FIBROBLAST HETEROGENEITY AND PULMONARY FIBROSIS

By

DIANE J. TORRY, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (1995) MCMASTER UNIVERSITY

(Medical Sciences) Hamilton, Ontario

TITLE: Fibroblast Heterogeneity and Pulmonary Fibrosis

AUTHOR: Diane J. Torry, B.Sc. (McMaster University)

SUPERVISOR: Dr. Jack Gauldie

NUMBER OF PAGES: xi, 141

FIBROBLAST HETEROGENEITY AND PULMONARY FIBROSIS

ABSTRACT

Idiopathic pulmonary fibrosis is a chronic inflammatory disease of the lung characterized by pathologic alterations with fibroblast proliferation and disordered deposition of extracellular matrix resulting in impairment of gas exchange often leading to respiratory failure. In the pulmonary interstitium, the fibroblast and the extracellular matrix products they produce, play a pivotal role in maintaining the structural and functional integrity of the lung.

In the study of lung fibrosis and other fibrosing diseases much attention has been directed at events which alter fibroblast activities. In this construct however, the lung fibroblast is viewed merely as a homogeneous target cell altering its behaviour as a result of immune and inflammatory events. Importantly, it has become apparent over the years that (1) fibroblasts are themselves effector cells capable of releasing a variety of growth factors and mediators and (2) that fibroblasts comprise a heterogeneous population, both within and between tissues. Therefore, an alternative, but not a mutually exclusive hypothesis considers the heterogeneous nature of the fibroblast population and the potential contribution of various subpopulations to disease expression.

Our first report that fibroblasts derived from chronically activated and

inflamed human lung tissue behaved differently than normal fibroblasts suggested that fibroblast populations present in fibrotic lung tissue exhibited accelerated growth rates. In order to further examine the growth characteristics of fibrotic lung fibroblasts, a soft agarose culture system was established in which we examined the ability of lung fibroblast primary lines to form colonies under anchorage-independent growth conditions, possibly equating to aggressive or "transformed" behaviour. Fibroblasts derived from the lungs of patients with idiopathic pulmonary fibrosis exhibited anchorage-independent growth in soft agarose culture whereas fibroblast lines derived from normal adult tissue did not. These colonies were fibroblast-like according to morphology and immunohistochemical stain characteristics, and the ability to grow under semi-solid growth conditions was maintained by the fibrotic derived cells even after selection and expansion of single colonies. Interestingly, fibroblast cell lines derived from neonatal lung tissue also exhibited the ability to grow as colonies under soft agarose growth conditions suggesting newly differentiated fibroblast populations may be prevalent in fibrotic lung tissue.

The effect of various growth and differentiating factors on the modulation of the anchorage-independent colony formation phenotype was examined in vitro. Treatment with various growth factors, including PDGF, FGF, EGF, TGFβ and corticosteroid were able to modify the colony forming

abilities of fibrotic and neonatal fibroblast lines. Importantly, none of the above treatments was able to induce fibroblasts derived from normal adult lung tissue to form colonies. The ability of IPF fibroblast lines and neonatal lung fibroblast lines to form colonies under soft agarose growth conditions was inhibited by treatment with retinoids, known differentiating agents, implying the modulation/differentiation of a particular fibroblast phenotype toward a more mature phenotype; one incapable of anchorage-independent growth.

Since the ability to form colonies under soft agarose growth conditions by fibrotic fibroblasts appeared to be a stable and disease specific phenotype and since in vitro we were able to successfully modulate this behaviour with retinoids, we examined the effect of orally administered retinoic acid on the modulation of the fibrotic lung response in a rat model of bleomycin induced pulmonary fibrosis. In this study, we report that the in vivo administration of all-trans retinoic acid significantly decreased the number of fibrotic lesions and dramatically altered the pattern of collagen deposition in the bleomycin treated lung.

These studies have confirmed and extended our earlier hypothesis concerning the contribution of fibroblast heterogeneity to the pathogenesis of pulmonary fibrosis and suggests avenues of intervention that may lead to alteration of the fibrotic tissue and return to normal lung function.

ACKNOWLEDGEMENTS

This thesis is dedicated to my mother, to my husband Steven, and to my children Albert and Gabrielle. Thanks for your love and support. Todd and Jane Ann I could have never made it without you guys. Thanks for being you. I would also like to thank Duncan, Zhou and Big Albert for their friendship over the past years (and years to come).

Finally, I wish to thank my supervisor Dr Jack Gauldie for allowing me the opportunity and freedom to make it to the finish line.

