstimulated or negative control, the data still failed to demonstrate significant between-group differences in fibroblast procollagen production. When the asthmatic populations were pooled and compared to the normal controls, analyses still revealed a lack of significant between-group differences in procollagen production in response to LTC_4 or IGF-I (alone or in combination). This held true for both the absolute numbers and when the data were expressed as a ratio to its negative control. Thus, our data do not indicate that any of our chosen concentrations of LTC_4 or IGF-I, alone or in combination, affects fibroblast procollagen production differentially between asthmatics and normal controls, or even

between asthmatic groups of different disease severity, under our experimental concentrations and conditions.

Table 6.4. Results of Fibroblast Procollagen Production with LTC_4/IGF Stimulation, Analyzed by Severity of Asthma.

	Normal Controls (n=8)	Mild/Moderate Asthma (n=10)	Severe Asthma (n=5)	*P* value
Baseline Procollagen (ng/mL)	3126 ± 1026	1838 ± 838	3948 ± 1124	0.32
Effects of LTC₄/IGF:				
LTC_4 (10^{-6} M)	3645 ± 95 †	1832 ± 824	2186 ± 1042	0.36
	1.03 ± 0.14 ‡	0.82 ± 0.12	0.72 ± 0.16	0.34
LTC_4 (10^{-8} M)	2489 ± 918	1738 ± 749	4291 ± 1005	0.15
	0.85 ± 0.07	0.95 ± 0.06	1.09 ± 0.08	0.11
LTC_4 (10^{-10} M)	2843 ± 763	1664 ± 623	3170 ± 836	0.30
	0.88 ± 0.05	0.94 ± 0.04	0.88 ± 0.06	0.58
LTC_4 (10^{-12} M)	3956 ± 1041	1825 ± 850	3804 ± 1140	0.23
	1.11 ± 0.09	1.01 ± 0.07	1.05 ± 0.11	0.69
IGF-I (100 ng/mL)	4557 ± 1503	2628 ± 1227	5841 ± 1646	0.33
	1.46 ± 0.14	1.34 ± 0.11	1.29 ± 0.15	0.69
LTC_4 (10^{-6} M) + IGF-I (100 ng/ml)	4712 ± 1626	2086 ± 1408	3437 ± 1782	0.49
	1.31 ± 0.34	0.86 ± 0.30	1.08 ± 0.37	0.61
LTC_4 (10^{-8} M) + IGF-I (100 ng/ml)	3564 ± 1511	2464 ± 1194	6249 ± 1511	0.17
	1.21 ± 0.3	1.21 ± 0.26	1.59 ± 0.32	0.62
LTC_4 (10^{-10} M) + IGF-I (100 ng/ml)	3574 ± 1384	2211 ± 1095	5662 ± 1384	0.18
	1.40 ± 0.14	1.32 ± 0.11	1.14 ± 0.14	0.41
LTC_4 (10^{-12} M) + IGF-I (100 ng/ml)	4765 ± 1625	2346 ± 1407	6898 ± 1780	0.16
	1.54 ± 0.13	1.39 ± 0.11	1.23 ± 0.14	0.29

All Data are Expressed as means ± SEM. † Data shown are absolute values; ‡ Data shown are fold inductions, i.e, condition as a ratio to unstimulated control. All P values are for between group comparisions.

Table 6.5. Results of Fibroblast Procollagen Production with LTC_4/IGF Stimulation Analyzed with Pooled Asthmatic Populations.

	Normal Controls (n=8)	Asthmatics* (n=15)	*P* value
Baseline Procollagen (ng/mL)	3126 ± 1061	2592 ± 695	0.68
Effects of LTC₄/IGF:			
LTC_4 (10^{-6} M)	3645 ± 925 †	1968 ± 628	0.15
	1.03 ± 0.14 ‡	0.78 ± 0.10	0.16
LTC_4 (10^{-8} M)	2489 ± 998	2650 ± 651	0.89
	0.85 ± 0.07	0.99 ± 0.05	0.11
LTC_4 (10^{-10} M)	2843 ± 786	2202 ± 515	0.50
	0.88 ± 0.05	0.92 ± 0.03	0.49
LTC_4 (10^{-12} M)	3956 ± 1068	2532 ± 699	0.28
	1.11 ± 0.09	1.02 ± 0.06	0.41
IGF-I (100 ng/ml)	4557 ± 1550	3704 ± 1015	0.65
	1.46 ± 0.14	1.32 ± 0.09	0.40
LTC_4 (10^{-6} M) + IGF-I (100 ng/ml)	4712 ± 1595	2605 ± 1084	0.29
	1.31 ± 0.33	0.94 ± 0.23	0.37
LTC_4 (10^{-8} M) + IGF-I (100 ng/ml)	3564 ± 1491	3919 ± 1013	0.85
	1.21 ± 0.29	1.35 ± 0.20	0.69
LTC_4 (10^{-10} M) + IGF-I (100 ng/mL)	3574 ± 1502	3538 ± 93	0.98
	1.40 ± 0.14	1.25 ± 0.08	0.38
LTC_4 (10^{-12} M) + IGF-I (100 ng/ml)	4765± 1763	4097 ± 1198	0.76
	1.54 ± 0.13	1.33 ± 0.09	0.18

** Includes mild, moderate, and severe asthmatics. All Data are Expressed as means ± SEM. † Data shown are absolute values; ‡ Data shown are fold inductions, i.e, condition as a ratio to unstimulated control. All P values are for between group comparisions.*

Production of IGFBP3

The results of fibroblast IGFBP3 production in response to LTC_4/IGF stimulation are shown in Tables 6.6 and 6.7 (see Appendix for graphical representations). As indicated, baseline fibroblast IGFBP3 production, without stimulation or exposure to LTC_4/IGF, was greatest in the severe asthmatics, although the differences between the groups were not statistically significant when analyzed by either the severity of asthma (severe: 3470 ± 835 ng/ml, mild/moderates: 1430 ± 457 ng/ml, normal controls: 2333 ± 546 ng/ml; p=0.11), or when the asthmatic populations were pooled (asthma: 1901 ± 439 ng/ml, normal controls: 2333 ± 598 ng/ml; p=0.57). However, to account for these differences in baseline IGFBP3 production and evaluate true alterations in IGFBP3 production that may have occurred upon exposure to LTC_4/IGF, the data are also presented as fold inductions, or ratios to the negative, unstimulated control.

When the data were normalized to account for the differences in baseline IGFBP3 production, analyses revealed that the fibroblasts from the normal controls produced significantly greater levels of IGFBP3 in response to stimulation with LTC_4 alone (10^{-10} M), as compared to the mild/moderate and the severe asthmatic groups (severe: 0.837 ± 0.10, mild/moderates: 0.944 ± 0.06, normal controls: 1.133 ± 0.07; p=0.05). However, this effect of LTC_4 alone was significant at only the one concentration (10^{-10} M). Analyses indicated that the fibroblasts from the normal controls also produced significantly greater levels of IGFBP3 when stimulated with IGF alone (severe: 1.13 ± 0.10, mild/moderates: 1.038 ± 0.06, normal controls: 1.297 ± 0.07; p=0.03), and the combination of IGF and LTC_4 (10^{-10} M) (severe: 1.04 ± 0.15, mild/moderates: 1.107 ± 0.08, normal controls: 1.410 ± 0.09; p=0.05). Thus, our data indicate that airway

fibroblasts derived from normal controls produce more IGFBP3 in response to some of our conditions of LTC_4/IGF stimulation.

Table 6.6. Results of Fibroblast IGFBP3 Production with LTC_4/IGF Stimulation, Analyzed by Severity of Asthma.

	Normal Controls (n=8)	Mild/Moderate Asthma (n=10)	Severe Asthma (n=5)	*P* value
Baseline IGFBP3 (ng/ml)	2333 ± 546	1430 ± 457	3470 ± 835	0.11
Effects of LTC$_4$/IGF:				
LTC_4 (10^{-6} M)	2871 ± 665 †	1759 ± 556	3169 ± 1015	0.33
	1.18 ± 0.07 ‡	1.06 ± 0.06	1.01 ± 0.11	0.34
LTC_4 (10^{-8} M)	2898 ± 723	1603 ± 605	2796 ± 1104	0.36
	1.08 ± 0.08	0.96 ± 0.07	0.85 ± 0.13	0.30
LTC_4 (10^{-10} M)	2887 ± 716	1532 ± 599	2943 ± 1094	0.30
	1.13 ± 0.07	0.94 ± 0.06	0.84 ± 0.10	**0.05**
LTC_4 (10^{-12} M)	2571 ± 547	1504 ± 457	3166 ± 835	0.16
	1.06 ± 0.06	0.95 ± 0.05	0.89 ± 0.09	0.29
IGF-I (100 ng/ml)	3177 ± 730	1724 ± 611	4046 ± 1115	0.14
	1.30 ± 0.07	1.03 ± 0.06	1.13 ± 0.10	**0.03**
LTC_4 (10^{-6} M) + IGF-I (100 ng/ml)	3292 ± 751	1875 ± 629	4450 ± 1148	0.13
	1.37 ± 0.08	1.16 ± 0.07	1.24 ± 0.12	0.17
LTC_4 (10^{-8} M) + IGF-I (100 ng/ml)	3728 ± 859	1844 ± 719	3611 ± 1312	0.22
	1.41 ± 0.99	1.14 ± 0.08	1.04 ± 0.15	0.08
LTC_4 (10^{-10} M) + IGF-I (100 ng/ml)	3776 ± 883	1768 ± 739	3670 ± 1348	0.20
	1.41 ± 0.09	1.13 ± 0.07	1.04 ± 0.15	**0.05**
LTC_4 (10^{-12} M) + IGF-I (100 ng/ml)	3488 ± 896	1801 ± 750	3762 ± 1369	0.27
	1.30 ± 0.09	1.14 ± 0.08	1.06 ± 0.15	0.32

All Data are Expressed as means ± SEM. † Data shown are absolute values; ‡ Data shown are fold inductions, i.e, condition as a ratio of unstimulated control. All P values are for between group comparisions.

Table 6.7. Results of Fibroblast IGFBP3 Production with LTC_4/IGF Stimulation Analyzed with Pooled Asthmatic Populations.