PREFACE

This dissertation consists of five chapters. The introduction to the thesis is Chapter one and reviews basic concepts and pertinent background to the thesis. It also encompasses the objectives of the study. Chapters 2 - 4, represent the core part of the thesis and comprise 1 individual published scientific paper, 1 manuscript accepted for publication and 1 manuscript submitted. Chapter 5 represents a summary chapter of the major findings and their relevance.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

13-cis	13-cis retinoic acid
AcA	acetic acid alcohol
AI	anchorage-independent growth
AtRA	all-trans retinoic acid
DEX	dexamethasone
ECM	extracellular matrix
EM	electron microscopy
FACS	fluorescence-activated cell sorting
FGF	fibroblast growth factor
GAG	glycosaminoglycan
GM-CSF	granulocyte-macrophage colony stimulating factor
IGF	insulin-like growth factor
IL-1	interleukin 1
IPF	idiopathic pulmonary fibrosis
LMP	low-melting point
LPS	lipopolysaccharide
PBM	peripheral blood monocyte
PDGF	platelet derived growth factor

PGE_2	prostaglandin E_2 series
R-al	retinal
R-ol	retinol
TGF	transforming growth factor
Thy 1	thymocyte 1
TNF	tumor necrosis factor

Chapter One

INTRODUCTION

1.1. THE LUNG FIBROBLAST

The lung fibroblast is a differentiated mesenchymal cell accounting for approximately 40% of the cells in the pulmonary interstitium (Rennard et al., 1982). Specific details as to the derivation of the mature fibroblast still remain to be determined. As such, fibroblasts are generally characterized by their location in connective tissues, their spindle shaped morphology, presence of cytoplasmic actin and vimentin filaments, and by their ability to synthesize the major extracellular matrix (ECM) proteins such as collagen I and III in the lung, fibronectin, and proteoglycan which determine the structure and influence the function of the lung (Clark et al., 1983; Raghu et al., 1991). The lung fibroblast not only influences the structure of the pulmonary parenchyma by its abundance but also influences the functioning of the lung by its contractile properties which help regulate air/blood flow (Kapanci et al., 1974). The lung fibroblast also plays a role in lung defense mechanisms owing to its phagocytic abilities (Raghu et al., 1991).

1.2. PULMONARY FIBROSIS

Pulmonary fibrosis is a devastating disease of the lung parenchyma characterized by fibroblast hyperplasia and the abnormal accumulation of extracellular matrix proteins in the interstitial and intra-alveolar compartments

of the lung (Karlinsky et al., 1982; Madri et al., 1980). Fibrosis of the lung may occur as a result of exposure of the lung to a variety of agents including (1) inhaled dusts such as asbestos and silica, (2) inhaled gases such as mustard gas, (3) therapeutic agents such as radiation therapy and systemically administered drugs such as bleomycin, (4) infectious agents and (5) autoimmune diseases such as rheumatoid arthritis and scleroderma (Weissler, 1989). However, approximately 2/3 of the cases diagnosed are of unknown etiology and are termed idiopathic pulmonary fibrosis (IPF)(Crystal et al., 1976).

Idiopathic pulmonary fibrosis is a chronic fibrotic disorder of the lung believed to be initiated by inflammatory reactions in the alveolar wall which proceed to chronicity by mechanisms not yet understood (Crystal et al., 1984). The resultant derangement of the lung architecture due to quantitative and qualitative alterations of the major extracellular matrix proteins eventually leads to decreased lung volumes and compliance with impairment of gas exchange (Basset et al., 1990; Katzenstein et al., 1985; Karlinsky et al., 1982; Madri et al., 1980). As such, pulmonary fibrosis is associated with a high mortality rate (a 5 year survival rate of approximately 50%)(Raghu, G. 1987).

As stated previously, the ECM is essential for providing both structural and functional integrity to the lung. The ECM constitutes approximately 25% of the dry weight of the lung and is composed of collagenous (60-70%) and non-collagenous proteins including elastin (25-30%), glycosaminoglycans (<

1%) and other glycoproteins such as fibronectin and laminin (Clark et al., 1983). In the pulmonary interstitium, the fibroblast is the main cell responsible for the organization of the connective tissue matrix (Fulmer et al., 1980; Bradley et al., 1980, Raghu et al., 1991). In the healthy lung, fibroblasts continuously remodel the lung matrix through a careful balance of protein synthesis and production of matrix degradative enzymes. Thus, when connective tissue sustains damage, the fibroblast is called upon to repair the damage and to restore the proper connective tissue profile unique to the lung. Lung fibrosis therefore, represents a loss of homeostasis which results in the excessive production of ECM elements by the tissue fibroblast.

1.3. COLLAGEN AND PULMONARY FIBROSIS

Collagen comprises approximately 60-70% of the total connective tissue of the adult human lung (Clark et al., 1983). The metabolism of collagen is a complex process involving a number of intracellular and extracellular steps. A procollagen molecule is formed intracellularly from the assembly of 3 alpha chains in a right handed helical arrangement and is then secreted by the cell (Crystal, 1975; Hance et al., 1976). Once outside the cell, the procollagen molecule is processed to the tropocollagen molecule (the fundamental unit of collagen) and owing to its ability to polymerize with other tropocollagen molecules forms the final collagen fibril (Crystal, 1975; Hance et al., 1976).

There are 25 known alpha chains in the human body; the various combinations of these composing the different types of collagen (van-der-Rest et al., 1991; Crystal, 1975; Hance et al., 1976). To date, there exist 14 genetically distinct collagens (Bienkowski et al., 1995), of which 12 have been identified in the lung (Adams, 1989). In the lung, type I collagen is the most abundant of the collagen types and is composed of two identical α1(I) chains and one α2(I) chain (Clark et al., 1983). Type I collagen accounts for approximately 65% of lung collagen content. Type III collagen is composed of 3 α1(III) chains and accounts for approximately 30% of lung interstitial collagen.

Pulmonary fibrosis is associated with an increase in the ratio of type I to type III collagen, with a predominance of type III collagen earlier in the disorder (Kirk et al., 1984) and type I collagen later in the disease course (Crystal et al., 1978). Of central importance to collagen deposition is the fact that in the lung, fibroblasts are the major cell type responsible for its synthesis. Not only do fibroblasts secrete collagen but in addition fibroblasts secrete degradative proteases responsible for collagen breakdown; as well as tissue inhibitors of metalloproteinase. The actual amount of collagen laid down therefore is the result of the balance between these activities (Raghu et al., 1991).

1.4. MECHANISMS OF LUNG FIBROSIS

1.4.1. The Fibroblast as a Target Cell

Previous studies focusing on the role of the fibroblast in idiopathic pulmonary fibrosis, largely focused on the notion of the fibroblast as a responder cell the behaviour of which could be regulated directly by soluble mediators released from a variety of cell types present in the inflamed tissue. Clearly, a number of cytokines and growth factors have been identified which mediate powerful effects on the function of fibroblasts by directly affecting the proliferation of fibroblasts and/or the synthesis/degradation of connective tissue elements. These mediators are for the most part synthesized and secreted by monocytes and activated macrophages, however other cell types including lymphocytes, mast cells, endothelial cells, and fibroblasts themselves can also produce these mediators.

1.4.2. Fibrogenic Cytokines

1.4.2.1. Interleukin-1

Interleukin-1 (IL-1) is a multifunctional cytokine with a variey of functions influencing inflammatory and immune processes (Dinarello et al., 1989). IL-1 protein exists in two forms, IL-1 α and β, which exhibit limited homology, but do express similar biological effects owing to their ability to bind to the same cell surface receptor (Sims et al., 1988). IL-1 is mitogenic for some fibroblast

cell lines (Schmidt et al., 1982; Thornton et al., 1990) and thus may contribute to fibrosis by stimulating fibroblast proliferation directly (Postlethwaite et al., 1988) or indirectly through PDGF induction (Raines et al., 1989). Moreover, depending on the cell line, IL-1 can either stimulate the production or promote the degradation of collagen (Matsushima et al., 1985; Postlethwaite et al., 1983; Goldring et al., 1987). IL-1 induces the production of fibronectin by fibroblasts (Krane et al., 1985). It also induces the production of matrix metalloproteinases (Postlethwaite et al., 1988).

1.4.2.2. Tumor Necrosis Factor α (TNFα)

TNF is a pleiotropic cytokine initially recognized for its capacity to induce necrosis of certain tumors (Carswell et al., 1975; Le et al., 1987; Beutler et al., 1988). TNFα is a proinflammatory cytokine, generally acting synergistically with IL-1β to orchestrate a wide variety of biological responses (Ulich, 1993). At low concentrations, TNFα is in itself mitogenic for fibroblasts, enhancing the proliferation of certain fibroblast lines, but at higher concentrations inhibiting fibroblast growth (Sugarman et al., 1985; Vilcek et al., 1986). However, the induction by TNFα of IL-1 in monocytes and endothelial cells may contribute to the fibroblast hyperplasia characteristic of fibrotic lung disease (Bachwich et al., 1986; Nawroth et al., 1986). In vitro, TNFα has been shown capable of modulating the production of extracellular matrix components, inhibiting

collagen secretion, increasing fibronectin synthesis, and stimulating increases in collagenase and glycosaminoglycan production (Dayer et al., 1985; Mauviel et al., 1988; Duncan et al., 1989; Elias et al., 1988). In vivo, TNFα has been demonstrated to be strongly fibrogenic. For example, Piquet et al., 1990 in a mouse model of bleomycin induced fibrosis demonstrated increased TNF mRNA levels and the protection from the development of bleomycin induced fibrosis by pre-treatment with anti-TNF antibody.

1.4.3. Growth Factors

1.4.3.1. Transforming Growth Factor-β (TGFβ)

The transforming growth factors (TGFβ) were initially identified by their ability to induce the transformed phenotype of anchorage-independent growth in non-neoplastic rat fibroblasts (Delarco et al., 1978). There are five known subtypes of TGFβs, but only three are known to be present in mammalian tissue (Kelley, 1992). Of these, TGFβ1 is the most prominent. TGFβs mediate numerous biological activities including a wide variety of responses that may potentially play a role in fibrosis. TGFβ can enhance the proliferation of certain fibroblast lines (Thornton et al., 1990) and is mitogenic for immature fibroblasts (Hill et al., 1986). TGFβ is chemotactic for fibroblasts, moreover, it is also chemotactic for monocytes and macrophages and can induce the synthesis of fibrogenic cytokines, including TGFβ itself in these cells (Wahl et al., 1987;

McCartney-Francis et al.,1990; Postlethwaite et al., 1987). TGFβ can directly modulate fibroblast ECM gene expression. For example, TGFβ stimulates an increase in the rate of collagen, fibronectin, proteoglycan, and tissue inhibitor of metalloproteinase (TIMP) synthesis and secretion (Roberts et al., 1986; Edwards et al., 1987; Varga et al., 1987; Overall et al., 1989) while inhibiting collagenase and stromelysin gene expression (Matrisian, 1994) and the synthesis of various proteases (Roberts et al., 1986; Ignotz et al., 1986, 1987).