	Normal Controls (n=8)	Asthmatics* (n=15)	*P* value
Baseline IGFBP3 (ng/ml)	2333 ± 598	1901 ± 439	0.57
Effects of LTC4/IGF:			
LTC_4 (10^{-6} M)	2871 ± 673 †	2085 ± 494	0.36
	1.18 ± 0.14 ‡	1.05 ± 0.10	0.15
LTC_4 (10^{-8} M)	2898 ± 721	1879 ± 529	0.27
	1.08 ± 0.08	0.94 ± 0.06	0.17
LTC_4 (10^{-10} M)	2887 ± 722	1857 ± 530	0.27
	1.13 ± 0.07	0.92 ± 0.05	**0.02**
LTC_4 (10^{-12} M)	2571 ± 577	1888 ± 423	0.35
	1.06 ± 0.06	0.94 ± 0.05	0.14
IGF-I (100 ng/ml)	3177 ± 776	2260 ± 569	0.35
	1.30 ± 0.07	1.06 ± 0.05	**0.01**
LTC_4 (10^{-6} M) + IGF-I (100 ng/ml)	3292 ± 809	2470 ± 594	0.42
	1.37 ± 0.08	1.18 ± 0.06	0.07
LTC_4 (10^{-8} M) + IGF-I (100 ng/ml)	3728 ± 868	2252 ± 637	0.19
	1.41 ± 0.09	1.12 ± 0.07	**0.03**
LTC_4 (10^{-10} M) + IGF-I (100 ng/mL)	3776 ± 896	2207 ± 657	0.17
	1.41 ± 0.09	1.09 ± 0.07	**0.01**
LTC_4 (10^{-12} M) + IGF-I (100 ng/ml)	3488 ± 910	2254 ± 668	0.29
	1.30 ± 0.09	1.12 ± 0.07	0.15

** Includes mild, moderate, and severe asthmatics. All Data are Expressed as means ± SEM. † Data shown are absolute values; ‡ Data shown are fold inductions, i.e, condition as a ratio of unstimulated control. All P values are for between group comparisons.*

DISCUSSION

We have shown that cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4), independently, as well as in combination with IGF-I, do not enhance airway fibroblast proliferation or procollagen production differently in asthmatics, as compared to normal controls. These results were confirmed when analyses were performed with both the absolute numbers and with the data expressed as ratios to the negative, unstimulated control, which account for between-group differences in baseline values. To our knowledge, there have been no studies that have investigated the independent effects of cysteinyl leukotrienes on proliferation and procollagen production in airway fibroblasts. However, Cohen et al. (43) reported that the cysteinyl leukotriene LTD_4 significantly enhanced proliferation of airway smooth muscle cells. Yet, it is difficult to extrapolate these data to airway fibroblasts, as smooth muscle cells and fibroblasts are two distinct cell populations with likely distinct responses to cysteinyl leukotrienes.

In contrast to the proliferation and procollagen outcomes, our data indicate that airway fibroblasts from normal controls produce significantly greater levels of IGFBP3 in response to stimulation with LTC_4 and IGF-I. These results were confirmed when analyses were performed with both the absolute numbers and the data expressed as ratios to the negative, unstimulated control, which accounts for the between-group differences in baseline IGFBP3 production. Consequently, given that the predominant role of IGFBP3 is to regulate the mitogenic effects of IGF-I (via restricting its bioavailability, transport to cells, and binding to membrane receptors) (49-52), it is tempting to speculate that the fibroblasts from the normal controls are better equipped for regulating tissue growth and repair. In this way, these data support the hypothesis of decreased fibroblast

IGFBP3 production as a mechanism involved in fibroblast remodeling activities. Along these lines, several studies have reported decreased IGFBP measurements in various contexts of tissue fibrosis (53-56). Furthermore, our data are in agreement with the studies of Conover et al. (51), in which the investigators report that IGF-I stimulation induced IGFBP3 production in both human and bovine fibroblasts.

In conclusion, while our data do not suggest a direct or independent role for cysteinyl leukotrienes and IGF-I in fibroblast-induced remodeling, our findings of an enhanced IGFBP3 production in response to these mediators may have some physiologic relevance. The components of the IGF axis, namely IGFBP3 in this context, may be sensitive to stimulation by other cytokines or growth factors elaborated in the milieu of the asthmatic airway, which could potentially alter fibroblast-induced remodeling. Thus, more studies investigating the mechanisms underlying the IGF axis in the fibroblast are needed to fully appreciate the meaning of the present findings. A greater understanding in this field could prove fruitful in providing insight into the mechanisms of action for many pertinent mediators present in the asthmatic airway (42).

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Chapter 7

Eosinophil-Fibroblast Interactions in Airway Remodeling

ABSTRACT

Significant eosinophil influx with evidence of activation and degranulation has been described in the airways of many asthmatics. Additionally, increased numbers of eosinophils and eosinophil products have been described in areas of fibrosis in various parts of the body, including the lung. We hypothesized that eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO) contribute to airway remodeling in asthmatics by enhancing airway fibroblast proliferation and procollagen production. Eight subjects with mild to moderate asthma, three with severe asthma, and nine non-asthmatic normal controls underwent bronchoscopy with endobronchial biopsy. Biopsies were placed in Dulbecco's Modified Eagle Medium and cultured. Fibroblasts were incubated with EDN (1, 10, 100 μg/ml) or EPO (1, 10, 100 U/ml), and [^{3}H]-thymidine incorporation was determined. Data were expressed as ratios to the negative control or baseline proliferation. Compared to baseline values, neither fibroblast proliferation nor procollagen production was enhanced with exposure to EDN or EPO. However, the contributions of these mediators to other aspects of fibroblast function, such as enhancement of cytokine production remain to be elucidated.

ABBREVIATIONS:

MBP	Major basic protein
EDN	Eosinophil-derived neurotoxin
EPO	Eosinophil peroxidase
ECP	Eosinophil cationic protein
DMEM	Dulbecco's Modified Eagle's Medium

INTRODUCTION

As a chronic inflammatory disease, asthma is invariably associated with tissue remodeling as the lungs continually undergo injury and repair (1). Thus, prominent structural alterations of the bronchial wall (collectively termed airway remodeling) can, and often do, accompany the disease, leading to the complication of fixed airway obstruction (2-4). Although remodeling is the result of several events, hyalinization and thickening of the epithelial basement membrane (i.e., subepithelial fibrosis) (5), which has been repeatedly described in the airways of many asthmatics, has received much attention. This morphologic change is believed to be due to the abnormal deposition of collagen, particularly types I, III, and V, by the airway fibroblasts found adjacent to the collagen layer (6,7). These fibroblasts are capable of producing an array of mediators and cytokines and undergoing phenotypic changes that may amplify and/or perpetuate inflammatory and fibrotic processes (8-10). Furthermore, given that the fibroblasts are anatomically positioned so as to form close contact with inflammatory cells as they migrate from the blood into the airway epithelium, there is great potential for interaction between these cells. In fact, such cell interactions have been strongly implicated in the

activation of fibroblast-induced remodeling, as many of the cytokines released by the inflammatory cells are able to modulate various fibroblast functions, including cell proliferation (6,11,12) and collagen synthesis (13-15).

The fibroblast and the eosinophil are two cell types whose interaction has been repeatedly implicated in inflammation and remodeling. For example, fibroblasts have been shown to synthesize and secrete the chemokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and eotaxin, both of which greatly enhance the recruitment and extend the half-lives of eosinophils (10,16). Studies have also demonstrated that fibroblasts are capable of inducing eosinophil degranulation (17,18), and that in turn, eosinophils can release fibrogenic factors (i.e., mitogens for fibroblasts) (19,19,20). Additionally, numerous studies have described an enhanced presence of eosinophils or eosinophil-derived products in regions of fibrosis in various disorders, including idiopathic pulmonary fibrosis (21), eosinophilic fasciitis (22), scleroderma (23), and even wound repair (24). While these studies are largely descriptive, providing only circumstantial evidence, they have paved they way for more direct investigations into the relationship of fibroblasts and eosinophils. Pincus et al. (19) and Shock et al. (25), have separately reported that eosinophil extracts stimulate fibroblast proliferation, suggesting that an eosinophil product directly causes fibrosis, though the precise mechanisms and/or mediators involved were not addressed. Similarly, Birkland et al. (26) reported that eosinophil-conditioned media stimulated both proliferation and collagen production in human dermal fibroblasts. Noguchi et al. (27) reported dermal fibroblast proliferation in response to the eosinophil granule protein known as the eosinophil-derived neurotoxin (EDN). Several investigators have suggested that the contribution of the eosinophil to

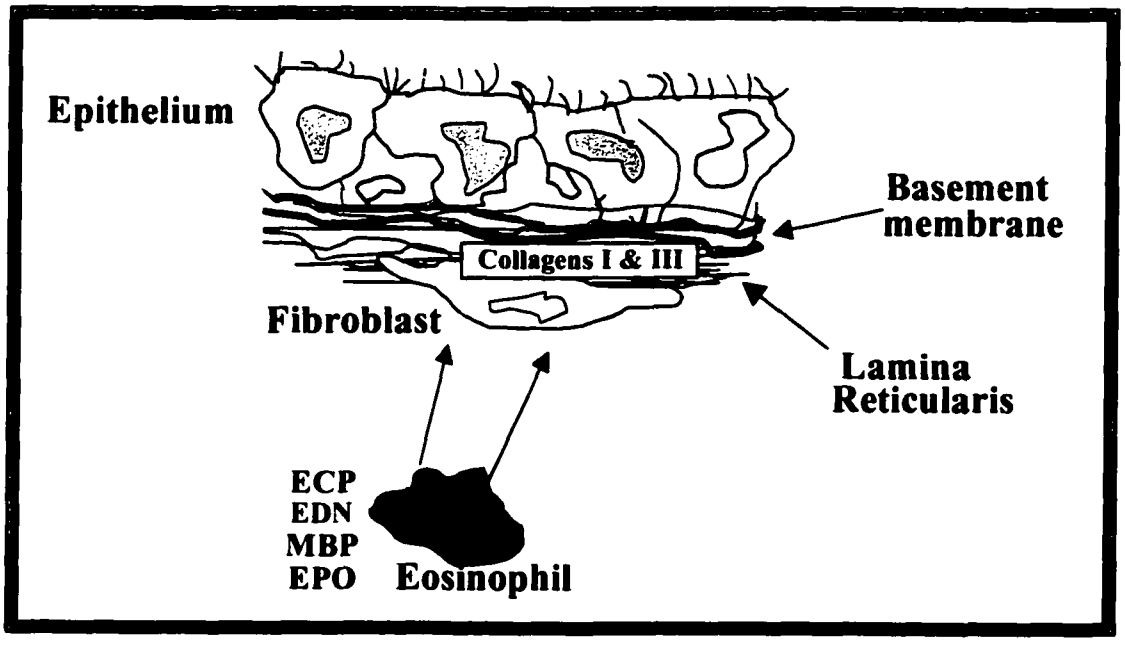
airway remodeling may be indirectly mediated through its production of TGF-β (28,29,30), which itself is a potent mitogen for fibroblasts (31). Thus, the eosinophil-fibroblast relationship appears to be relevant in the context of airway remodeling, although definitive mechanisms and/or mediators have not yet been identified.

There is strong circumstantial evidence that implicates eosinophils as important proinflammatory cells involved in the pathogenesis of asthma. Eosinophilia of the blood (32-34), bronchial tissues (32,35-40), bronchoalveolar lavage fluid (41-45), and sputum (46,47) has historically been recognized as one of the classic hallmarks for many, although not all (48), asthmatics. However, it has not yet been clearly established as to whether the eosinophils in this situation operate to the advantage or the detriment of the host (49). Many studies suggest the latter, as eosinophils have been shown to cause pronounced desquamation of the respiratory epithelium (38,47,50-52); inhibit ciliary activity (47,51,53); induce histamine release from basophils and mast cells (54); as well as alter tight junctions and widen intercellular spaces (55). Furthermore, the extent of peripheral blood eosinophilia has been shown to be inversely correlated to both the decrease in FEV_1 (34,56) observed in bronchial asthma and to the clinical severity of bronchial hyperresponsiveness (32,34,42,45,46,57,58). Thus, in this context, the eosinophil is believed to be a significant contributor to the pathogenesis of asthma, via its ability to act as a proinflammatory and epithelial-damaging effector cell (38,53,55,59-61). However, very little is understood about its role, if any, in the repair and/or remodeling process in the asthmatic airway. Yet, eosinophils are able to synthesize and release an array of products upon activation, including membrane-bound granular proteins (major basic protein, MBP; eosinophilic cationic protein, ECP; eosinophil

peroxidase, EPO; eosinophil-derived neurotoxin, EDN) (38,53,62-64); a range of lipid mediators, including prostaglandins and leukotrienes (65,66); and a tremendous variety of cytokines and chemokines (interleukins 1, 2, 3, 5, 6, 10, 12, TGF-β, TNF-α, GM-CSF, MIP, and RANTES) (67-73).