In response to TGFβ, fibroblast cell lines derived from patients with IPF upregulate procollagen gene expression and decrease rates of newly synthesized procollagen degradation (Raghu et al., 1989; Harrison et al., 1991). In vivo, TGFβ has been located in the peripheral lung tissue of patients with IPF (Broekelmann et al., 1991; Khalil et al., 1991) and increases of TGFβ mRNA and protein have been demonstrated in the lung of bleomycin treated rats prior to enhanced collagen synthesis (Hoyt et al., 1988; Khalil et al., 1989).

1.4.3.2. Platelet-derived Growth Factor (PDGF)

PDGF, originally isolated from platelets (Ross et al., 1974), is a 29-33 kDa dimeric glycoprotein composed of two polypeptide chains linked by multiple disulfide bridges (Deuel et al., 1984). The two polypeptide chains, α and β, are not active as monomers, however the intact dimeric forms ie $\alpha\beta$, $\alpha\alpha$,

or $\beta\beta$ all exhibit biologic activity (Kelley et al., 1990). PDGF has a multitude of activities. As a growth factor, PDGF is a potent mitogen for fibroblasts (Heldin et al., 1988; Clark et al., 1993). It has also been shown to be chemotactic for fibroblasts (Seppa et al., 1982) and for smooth muscle cells which also accumulate in the pulmonary interstitium during the course of IPF (Grotendorst et al., 1982). As a fibrogenic cytokine, PDGF has been reported to enhance the rate of collagen secretion (Thornton et al., 1990) and to stimulate fibroblasts to release collagenase (Bauer et al., 1985). In vivo, there is increasing evidence for the association of PDGF with IPF. For example, alveolar macrophages isolated from IPF patients exhibit increased PDGF transcription rates (Nagaoka et al., 1990), increased amounts of PDGF mRNA (Shaw et al., 1991) and the spontaneous release of PDGF protein (Martinet et al., 1987). Anti-PDGF antibody staining has been demonstrated in interstitial macrophages present in fibrotic lung biopsy specimens (Vignaud et al., 1991)

1.4.3.3. Fibroblast Growth Factors (FGFs)

The fibroblast growth factors are structurally related polypeptides that have been divided into acidic and basic FGFs based on their differing affinities for heparin (Lobb et al., 1986; Kelley, 1990). Acidic and basic FGFs are encoded by different genes but share 55% homology, and are similar in their biologic effects. Both types are mitogenic for mesenchymal cells, induce

collagen and fibronectin synthesis, and induce proteoglycan synthesis (Sprugel et al., 1987; D'Amore, 1990)

1.4.2.4. Insulin-like Growth Factors (IGFs)

The insulin-like growth factors (IGFs) are a family of small peptides having molecular weights of approximately 7.6 kDa (Stiles et al., 1993). IGFs were initially recognized for their ability to act as mitogens for a variety of cell types (Stiles et al., 1993). IGFs potentially play a role in inflammation and fibrosis through their ability to stimulate proliferation, chemotaxis and collagen production by lung fibroblasts (Goldstein et al., 1989). There is also increasing evidence implicating IGFs in the pathogenesis of fibrotic lung disease. Alveolar macrophages have been isolated from patients with IPF and have been demonstrated to secrete increased amounts of IGF-1 (Rom et al., 1988). Increased amounts of IGF-1 have also been detected in the bronchoalveolar lavage fluid from patients with systemic sclerosis (Harrison et al., 1994).

1.4.4. Extracellular Matrix Components

Not only is the extracellular matrix (ECM) an important structural support for cellular constituents, but several components of the extracellular matrix have been shown capable of modulating inflammatory and immune cell function. The degradation products of elastin and fibronectin such as would

occur at sites of tissue injury have been documented to be chemotactic for fibroblasts (Senior et al., 1982; Postlethwaite et al., 1981). Moreover, components of the fibronectin molecule are able to stimulate the proliferation of resting fibroblasts (Bitterman et al., 1983). In vivo, macrophages derived from patients with pulmonary fibrosis spontaneously release more fibronectin than those derived from healthy lungs (Rennard et al., 1981). Fibronectin production has also been shown to be increased in cells from patients with interstitial lung disease (Silver et al., 1990). The ECM may also serve as a source/anchor for signals capable of modulating fibroblast growth. For example, heparin and proteoglycans are known to bind many growth factors and cytokines (ie FGF, TGFβ and TNFα)(Roche, 1995; Nathan and Sporn, 1991). The importance of this function of the ECM may be to concentrate or store mediators at specific sites or to prevent their degradation (Schubert, 1992).