Thus, given the strong interaction between the eosinophil and fibroblast in airway inflammation and remodeling, as evidenced by the numerous studies outlined herein, we hypothesized, as schematically depicted in Figure 7.1, that the eosinophil directly potentiates the airway remodeling process in asthmatics by stimulating both the proliferative and the synthetic capabilities of the fibroblast. More specifically, we hypothesized that the eosinophil granular proteins eosinophil-derived neurotoxin and eosinophil peroxidase themselves directly induce fibroblast activation and fibroblast-induced airway remodeling.

Figure 7.1. **Potential Eosinophil-Fibroblast Interaction in Airway Remodeling.**



MATERIALS & METHODS

Subjects

A group of subjects consisting of three severe asthmatics, eight mild asthmatics (severity determined by American Thoracic Society standards), and nine normal, healthy individuals without asthma (non-asthmatic) were recruited from the general Denver community. All asthmatic subjects met diagnostic criteria for asthma (74), exhibiting a methacholine PC_{20} of less than 8 mg/ml, and were maintained on inhaled β_2 agonists only. Exclusion criteria included use of cromolyn, nedocrimil, inhaled or oral corticosteroids, leukotriene modifiers and/or theophylline within 6 weeks prior to the study; an upper respiratory tract infection within 6 weeks prior to the study; immunotherapy within 3 months prior to the study; cigarette use; and, significant non asthma pulmonary disease or other medical illnesses. Informed consent was obtained from all subjects participating in the study, in accordance with the standards of the institutional review board.

Protocol

Subjects underwent bronchoscopy with endobronchial biopsy as previously described (75). The site of biopsy was randomized to either the right or left lower lobe, and 5 to 6 endobronchial biopsy specimens were taken from the tertiary carinae of the right or left lower lobes under direct visualization. Prior to bronchoscopy, spirometry was performed before and after the administration of 0.18 mg of albuterol from a metered dose inhaler and 0.4 mg atropine, introduced intravenously. Xylocaine (4%) was used to anesthetize the upper airway, and Xylocaine (1%) was applied to the laryngeal area,

trachea, and orifice of the right lower or left lower lobe bronchi via the bronchoscope. Supplemental oxygen was administered throughout the procedure along with monitoring of heart rate and oxygen saturation. Subjects' vital signs were monitored in our laboratory after the procedure, and subjects were discharged once their FEV_1 was 90% of their pre-bronchoscopy, post-bronchodilator value.

Fibroblast Culture

Fibroblasts were cultured from endobronchial biopsy tissue as previously described (76). Briefly, biopsy specimens were rinsed and cut into several small pieces (approximately 5-6 per biopsy) and placed into wells (2 pieces/well) of a 24-well tissue culture plate, containing Dulbecco's Modified Eagle's Medium (DMEM), supplemented with fetal bovine serum (10%), streptomycin (100 μg/ml), penicillin (10,000 U/ml), and gentamicin (100 μg/ml). The tissue pieces were incubated at 37°C/5% CO_2 and cultured until cell growth established >50% confluency (approximately 8-20 days), with the media changed every 2-3 days. Upon reaching >50% confluency, the tissue pieces were removed, and the cells were trypsinized, counted, tested for viability, stained for identification, and placed into 96- and 24-well plates for proliferation and collagen experiments, respectively. Additionally, cells were seeded in 15 X 60-mm culture dishes for subsequent cell passages (Becton Dickinson, Franklin Lakes, NJ). All cells were stained with monoclonal mouse anti-human antibodies against fibroblast antigen (Ab-1; Calbiochem, San Diego, CA), vimentin (DAKO, Carpinteria, CA) and α-smooth muscle actin (DAKO) to confirm fibroblast, myofibroblast, and mesenchymal cell specificity, respectively. First passage cells generated directly from the biospy tissue were stained on

cytospin slides while second through fourth passage fibroblasts were grown directly onto 18 mm coverslips (Fisher Scientific, Pittsburgh, PA) that were placed in the culture dishes upon repassage. Only cells from the first through fourth passages were used for experiments, given the potential concern for changes in cell function including enhanced senescence that may occur after several passages (77).

Fibroblast Proliferation

To determine cell proliferation, [^{3}H]-thymidine incorporation was measured, as previously described (19). Fibroblasts were seeded in 96-well culture plates at a density of 10^4 cells/well in 200 µl of DMEM supplemented with FBS (10%) and incubated for 24 h to allow cell attachment. The adherent cells were then rinsed with PBS, and fresh DMEM supplemented with 0.5% FBS was added to the wells for an additional 24 h incubation to establish quiescence. After this time, purified human eosinophil-derived proteins EDN (1, 10, 100 µg/ml) or EPO (1, 10, 100 U), and [^{3}H]-thymidine (5 µCi/ml; ICN) were added to each well in triplicate and incubated for an additional 24 h. Following a 24 h incubation with the eosinophil proteins and [^{3}H]-thymidine, the cell media was removed, and the cells lysed with 0.2 M NaOH. Cellular DNA was harvested onto glass fiber filter papers and the ^{3}H counted via a scintillation counter. Results are expressed as mean counts per minute (cpm) ± SEM.

Quantification of Procollagen I Production

For this assay, cells were seeded at a density of 5 x 10^4 cells/well in 24-well culture plates containing 500 µl of DMEM supplemented with FBS (10%) and incubated

for 24 h to permit cell attachment. The cells were then rinsed with PBS and fresh DMEM (without FBS) was added to the wells for an additional 24 h incubation to establish quiescence. After this time, purified human eosinophil-derived proteins EDN (1, 10, 100 μg/ml) or EPO (1, 10, 100 U), and ascorbic acid (50 μg/ml) were added to each well in triplicate and incubated for an additional 24 h. After 24 h, fibroblast culture supernatants were removed, diluted 1:100 in EIA buffer and analyzed for procollagen type I using a standard sandwich ELISA.

Briefly, microtiter plates (96-well; NUNC, Roskilde, Denmark) were coated (100 μl into each well) with mouse anti-human procollagen I (Takara PC-8-7 through Panvera) and diluted in PBS (pH 7.4) to a concentration of 1.0 μg/ml. This medium was allowed to sit 16 to 24 h at room temperature, at which time 225 μl of a saturation buffer (0.1 M potassium phosphate, 1.5 mM sodium azide, 0.4 M sodium chloride, 0.1 mM EDTA, and 0.3% bovine serum albumin) was added to each well. After blocking, ELISA plates were washed (PO_4/Tween buffer) and samples (100 μl) were added to wells in triplicate and incubated for 3 h at 37°C. At this time, plates were again washed and 100 μl of detection antibody (Takara MK101) were added to each well. After an overnight incubation at 4°C, the plates were washed and TMB substrate added. Plates developed for 10-30 min and then development was halted by the addition of 100 μl of 1M phosphoric acid. Plates were read at 450 nm and standard curves were generated with 4-parameter function on Delta Soft software. The sensitivity of the assay is from 0.2 to 100 ng/ml.

RESULTS

Subjects

Subject Characteristics are shown in Table 7.1.

Table 7.1. Subject Characteristics.

	Non-Asthmatic Controls	Mild/Moderate Asthmatics	Severe Asthmatics	P Value
Sex (M/F)	4/5	3/5	2/1	–
Age (y)	31 ± 3.2	36.8 ± 2.63	32.6 ± 3.7	0.48
Medicines	None	β_2-agonists	β_2-agonists	–
FEV_1 (L)	3.8 ± 0.26	3.22 ± 0.25	2.6 ± 0.35	0.03
FEV_1(% Predicted)	102.5 ± 4.24	89.8 ± 4.10	64.7 ± 5.79	0.0001

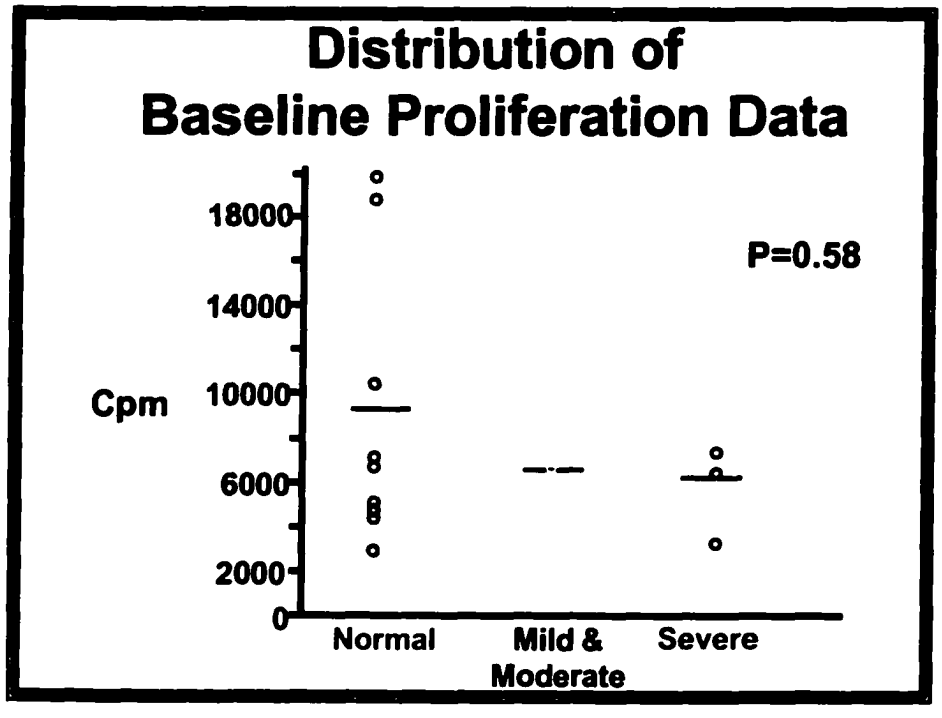
Data are presented as means ± standard error of the mean.

Fibroblast Proliferation

Immunostaining with vimentin, Ab-1, and α-smooth muscle actin confirmed the fibroblast phenotype, with 100% of the cells positive for vimentin, > 95% positive for Ab-1 expression, and < 5% positive for α-smooth muscle actin. The results of fibroblast proliferation in response to EDN and EPO are shown in Tables 7.2 and 7.3. As shown in Table 7.2 and Figure 7.1, baseline fibroblast proliferation, without stimulation or exposure to eosinophil proteins, was greatest in the non-asthmatic group, although the differences between the groups were not statistically significant when analyzed by either the severity of asthma (severes: 7006 ± 3147 cpm, mild/moderates: 7270 ± 1927 cpm, normal controls: 9783 ± 1817 cpm; p=0.58), or when the asthmatic populations were pooled (asthma: 7198 ± 1598, normal controls: 9783 ± 1766 cpm; p=0.29). However to account for these differences in baseline proliferation and evaluate true proliferative

changes that may have occurred upon exposure to eosinophil proteins, the data are also presented as fold inductions, or ratios to the negative, unstimulated control.