1.5. THE FIBROBLAST AND LUNG FIBROSIS

Several lines of evidence suggest that fibroblasts may play a key role in the development of lung fibrosis. First, their numbers are shown to increase in areas of active fibrosis (Kuhn et al., 1989) which ultimately represents a potential increase in the number of cells producing extracellular matrix proteins. Secondly, the lung fibroblast is the predominant cellular source of collagen I

which is increased in patients with IPF (Hance et al., 1976; Crystal et al., 1978). Third, fibroblasts examined from IPF lung biopsy specimens reveal an increase in the proportion of activated fibroblasts. This in contrast to fibroblasts present in normal lungs which are essentially in a quiescent state (Kuhn et al., 1989) Thus, an increase in the number of fibroblasts actively synthesizing collagen could account for alterations in the lung matrix. Fourth, although alterations in the behaviour of inflammatory/immune cells present in IPF have been well documented, cells such as the alveolar macrophage and neutrophil are somewhat short-lived and thus may not alone account for the chronicity present. Moreover, treatment of IPF patients with drugs believed to alter the activity of effector immune cells has not proven to be of great benefit in the treatment of pulmonary fibrosis. Fifth, fibroblast mitogens are released spontaneously by alveolar macrophages isolated from patients with fibrosing alveolitis (Bitterman et al., 1982). Sixth, in lung biopsy specimens from IPF patients, decreased collagen degradation has been observed which could eventually result in increased lung collagen content (Selman et al., 1986). A reduction in the degradation of newly synthesized collagen has also been reported in rabbits treated with bleomycin (Laurent et al., 1983) and finally, fibroblast cell lines derived from fibrotic lung tissue have been found to rapidly proliferate in culture (Jordana et al., 1988) leading these latter authors to propose that the fibroblast hyperplasia characteristic of fibrosis may result from

conditions present during the process of inflammation and fibrosis which select for rapidly replicating clones of fibroblasts.

1.6. THE FIBROBLAST AS AN EFFECTOR CELL

As stated previously, fibroblasts as responder cells are the key producers of matrix proteins and their function can be modulated by the release of mediators from macrophages, neutrophils and lymphocytes. However, it has become increasingly evident that fibroblasts are effector cells in their own right. For example, fibroblasts are able to produce a wide variety of cytokines such as IL-1, IL-6, and IL-8 (Elias et al., 1989,1990; Rolfe et al., 1991; Strieter et al., 1989a,1989b; Scott et al., 1990; Vancheri et al., 1989) and eicosanoids (Elias et al., 1987b) which can influence the inflamed state. Fibroblasts are capable of producing mitogenic cytokines and can synthesize their own progression factors in response to PDGF (Clemmons et al., 1985; Fabisiak et al., 1992). Moreover, through their production of extracellular matrix proteins, fibroblasts are able to modulate the function of inflammatory cell populations that infiltrate the lung during disease progression. Finally, to add to the complexity of the role of the fibroblast in fibrosis, a large volume of evidence has now accumulated documenting the existence of heterogeneity among fibroblast populations.

1.7. FIBROBLAST HETEROGENEITY

Until recently, it was assumed that fibroblasts represented a homogeneous population of cells which functioned merely as target cells, the behaviour of which could be modified upon direct stimulation by mediators secreted by other cell types. While fibroblasts as a group do share common characteristics such as structure, anatomical location, and extracellular matrix production, a number of studies have documented significant differences in the phenotype and function of fibroblasts.

1.7.1. Proliferation

It has been suggested that in the normal lung, only a small proportion of fibroblasts are actually proliferating at any one time (Jordana et al., 1992). Although the mere presence of proliferating/non-proliferating cells in the lung could be viewed as a measure of heterogeneity, studies on the growth potentials of cultures of fibroblasts reveals that when fibroblasts divide, they produce clones with a wide variety of doubling potentials, including clones which divide very little and are referred to as the terminally differentiated pool (Martin et al., 1974). Moreover, continuous cloning of these populations results in a shift in the distribution of rapidly proliferating cells towards those with slower growth rates. The observation by Hayflick and Moorhead (1961) that clones of fetal lung fibroblasts exhibit a limited proliferative capacity i.e.

only 5% of cells are still capable of cell division 50 passages later, led to the hypothesis that a decrease in growth potentials of fibroblast cultures was a consequence of terminal differentiation (Smith et al., 1974). It was hypothesized by two groups (Kirkwood et al., 1975; Jones et al., 1982) that there exists in culture a limited number of stem-like cells and that everytime a cell undergoes division, its chances of becoming a committed cell with a limited growth potential increases. Thus, true fibroblast growth heterogeneity is more likely reflective of differences in the proportions of cycling/non-cycling cells in the tissue at any one time.

Variations in the proliferative rates of fibroblasts isolated from different tissues has been observed by a number of investigators. In the early 1960s, comparisons of fibroblasts obtained from the skin, lung, synovial tissue, and peritoneum revealed differences in proliferative potential and mucopolysaccharide synthesis (Castor et al., 1962). In the 1970s, Schneider et al. (1977) compared fetal fibroblasts derived from lung and skin tissues and observed that lung fibroblasts displayed faster growth rates, lengthened in vitro life spans, higher cell numbers at confluency, and differences in cellular morphology.