Figure 7.2. **Baseline Fibroblast Proliferation Among Subject Groups.**



Analyses of the absolute values of [^{3}H]-thymidine detected in the proliferation experiments, expressed in cpms, revealed that there were no significant between-group differences in fibroblast proliferation when stimulated with either EDN or EPO at any of the concentrations (1, 10, 100 μg/ml; 1, 10, 100 U/ml, respectively). Furthermore, when the proliferation numbers resulting from exposure to these proteins were expressed as a ratio to the negative or unstimulated control to account for the unequal baseline proliferation numbers between the groups, there were still no significant between-group differences detected in the proliferative responsiveness to EDN and EPO. Finally, when the data were analyzed with the pooled asthmatic populations (Table 7.3), neither the

absolute numbers nor the fold inductions calculated from those numbers demonstrated significant between-group differences in fibroblast proliferation. Thus, our data indicate that the eosinophil proteins EDN and EPO do not affect airway fibroblast proliferation, either positively or negatively, in a way that differentiates asthmatics from non-asthmatic controls.

Table 7.2. Results of Fibroblast Proliferation with Eosinophil-Derived Proteins, Analyzed by Severity of Asthma.

	Normal Controls (n=9)	Mild/Moderate Asthma (n=8)	Severe Asthma (n=3)	P value
Baseline Proliferation (cpm)	9783 ± 1817	7270 ± 1927	7006 ± 3147	0.58
Effects of proteins:				
EDN (1 μg/ml)	9320 ± 2189 †	7226 ± 2189	6207 ± 3574	0.70
	0.88 ± 0.05 ‡	0.91 ± 0.05	0.89 ± 0.09	0.94
EDN (10 μg/ml)	10749 ± 2441	7141 ± 2441	6622 ± 3986	0.52
	0.94 ± 0.07	0.94 ± 0.07	0.95 ± 0.11	0.99
EDN (100 μg/ml)	10991 ± 2495	6881 ± 2495	6113 ± 4074	0.43
	0.97 ± 0.07	0.89 ± 0.07	0.88 ± 0.12	0.70
EPO (1 U/ml)	10906 ± 2134	7589 ± 2263	8822 ± 3696	0.57
	1.08 ± 0.08	1.08 ± 0.08	1.29 ± 0.14	0.37
EPO (10 U/ml)	11496 ± 2055	6786 ± 2180	9072 ± 3559	0.32
	1.26 ± 0.15	0.97 ± 0.16	1.34 ± 0.25	0.31
EPO (100 U/ml)	7315 ± 1674	5599 ± 1776	5498 ± 2899	0.75
	0.70 ± 0.12	0.79 ± 0.13	0.82 ± 0.21	0.84

All Data are Expressed as means ± SEM. † Data shown are absolute values; ‡ Data shown are fold inductions, i.e, condition as a ratio of unstimulated control. All P values are for between group comparisons.

Table 7.3. Results of Fibroblast Proliferation with Eosinophil-Derived Proteins, Analyzed with Pooled Asthmatic Populations.

	Normal Controls (n=9)	Asthmatics* (n=11)	*P* value
Baseline Proliferation (cpm)	9783 ± 1766	7198 ± 1598	0.29
Effects of proteins:			
EDN (1 μg/ml)	9320 ± 2127 †	6948 ± 1814	0.41
	0.88 ± 0.05 ‡	0.90 ± 0.05	0.76
EDN (10 μg/ml)	10749 ± 2369	7000 ± 2020	0.25
	0.94 ± 0.06	0.94 ± 0.07	0.97
EDN (100 μg/ml)	10991 ± 2422	6671 ± 2066	0.19
	0.97 ± 0.07	0.89 ± 0.06	0.39
EPO (1 U/ml)	10906 ± 2079	7925 ± 1880	0.30
	1.08 ± 0.08	1.14 ± 0.07	0.63
EPO (10 U/ml)	11496 ± 2015	7409 ± 1822	0.15
	1.26 ± 0.15	1.07 ± 0.13	0.35
EPO (100 U/ml)	7315 ± 1627	5571 ± 1472	0.44
	0.70 ± 0.12	0.79 ± 0.11	0.56

** Includes mild, moderate, and severe asthmatics. All Data are Expressed as means ± SEM. † Data shown are absolute values; ‡ Data shown are fold inductions, i.e, condition as a ratio to unstimulated control. All P values are for between group comparisions.*

Procollagen Production

The results of fibroblast procollagen I production in response to stimulation with EDN and EPO are shown in Tables 7.4 and 7.5 (see Appendix for graphical representations). As shown in Figure 7.2 and Table 7.4, there were no significant differences in baseline fibroblast production of procollagen I between the groups when analyzed by either the severity of asthma (severes: 2591 ± 896 ng/mL, mild/moderates: 2825 ± 634 ng/mL, normal controls: 2645 ± 567 ng/mL; p=0.97), or when the asthmatic

populations were pooled (asthma: 2747 ± 489, normal controls: 2645 ± 536 ng/mL; p=0.89). However, for consistency, the data are shown as both absolute values as well as fold inductions, or ratio of unstimulated control, just as with the proliferation data, to account for true EDN- and/or EPO-induced alterations in procollagen production.

Analyses of the absolute values of procollagen I measured (expressed in ng/mL) revealed that there were no significant between-group differences in fibroblast procollagen with either EDN or EPO stimulation at any of the concentrations (1, 10, 100 μg/ml; 1, 10, 100 U/ml, respectively). Furthermore, when the procollagen data were expressed as a ratio to the unstimulated control, there were still no significant between-group differences detected in the proliferative responsiveness to EDN and EPO. Finally, when the data were analyzed with the asthmatic populations pooled together (Table 7.3), neither the absolute numbers nor the fold inductions calculated from those numbers demonstrated significant between-group differences in fibroblast proliferation. Thus, our data indicate that the eosinophil proteins EDN and EPO do not affect airway fibroblast proliferation, either positively or negatively, in a way that differentiates asthmatics from non-asthmatic controls.

Figure 7. 3. **Baseline Fibroblast Procollagen I Production Among Subject Groups.**

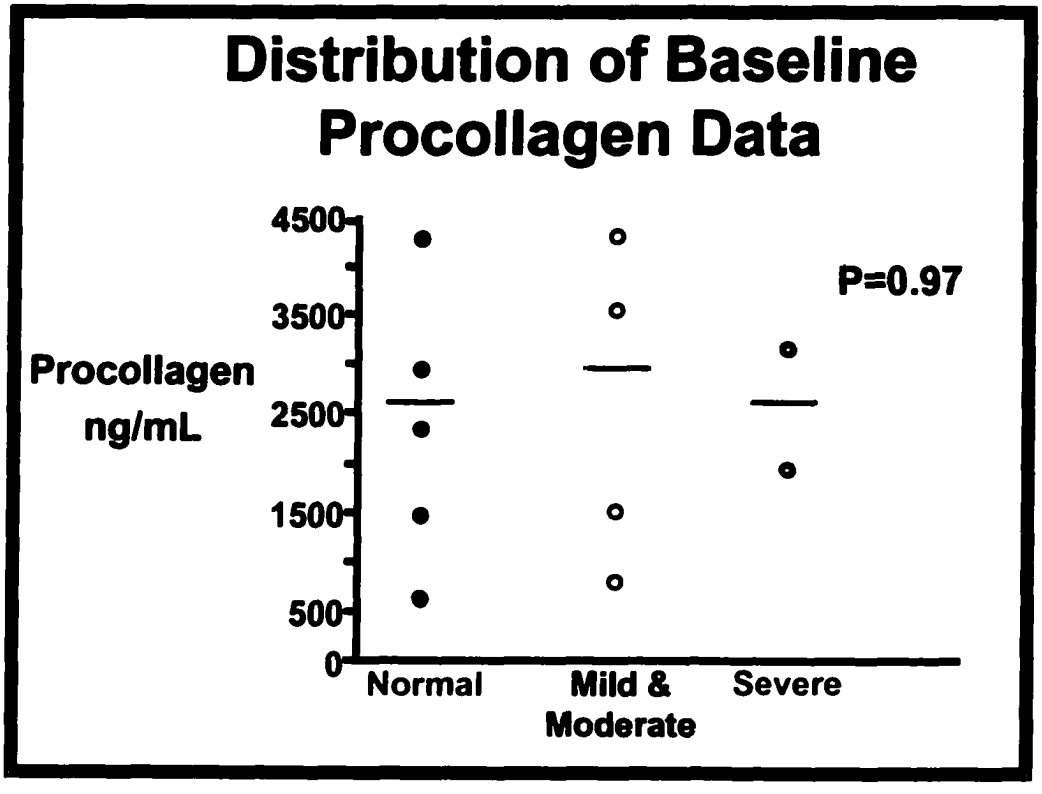


Table 7.4. Results of Fibroblast Procollagen Production with Eosinophil-Derived Proteins, Analyzed by Severity of Asthma.

	Normal Controls (n=5)	Mild/Moderate Asthma (n=4)	Severe Asthma (n=2)	P value
Baseline Procollagen (ng/mL)	2645 ± 567	2825 ± 634	2591 ± 896	0.97
Effects of proteins:				
EDN (1 μg/ml)	2873 ± 653 †	2644 ± 754	2398 ± 924	0.91
	0.99 ± 0.08 ‡	0.79 ± 0.09	0.95 ± 0.11	0.27
EDN (10 μg/ml)	2328 ± 662	1952 ± 740	2208 ± 1046	0.93
	0.84 ± 0.12	0.63 ± 0.13	0.87 ± 0.19	0.46
EDN (100 μg/ml)	2485 ± 668	2347 ± 747	2213 ± 1056	0.97
	0.92 ± 0.10	0.80 ± 0.12	0.88 ± 0.16	0.75
EPO (1 U/ml)	2277 ± 794	2276 ± 887	2530 ± 1255	0.98
	0.80 ± 0.15	0.72 ± 0.16	0.94 ± 0.23	0.75
EPO (10 U/ml)	2238 ± 522	1457 ± 522	2105 ± 738	0.57
	0.76 ± 0.09	0.52 ± 0.09	0.84 ± 0.13	0.16
EPO (100 U/ml)	1617 ± 632	1799 ± 632	1701 ± 894	0.98
	0.53 ± 0.11	0.56 ± 0.11	0.67 ± 0.15	0.78

All Data are Expressed as means ± SEM. † Data shown are absolute values; ‡ Data shown are fold inductions, i.e, condition as a ratio of unstimulated control. All P values are for between group comparisions.

Table 7.5. Results of Fibroblast Procollagen Production with Eosinophil-Derived Proteins, Analyzed with Pooled Asthmatic Populations.

	Normal Controls (n=5)	Asthmatics* (n=6)	*P* value
Baseline Procollagen (ng/mL)	2645 ± 536	2747 ± 489	0.89
Effects of proteins:			
EDN (1 μg/ml)	2873 ± 607 †	2546 ± 543	0.70
	0.99 ± 0.08 ‡	0.85 ± 0.07	0.22
EDN (10 μg/ml)	2328 ± 626	2037 ± 571	0.74
	0.84 ± 0.12	0.71 ± 0.11	0.43
EDN (100 μg/ml)	2485 ± 630	2302 ± 575	0.83
	0.92 ± 0.10	0.83 ± 0.09	0.50
EPO (1 U/ml)	2277 ± 749	2361 ± 684	0.94
	0.80 ± 0.14	0.79 ± 0.13	0.94
EPO (10 U/ml)	2238 ± 506	1673 ± 413	0.41
	0.76 ± 0.11	0.63 ± 0.09	0.38
EPO (100 U/ml)	1617 ± 592	1766 ± 483	0.85
	0.53 ± 0.10	0.60 ± 0.08	0.63

** Includes mild, moderate, and severe asthmatics. All Data are Expressed as means ± SEM. † Data shown are absolute values; ‡ Data shown are fold inductions, i.e, condition as a ratio to unstimulated control. All P values are for between group comparisions.*

DISCUSSION

We have shown that neither eosinophil-derived neurotoxin (1, 10, 100 μg/ml), nor eosinophil peroxidase (1, 10, 100 U/ml) affect airway fibroblast proliferation differently in asthmatics, as compared to normal controls. These results were confirmed when analyses were performed with both the absolute numbers and the data expressed as ratios

to the negative, unstimulated control, which accounts for the differences in baseline proliferation observed between the groups.

Our proliferation results are not in agreement with those presented by Noguchi et al. (27), in which the investigators highlight a trend of significance in the proliferative response of human lung fibroblasts to EDN, but not EPO, at similar concentrations. However, the differences between their findings and ours may be a consequence of the differences in the cell models employed. In the aforementioned study, the investigators utilized human dermal foreskin fibroblasts for their studies, which could conceivably respond differently than our lung fibroblast cultures. This concept of "tissue-specific fibroblasts" is supported by numerous studies, as dermal fibroblasts have previously been shown to differ from pulmonary fibroblasts with respect to morphology, proliferation rates, and synthesis of cytokines and extracellular components (78,79). Consequently, it is difficult to interpret and extrapolate the physiologic relevance of these differences. With the exception of this one abstract, to our knowledge, there have been no reports investigating the specific effects that each of these eosinophil granule proteins may have on airway fibroblasts in this context. However, Gleich et al. (53) have tested and compared the effects of all the major eosinophil proteins on guinea pig airway epithelium. In doing so, they demonstrated that MBP and ECP were highly cytotoxic, with concentrations as low as 10 μg/ml causing epithelial desquamation. Conversely, they found that EDN had no cytotoxic effect on the airway epithelium, even at concentrations as high as 200 μg/ml and that EPO caused partial ciliostasis and injury to the airway epithelium at concentrations in excess of 50 U/ml. However, it is difficult to

extrapolate these data to airway fibroblasts, as epithelial cells and fibroblasts are two distinct cell populations with likely distinct responses to eosinophil granule proteins.

As previously cited in the introduction, several investigators have demonstrated eosinophil-related fibroblast proliferation. Pincus et al. (19) and Shock et al. (25) have separately reported that eosinophil extracts stimulate fibroblast proliferation, suggesting that an eosinophil product directly causes fibrosis, though the precise mechanisms and/or mediators involved were not addressed. Similarly, Birkland et al. (26) reported that eosinophil-conditioned media stimulated both proliferation and collagen production in human dermal fibroblasts. However, the precise mediators and/or mechanisms involved in the eosinophil-induced fibroblast proliferation were not determined or addressed.

Similar to the proliferation data, we have shown that EDN and EPO do not affect airway fibroblast procollagen I production differently in asthmatics, as compared to normal controls. These results were confirmed when analyses were performed with both the absolute numbers and the data expressed as ratios to the negative, unstimulated control, which accounts for the differences in baseline proliferation observed between the groups. Our data are not in agreement with those previously highlighted, which strongly implicate the eosinophil as a key orchestrator in the development of fibrosis. However, the aforementioned studies are largely descriptive, providing only circumstantial evidence of the association between eosinophil infiltration and the development of fibrosis. Within the context of asthma, Djukanovic et al. (80) have reported significant associations between the degree of subepithelial fibrosis and eosinophil infiltration in biopsy tissue. However, there were no mechanisms of action addressed to account for such an association. To our knowledge, there are currently no studies that have examined

the effects of eosinophil granule proteins on airway fibroblast procollagen I production. Thus, while our data indicate that the eosinophil granule proteins do not independently enhance fibroblast procollagen production in asthma, the relationship between the eosinophil and fibroblast-induced remodeling activities cannot be dismissed. Eosinophils are capable of producing several cytokines and growth factors upon activation, many for which the responses in various airway cell types are not presently understood. Thus, it is conceivable that one, or even several, of the eosinophil-derived mediators not addressed in this study could account for significant eosinophil-induced fibroblast remodeling activities.

There is strong circumstantial evidence *in vivo* and *in vitro* suggesting that eosinophils are important contributors to asthma pathogenesis, particularly in relation to the development of airway inflammation and hyperresponsiveness. As eosinophil infiltration and bronchial epithelial desquamation are both salient features of asthma pathogenesis, the approach that the eosinophil's signature lies in its ability to induce injury to the bronchial epithelium has been a recent point of exploration (32,81). Undoubtedly, among the eosinophil-derived granule proteins, it is the MBP that has received most of the attention with respect to the eosinophil's role in this context. To start with, both the sputum (47) and bronchoalveolar lavage fluid (42) from patients with asthma have been shown to contain increased concentrations of MBP. Furthermore, MBP concentrations, comparable with those found in the sputum of asthmatics, have been shown to induce desquamation and destruction of the bronchial epithelium in human (47,50), guinea pig (51), and murine (52) airways, in a way that closely mimics the morpology of damage to the bronchial mucosa in asthma (50). In addition, localized

deposits of MBP have been detected via immunofluorescence within ulcerated areas of the bronchial mucosa and necrotic portions of the bronchial wall in patients who have died of asthma (37). Collectively, these data suggest that the eosinophil granule MBP is significantly involved in eosinophil-induced injury to the airway epithelium. Therefore, while isolated studies within the body of literature in this field suggest that eosinophils are important participants in fibroblast-mediated remodeling activities, such as cell proliferation and extracellular matrix production, it is conceivable that eosinophil-induced injury to the bronchial epithelium represents the contribution of the eosinophil to airway remodeling. In this way, the eosinophil-induced tissue injury would precipitate repair and wound healing processes. However, this link, be it direct or indirect, remains to be elucidated.

In conclusion, there is currently little conclusive evidence as to how the eosinophil participates in the airway remodeling process. However, since eosinophils are known to produce many mediators involved in the normal regulation of fibroblast proliferation and matrix production, including growth factors (i.e., TGF-β, PDGF, IGF), metalloproteinases, and collagenases, there is reason to believe that they play an important role. Future studies that would clarify the ways in which eosinophils and/or eosinophil-derived mediators can elicit both injury and repair processes, be they direct or indirect, will be useful in enhancing our understanding of the significance of the eosinophil-fibroblast interaction in the context of airway remodeling in asthmatics.

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Chapter 8

Conclusions and Future Directions

8.1. Conclusions

Throughout the course of this journey into the world of the bronchial fibroblast, we have observed how a few bronchial cytokines and growth factors elaborated in the asthmatic airway can alter fibroblast activities significant to airway remodeling, whereas others do not appear to have any effect.

In Chapter 4, we learned that IL-4, IL-13 (each alone and in combination), as well as dexamethasone (alone and in combination with IL-4 and IL-13), significantly increased fibroblast proliferation in mild/moderate asthmatics, as compared to severe asthmatics and normal controls. Furthermore, treatment of the fibroblasts with dexamethasone did not appear to alter the ability of IL-4 and IL-13 to enhance proliferation of fibroblasts. In this study, we found the mild/moderate asthmatics to exhibit the greatest proliferative responsiveness to the cytokines under investigation. In Chapter 5, we learned that stimulation with platelet-derived growth factor resulted in significant procollagen production in fibroblasts from severe asthmatics, compared to mild/moderate asthmatics and normal controls, while having no significant effect on fibroblast proliferation. In Chapter 6, we found that cysteinyl leukotrienes and insulin-like growth factor-I, either alone or in combination, had no effect on fibroblast proliferation or collagen synthesis that differentiated asthmatic populations from each

other or from healthy controls. However, fibroblast production of IGFBP3 was significantly enhanced in normal controls upon stimulation with these mediators. Finally, in Chapter 7, we learned that the eosinophil-derived proteins, eosinophil peroxidase and eosinophil-derived neurotoxin, did not affect fibroblast proliferation or procollagen synthesis in any discernable manner that is informative as to the responsiveness of fibroblasts from asthmatics from those of non-asthmatic controls.

It is clear from the spectrum of investigations reported herein that the contribution of the fibroblast to airway remodeling activities is not so simple to define. In conclusion, the studies presented are perhaps suggestive of potentially heterogeneous fibroblast populations in asthmatic populations, that are as of yet incompletely characterized. It is conceivable that the asthmatics in these studies, as well as the fibroblasts derived from their airways, may constitute significant subpopulations in addition to those already defined based on their clinical disease. The existence of such subpopulations may account for the differences in fibroblast responsiveness, and/or even the lack of responsiveness, among the asthmatic populations to the various types of cytokines and growth factors investigated in these studies.

8.2. Limitations of the Study

Perhaps the single greatest issue of limitation in the presented research is the utilization of an *in vitro* model as a representative for *in vivo* conditions, which arguably may not be a precise mimicry. It is feasible that fibroblasts existing within the milieu of the asthmatic airway, characterized by persistent inflammation and elevated cytokine levels, have, as a product of their environment, undergone phenotypic alterations that

cause them to behave differently from those of healthy non-asthmatic controls. However, it is unclear whether such phenotypic alterations persist once the cells have been removed from the inflammatory environment, such as with biopsy acquisition, or whether they are merely products of their environment, and once removed, similarly undergo phenotypic alterations arising from the lack of inflammatory stimulus. This breach in our current understanding of the pathologic mechanisms driving both airway inflammation and remodeling in asthma inevitably prevents an adequate defense of this limitation to the presented studies. Nevertheless, it is acknowledged.

Additionally, as with any cell culture studies, one has to appreciate the finicky nature of cell culture, particularly that involving primary cells that have not been virally transformed, as an inevitable limitation to a model such as ours. For example, one must be cautious that the experimental handling is executed so as to minimize any experimentally-induced phenotypic changes that can be incurred, such as is the case with different confluency states of cell cultures (1). Fortunately, in our studies, I, the author, was the sole experimentalist for the work, and therefore, we are confident that such potential concerns were at least minimized in our studies. Nevertheless, it is possible that fibroblast receptor numbers or function changed *in vitro*, resulting in reduced or increased responses to stimulation, thereby providing us with an incomplete understanding of the *in vivo* mechanisms that account for fibroblast-induced airway remodeling activities in asthmatics. Such a scenario could potentially result from either the former or the latter mentioned limitations, i.e., either an *in vivo* inflammation-induced phenotypic alteration or an *in vitro* culture-induced phenotypic alteration.