A large body of evidence has also accumulated demonstrating the existence of growth heterogeneity within the fibroblast population of a specific tissue. Differences in cell proliferative rates have been documented by Harper

et al. (1979) who examined normal adult fibroblasts isolated from the papillary layer and the reticular layer of the dermis and observed that papillary fibroblasts exhibited greater proliferative rates. Azzarone et al. (1982) extended these studies and observed that fibroblasts derived from the papillary regions of the skin exhibited a greater proliferative potential, saturation density, and in vitro lifespan as compared to fibroblasts isolated from the reticular region. Differences in cell growth rates have also been documented in fibroblast cultures derived from gingival tissue. Moreover, these differences appeared to be stable since the observed heterogeneity persisted throughout numerous in vitro cell replications (Hassell et al., 1983).

Studies examining the growth rates of fibroblasts derived from fibrotic tissue have demonstrated differences in the proliferative rates of fibroblasts derived from fibrotic as compared to normal tissue. Rodemann et al. (1990) isolated fibroblasts from kidneys affected with interstitial fibrosis and observed that these fibroblasts exhibited abnormal hyperproliferative growth characteristics as compared to fibroblasts obtained from normal tissues. In addition, these data illustrated for the first time increased fibroblast numbers in fibrotic kidneys.

Chen et al. (1992) isolated mesenchymal cells from patients dying with acute lung injury and observed that these cells displayed enhanced proliferative rates as compared to control cells when analyzed in serum-free defined medium

containing no exogenous peptide growth factors. Moreover, this proliferative phenotype was observed to be stable for at least 5 passages in vitro.

In studies documented by Jordana et al. (1988) it was observed that fibroblast cell lines and clones of fibroblast lines derived from fibrotic lung tissue exhibited greater mean proliferative rates than fibroblasts derived from normal lung tissue. Raghu et al. (1988) also studied the behaviour of fibroblasts obtained from normal and fibrotic lung and observed that fibroblasts derived from early fibrosis proliferated quicker than fibroblasts obtained from normal lung. Moreover, these authors also demonstrated differences in the proliferative potential of fibroblasts derived from early versus late fibrosis, such that fibroblasts obtained from early disease displayed a greater proliferative potential than fibroblasts from late fibrosis. These results substantiate those of Absher et al. (1984) who demonstrated in a rat model of bleomycin induced pulmonary fibrosis that lung fibroblasts derived from end stage fibrotic tissue displayed decreased growth rates and a decreased cell yield as compared to normals. Thus, from these data, we suggest that a "growth aggressive" fibroblast subpopulation preexists in the fibrotic lung and that it is somehow selected and expanded during the pathogenesis of fibrosis or evolves as a result of a differentiating event(s), thus altering the overall behaviour of the lung.

Interestingly, Jordana et al. (1988) has observed that primary lines of neonatal lung fibroblasts exhibited faster proliferative rates when compared to

adult lung fibroblasts. Although this observation is not entirely unexpected owing to the previous discussion of growth potentials in mass cultures, it is interesting in that it predicts the emergence of newly differentiated cells (such as would be mobilized during normal injury and repair processes) early in disease progression exhibiting greater proliferative potentials. In addition, as the disease progresses to a more advanced stage, changes would eventually occur within the lung parenchyma as the pool of 'younger' fibroblasts shift to a more differentiated / less proliferative state, as would be suggested by the studies of Raghu et al. (1988) and Absher et al. (1988).

The notion that disease development might be related to the persistent expression of a fetal or newly differentiated fibroblast phenotype or due to the emergence of a newly differentiated stem-like cell has also been hypothesized in neoplastic disease (Schor et al., 1987) and in atherosclerosis (Schwartz et al., 1985). Indeed, fetal fibroblasts and adult fibroblasts do differ from each other in a number of ways. These include differences in morphology (Maksvytis et al, 1984); ability to grow under conditions of anchorage-independent growth (Nakano et al., 1981); differences in expression of specific growth factors such as IGFs and PDGF (Clemmons et al., 1983; Daughaday et al., 1989); differences in response to various mediators (Hill et al., 1986; Edwards et al., 1987; Romaris et al., 1991); and differences in the production of extracellular matrix elements, specifically glycosaminoglycan synthesis

(Caniggia et al., 1992; Matsuura et al., 1985). Therefore, the development of fibrosis may in fact involve a differentiating event resulting in the presence of new young fibroblasts behaving more in keeping with a growing and proliferating tissue such as found in the neonatal developing lung. It is therefore possible that the fibrotic aspect of IPF is a consequence of not being able to shut off the behaviour of this newly differentiated cell population or to switch the cells to an adult behaviour.

1.7.2. Morphology

Two studies have documented the separation of fibroblast subsets on the basis of cell size. Mitsui et al., (1976) have fractionated human lung fibroblasts into two populations by gravity sedimentation through fetal bovine serum gradient. This separation procedure yielded fibroblast populations containing either large, slowly replicating cells, or small rapidly dividing cells. Elias et al. (1987) isolated 3 main fractions of human lung fibroblasts by gravity sedimentation through Percoll which were heterogeneous in cell volume and growth. However, in both the study of Mitsui et al. (1976) and Elias et al. (1987) the fractionated phenotypes were not retained in culture.