Finally, in addition to the aforementioned limitations, it is possible that within our asthmatic populations, there existed phenotypic subpopulations which have not yet been characterized. As previously mentioned, numerous studies have revealed heterogeneous asthmatic phenotypes (e.g., nocturnal, fatal, eosinophilic, neutrophilic, etc.). However, this represents an area of asthma research that is largely uncharacterized. Nevertheless, it is feasible that the asthmatic subjects within our studies, while classified on the basis of similarities in the severity of their disease, may possess different, though not yet well understood, pathologic characteristics that would, if understood, be more informative of the mechanisms driving fibroblast activities. Such a lack of knowledge is of course a limitation to our true understanding of the nature of the fibroblast in airway remodeling.

8.3. Recommendations for Future Directions

The complexity of the remodeling response and our inadequate knowledge of the *in vivo* effector functions of mediators, that have been implicated in the many facets of this dynamic process, have introduced a variety of challenges for investigators in the field of airway remodeling. Although many studies have been successful in characterizing the early cellular events involved in airway inflammation, the gap of understanding between chronic airway inflammation and repair is still quite large. Thus, future directions that would be of great interest and may enhance our understanding of fibroblast-induced airway remodeling activities may include exploratory DNA-based approaches, such as gene chip and array analyses, that could essentially guide us to a more focused pathway. By acquiring such a "window" into the genetic expression of fibroblasts, perhaps even under a variety of conditions, investigators could focus on a select group of pertinent cytokines and growth factors derived from the seemingly infinitesimal pool that exists in

asthma. While such techniques have begun to be applied to asthma research, their utilization in this context is virtually unexplored.

In addition to the exploratory studies, investigators in the field of airway remodeling would clearly benefit from the development of sound animal models of airway remodeling. Currently, there are only a limited number of such models, although it is a growing field. Fortunately, the surge of knowledge that has been generated from the human genome project has led to the development of many transgenic mouse models that have been, and continue to be, useful in the investigations of airway remodeling. The utilization of mice with targeted genetic mutations is now routine for many laboratories studying airway inflammation and fibrosis. For example, a number of laboratories have demonstrated that the Clara cell 10 kD protein promoter (CC10; also called Clara cell secretory protein) can be used to overexpress known or newly discovered genes in the murine airway. This is of great value, as it allows investigators to define and correlate pathologic, immunologic, and physiologic *in vivo* effector functions of a given transgene. This approach has successfully revealed the ability of IL-4 to generate airway mucus metaplasia (2), IL-5 to generate eosinophilic inflammation and airway remodeling (3), IL-9 to induce mast cell hyperplasia (4), and IL-11 to induce airway fibrosis, myocyte, and myofibroblast hyperplasia (5).

Thus, in conclusion, it seems worthwhile to employ molecular biology tools to facilitate the difficult task of characterizing the many potential ways in which the bronchial fibroblast may contribute to airway remodeling in asthma.

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Appendix

Figure A.1. **Fibroblast Proliferation with PDGF-AA Stimulation Analyzed by Severity of Asthma.**

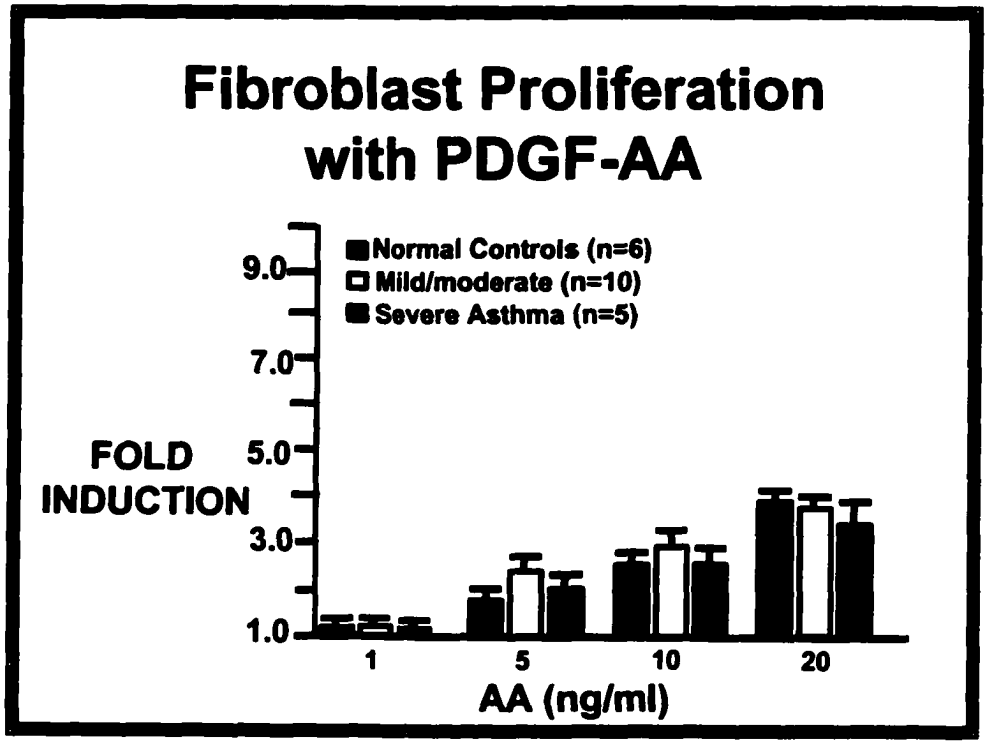

Figure A.2. **Fibroblast Proliferation with PDGF-BB Stimulation Analyzed by Severity of Asthma.**

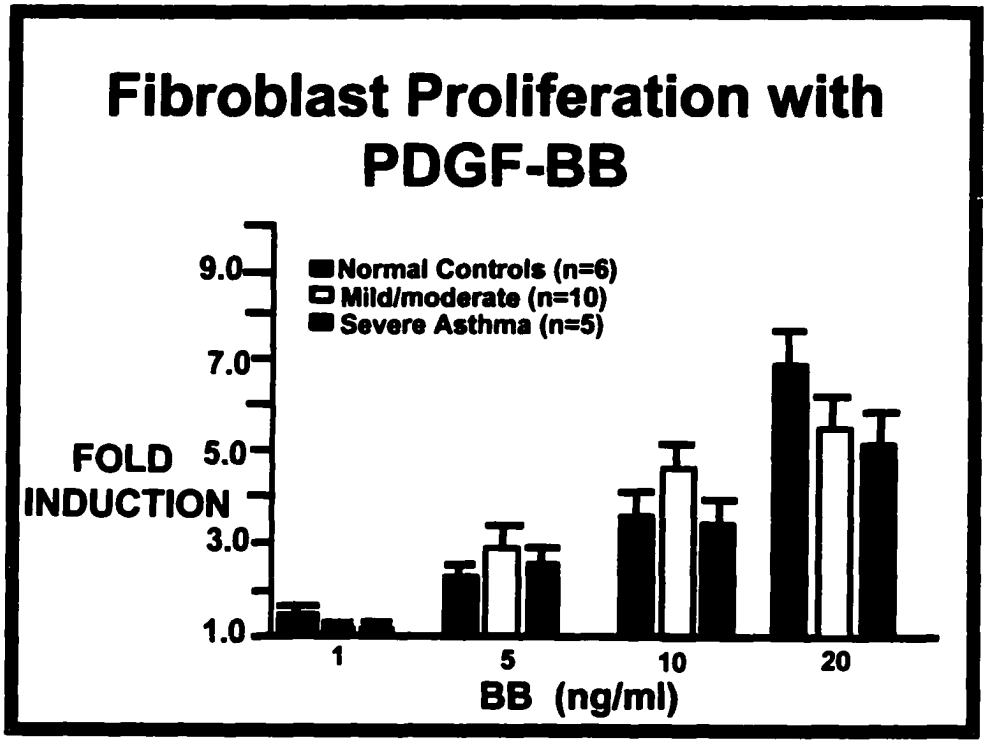

Figure A.3. **Fibroblast Proliferation with PDGF-AB Stimulation Analyzed by Severity of Asthma.**

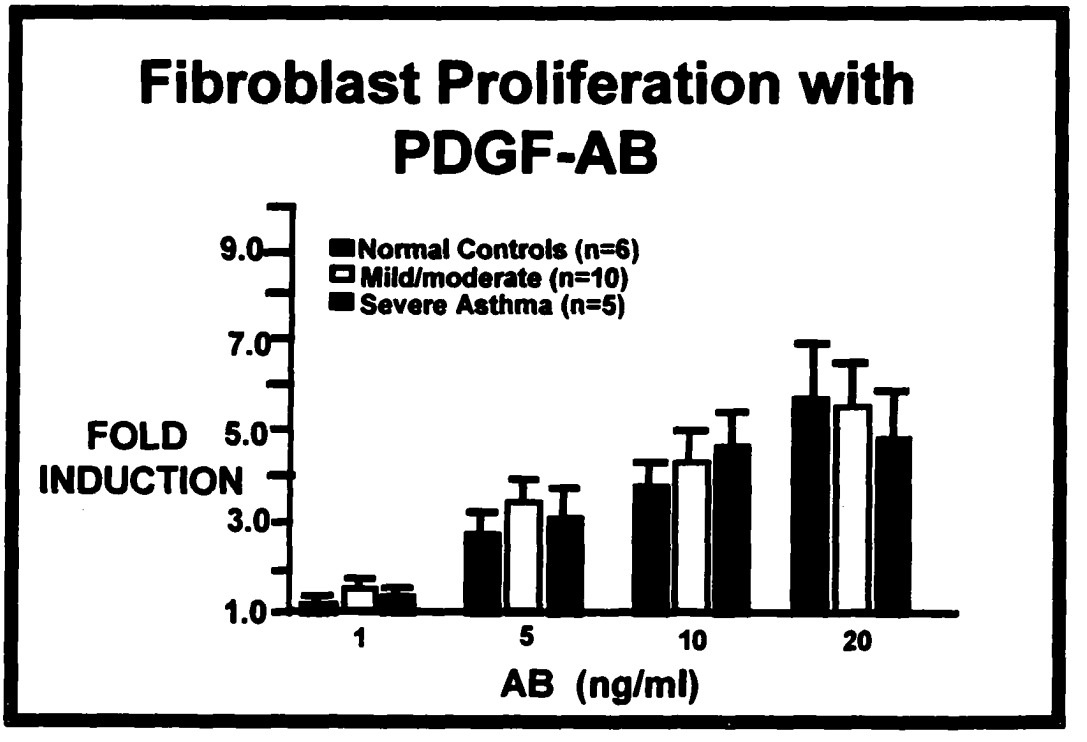

Figure A.4. **Fibroblast Proliferation with LTC_4 Stimulation by Severity of Asthma.**

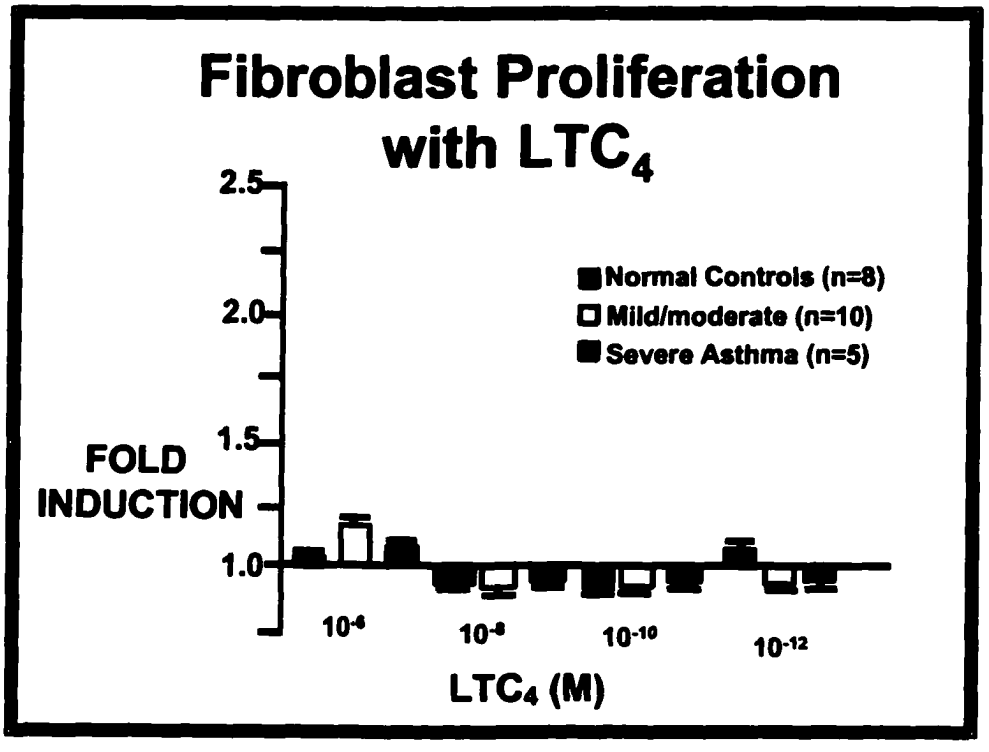

Figure A.5. **Fibroblast Proliferation with LTC_4 /IGF Stimulation by Severity of Asthma.**

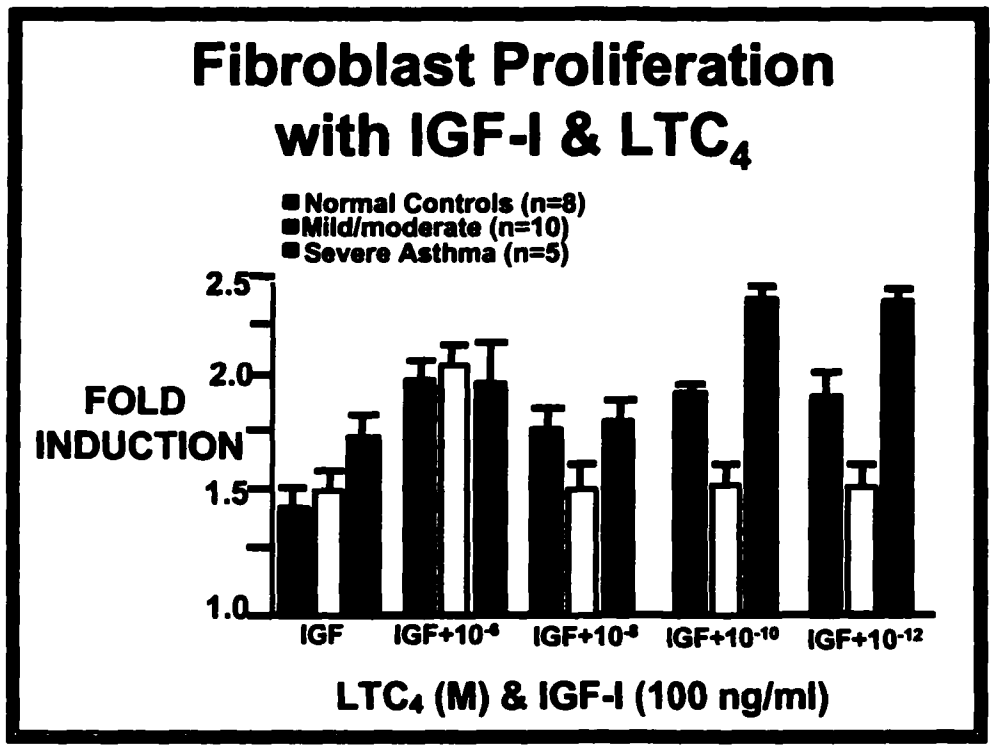

Figure A.6. **Fibroblast Procollagen Production with LTC_4 Stimulation by Severity of Asthma.**

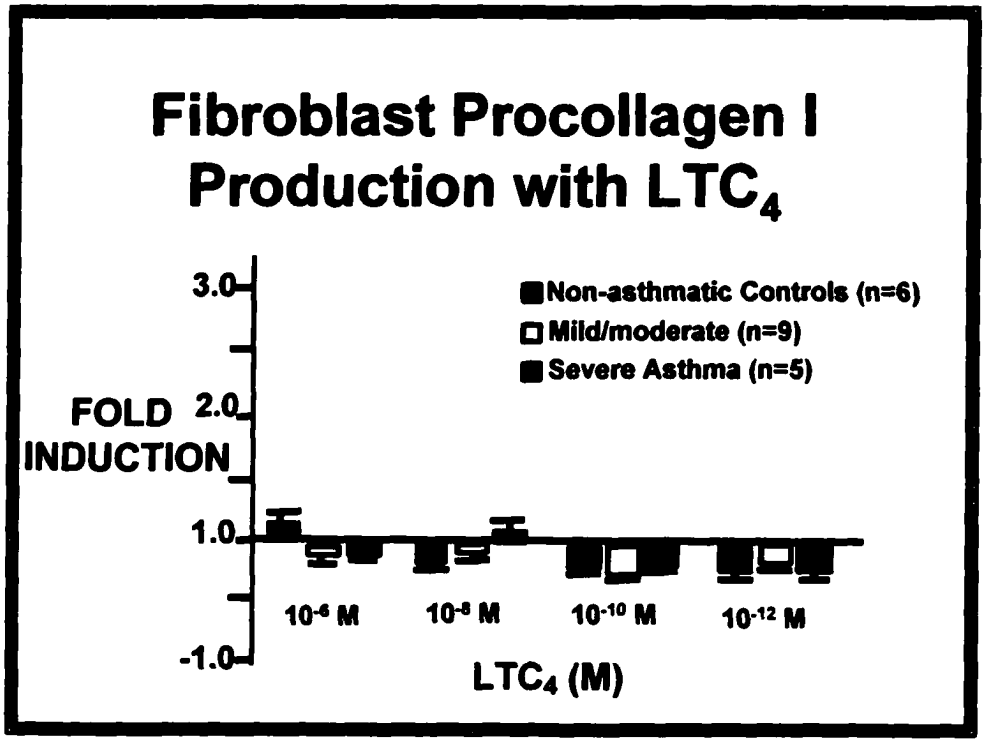

Figure A.7. **Fibroblast Procollagen Production with LTC_4 /IGF Stimulation by Severity of Asthma.**

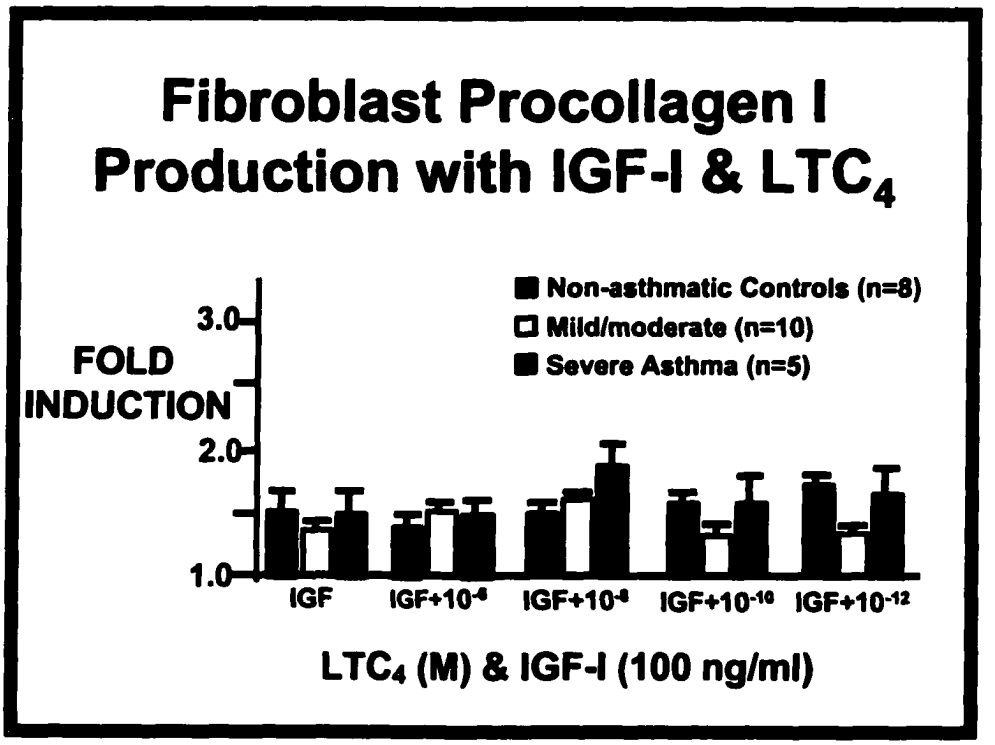

Figure A.8. **Fibroblast IGFBP3 Production with LTC_4 Stimulation by Severity of Asthma.**

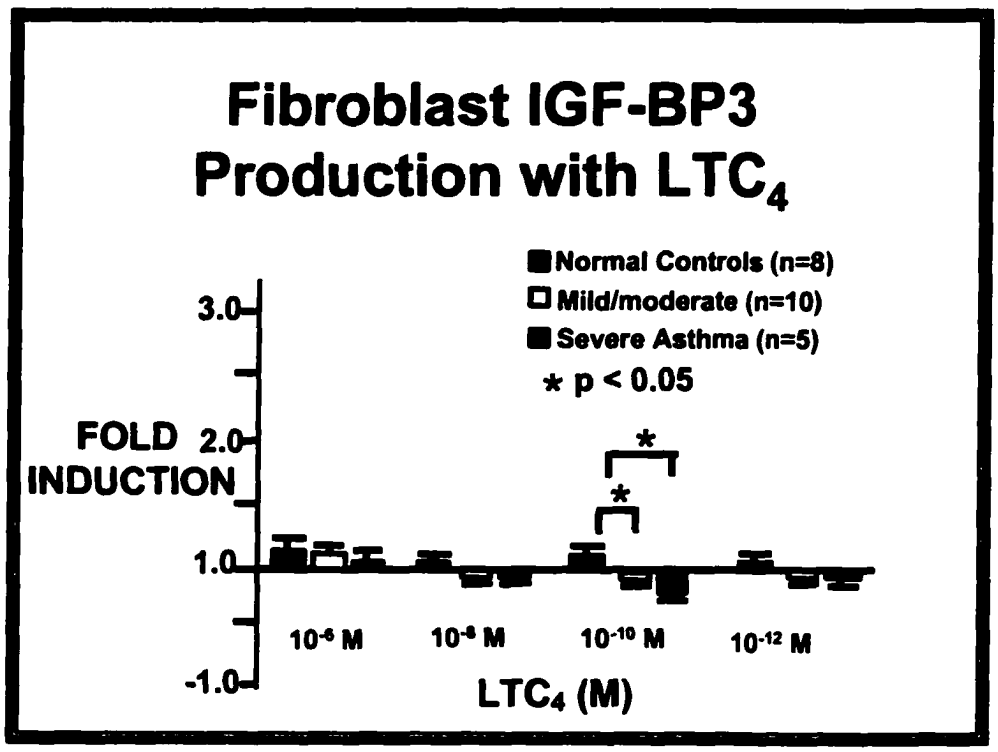

Figure A.9. **Fibroblast IGFBP3 Production with LTC_4 /IGF Stimulation by Severity of Asthma.**