Lung fibroblast subpopulations have also been characterized on the basis of cell shape, distribution of cellular organelles, and on whether or not they contained lipid droplets. Wolosewick et al. (1976) have documented the

existence of two morphologically distinct fibroblast populations of intermediate passage which differed in terms of cell size and shape. One fibroblast subset displayed features of the typical fibroblast type which was small, fusiform in shape with a centrally placed nucleus. The other fibroblast subset contained larger cells, was nonfusiform in shape with a nucleus placed to one side of the cytoplasm. These two subpopulations were also observed to differ in the size of nuclei and the distribution of internal organelles and inclusions.

Voccaro and Brody (1978) examined postnatal alveolar septal formation and described the presence of an interstitial fibroblast filled with neutral lipid. The subsequent isolation and characterization of the lipid-containing interstitial cell has revealed that (i) lipid and nonlipid containing interstitial lung cells are heterogeneous with respect to growth rates (Maksvytis et al. 1981; Brody et al., 1983), (ii) lipid containing interstitial cells are the most abundant in the neonatal rat lung, however they are present in smaller numbers in the adult rat lung (Kaplan et al., 1985), (iii) lipid interstitial cells contain dense bundles of cytoplasmic microfilaments (Maksvytis et al., 1981) and (iv) at no time was any evidence observed of conversion from a lipid to a nonlipid phenotype (Brody et al., 1983). A number of related studies have since examined further the nature of the lipid interstitial fibroblast and are reviewed by Berk et al., (1992).

A second type of interstitial cell found in the lung which is found to express contractile features is the myofibroblast (Gabbiani et al., 1971; Darby

et al., 1990). The myofibroblast is unique in that morphologically it resembles a fibroblast, but under EM examination, it has been shown to possess contractile elements similar to smooth muscle cells. Specifically, a number of these cells exhibit staining for α smooth muscle cell actin (Darby et al., 1990). Cells expressing the myofibroblast phenotype have been identified in (1) a variety of normal tissues including the lung, (2) the granulation tissue of wound healing, (3) in IPF tissue, and (4) in other chronically inflamed airways tissue (Ryan et al., 1974; Adler et al., 1981; Schmitt-Graff et al., 1994; Tremblay et al., 1994). Functionally, it is believed that the myofibroblast phenotype plays an important role in wound contraction in both normal repair and pathological processes (Majno et al., 1971; Kuhn et al., 1991). Therefore, it would appear that fibroblast subpopulations exist in normal tissue which express distinct cytoskeletal features conferring upon them unique functional capabilities. Current evidence suggests that the myofibroblast is locally derived from surrounding mesencymal cells although its specific cell of origin is unknown (Juliano et al., 1993). Whether the myofibroblast represents an induced phenotype or is representative of a stage of fibroblast development is unknown (Schmitt-Graff et al., 1994; Tremblay et al., 1994).

A third type of adult lung cell which is found to express contractile features is the contractile interstitial cell (CIC)(Kapanci et al., 1974; Adler et al., 1989). The CICs also exhibit ultrastructural characteristics of both fibroblasts

and smooth muscle cells. Interestingly, EM and immunofluorescence studies reveal the presence of actin filaments in their cytoplasm and it is believed that the contraction of these cells may play a role in normal lung ventilation and perfusion of the alveoli (Kapanci et al., 1974). Ultrastructurally, these cells appear similar to the myofibroblast and are now believed to be identical to the lipid interstitial cell characterized in mature rodent lungs (Kaplan et al., 1985).

Finally, studies by Conrad et al. (1977) provide evidence for heterogeneity between tissues by describing fibroblast cell lines derived from cornea, heart and skin which differ in morphology, saturation densities and sensitivity to trypsin and EDTA.

1.7.3. Responses to Cellular Mediators

Fibroblasts have been observed to differ in their response to cellular mediators. Kondo et al. (1985) observed that fetal lung and skin fibroblasts differed on the basis of cell growth in response to hydrocortisone and by differences in the binding of dexamethasone. In dermal fibroblasts, evidence provided by Smith et al. (1989) demonstrated that while dermal fibroblast glycosaminoglycan (GAG) synthesis was inhibited by thyroid hormone and glucocorticoid, fibroblasts derived from retroocular tissues were not responsive to either. Moreover, GAG production was demonstrated to be upregulated by interferon gamma in retroocular fibroblasts but not in dermal fibroblasts (Smith

et al., 1991).