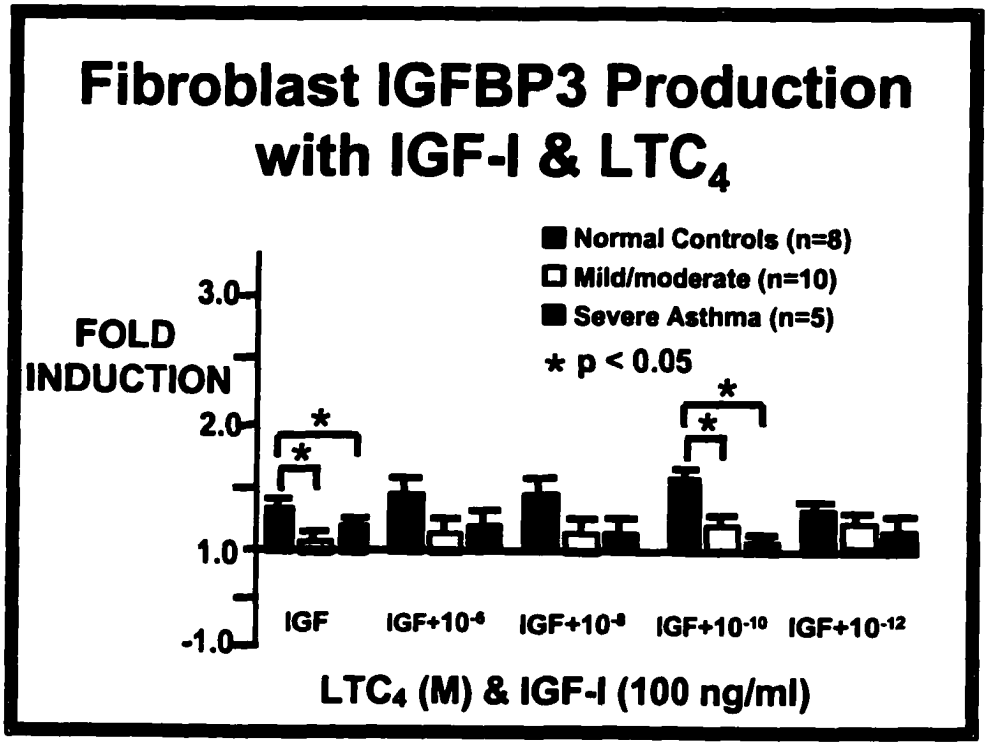

Figure A.10. **Fibroblast Proliferation with Eosinophil-Derived Neurotoxin Stimulation by Severity of Asthma.**

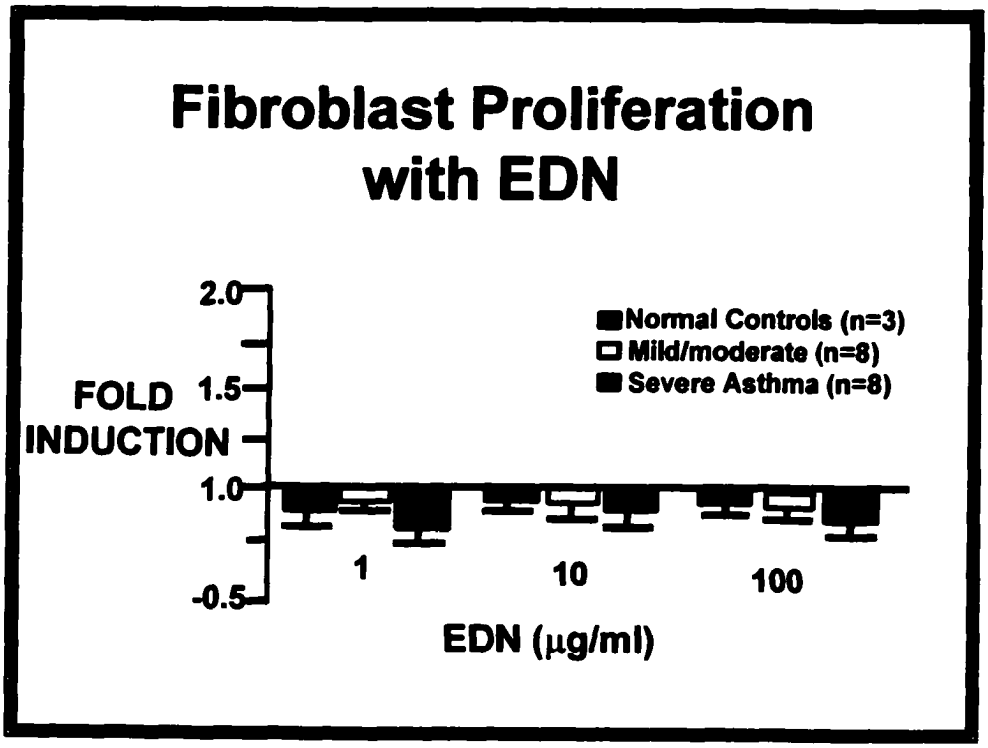

Figure A.11.Fibroblast Proliferation with Eosinophil Peroxidase Stimulation by Severity of Asthma.

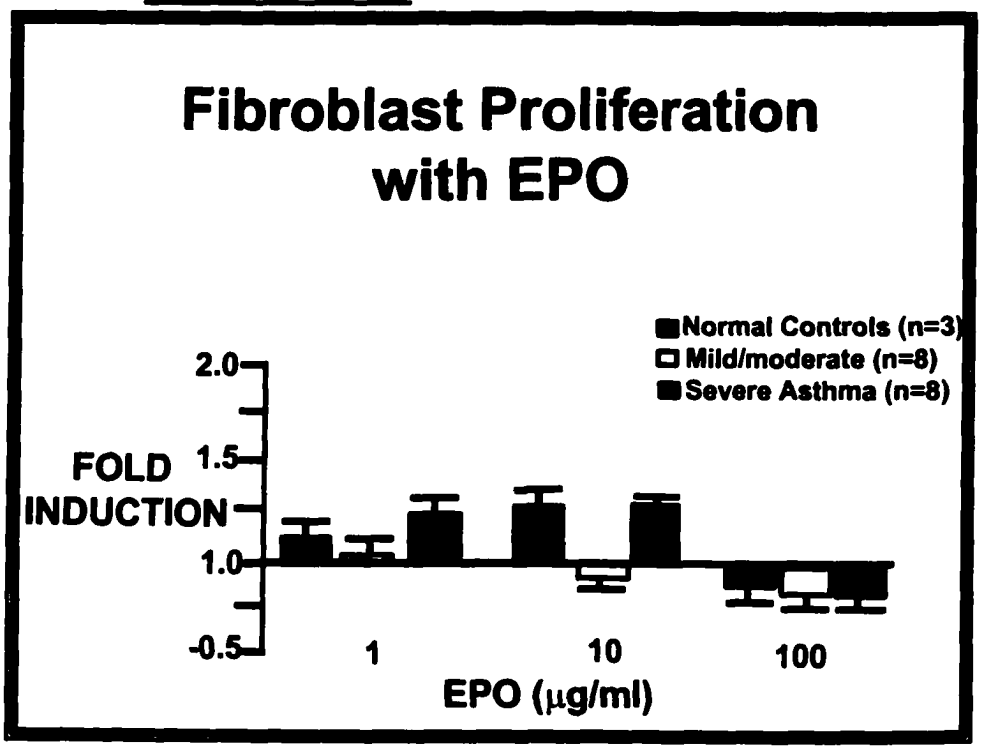

Figure A.12.Fibroblast Procollagen Production with Eosinophil-Derived Neurotoxin Stimulation by Severity of Asthma.

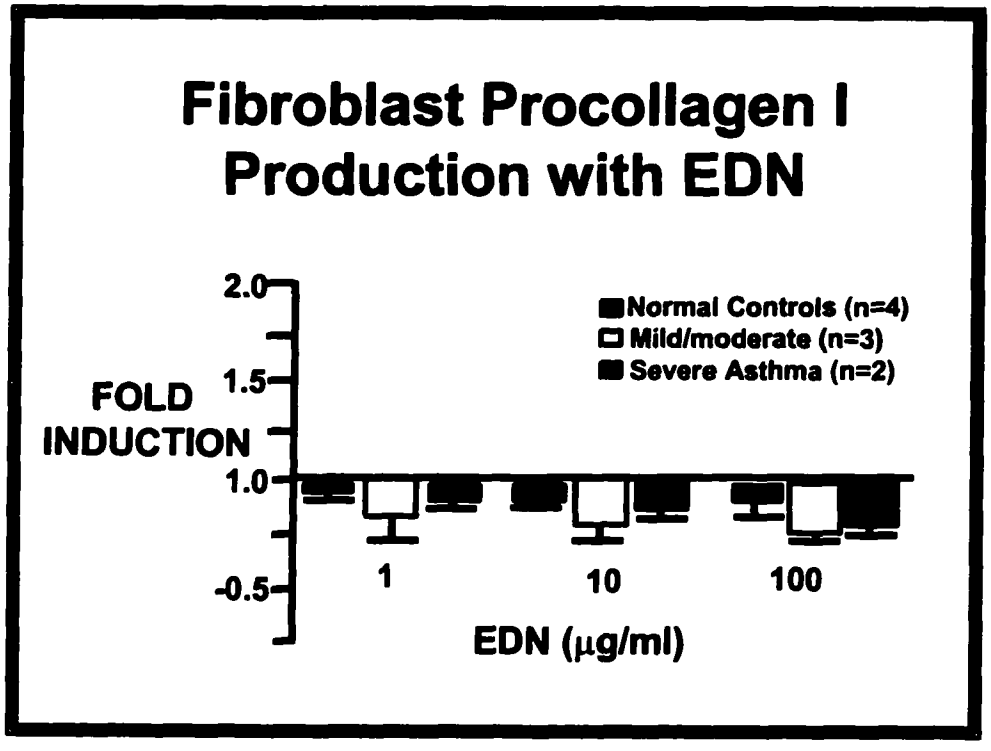

Figure A.13.Fibroblast Procollagen Production with Eosinophil Peroxidase Stimulation by Severity of Asthma.