A number of investigators have also reported differences in the response of lung fibroblasts to various mediators. Xing et al. (1992) demonstrated heterogeneity in the responsiveness of different fibroblast populations within the respiratory tract to lipopolysaccharide (LPS). The authors examined fibroblasts derived from lung (either normal or fibrotic), bronchial, and nasal (normal or nasal polyp) tissue and reported that whereas IL-1 was able to induce the synthesis of GM-CSF, IL-8, and IL-6 by all respiratory fibroblasts, exposure to LPS directly stimulated the synthesis of these cytokines only in the nasal fibroblast populations. Studies by Jordana et al. (1992) examined the ability of normal adult lung fibroblasts and normal neonatal lung fibroblasts to proliferate in response to fibroblast conditioned media derived from IPF fibroblasts, normal neonatal fibroblasts, and control fibroblasts. Specifically, these authors demonstrated that while neonatal derived supernatants yielded the greatest effect on fibroblast proliferation, the stimulation of proliferation was largely dependent upon the target cell employed such that neonatal cells consistently displayed greater proliferative responses to the same medium when compared to control fibroblasts. These results are in agreement with studies by Plisko et al. (1983) who observed that the growth responsiveness of neonatal skin fibroblasts was greater than adult skin fibroblasts in response to the same serum mitogens. Importantly, these studies suggest that the

proliferative events occuring during the process of inflammation and fibrosis are not only affected by the presence of cellular mediators and mitogens, but also by the responsiveness of the target cell.

Since pulmonary fibrosis is a chronic condition characterized by an ongoing inflammatory response, the effect of prolonged exposure of fibroblasts to various cellular mediators yields interest. To this end, Ko et al. (1977) observed two subpopulations of normal human gingival fibroblasts based on their sensitivity or insensitivity to the growth inhibiting effects of prostaglandin such that the incubation of these fibroblasts for 2 weeks in media supplemented with prostaglandin E_2 (PGE_2) resulted in the eventual outgrowth of a PGE_2 unresponsive population. The proliferative response of clonally derived lung fibroblast lines chronically exposed to peripheral blood monocyte (PBM) supernatants was also examined by Jordana et al. (1992). These authors demonstrated that following prolonged exposure of lung fibroblasts to LPS stimulated PBM supernatant, fewer clones were capable of being inhibited when reexposed to PGE_2. Interestingly, these authors also demonstrated that clones of rapidly proliferating IPF fibroblasts were only minimally inhibited by acute exposure to PBM supernatants as compared to control lung fibroblasts (Jordana et al., 1992). Similarly, Mio et al. (1992) examined the growth characteristics of fibroblasts derived from IPF lung tissue and control fibroblasts and reported that fibroblasts obtained from higher-intensity fibrotic lesions were

less inhibited then either control or fibroblasts obtained from lower-intensity fibrotic lesions in their proliferative response to PGE_2.

Korn et al. (1984) observed that fibroblasts derived from neonatal foreskin were also heterogeneous in their response to stimulation by mitogen activated mononuclear cell supernatants. Eleven fibroblast substrains revealed differences in proliferative rates and prostaglandin E_2 synthesis. Since the growth suppression observed among the fibroblast clones coincided with the stimulation of prostaglandin synthesis in these clones, the differences in proliferative rates observed most likely reflected inherent differences in the prostaglandin synthetic response of the fibroblast clone itself. Moreover, in a related study, Korn et al. (1985) demonstrated that short-term exposure of neonatal skin fibroblasts to mitogen-activated mononuclear cell supernatants resulted in the selection of fibroblasts that were hyperresponsive in their prostaglandin synthetic response upon re-stimulation by mononuclear cell products. Furthermore, this response was observed to persist for as long as 4 months in culture (Korn et al., 1983).

1.7.4. Cell Surface Receptor/Protein Expression

Studies by Ndumbe et al. (1985) have utilized monoclonal antibodies in order to characterize surface antigen differences in fibroblasts derived from bone marrow, skin, and human embryo lung tissue. Subpopulations of rat lung

fibroblasts have been separated by the use of fluorescence-activated cell sorting (FACS) based on the relative cell surface associated expression of collagen type I or III (Breen et al., 1990). One subset displayed a high density of type I collagen receptors and a low density of type III collagen receptors, while the other subpopulation expressed high type III collagen receptors and low type I collagen receptor intensities. Differences in the intensity of staining correlated with differences in the cellular steady state levels of type I and type III procollagen mRNA. These subpopulations were able to maintain this phenotypes for at least 4 passages.

Bordin et al. (1984) defined by FACS analysis a subset of normal gingival fibroblasts characterized by high levels of C1q receptors. In addition, these cells displayed an enhanced proliferative rate after exposure to C1q, as well as an increased protein synthetic rate, of which approximately 40% was directed to collagen production. These properties were found to be maintained in culture for at least 12 population doublings. In a similar study, Maxwell et al. (1987) examined skin fibroblasts derived from patients with early or chronic Scleroderma for C1q binding and demonstrated that fibroblasts isolated from early lesions bound significantly higher amounts of C1q.

Phipps et al. (1989) have isolated two distinct and stable populations of fibroblasts derived from normal murine lungs based on the surface expression of thymocyte-1 (Thy1) antigen. Thy1+ and Thy1- populations were found to

exhibit distinct morphologies and functions. Thy1+ fibroblasts are spindle shaped, contain intracellular lipid, and proliferate more rapidly than the Thy1- populations. In response to treatment with interferonγ, both subsets express class I major histocompatibility antigens (MHC), however class II MHC expression and the presentation of antigen to T lymphocyte clones was only inducible in Thy1- fibroblasts. Thy1- fibroblast populations also synthesized IL-1α, with TNFα treatment further enhancing IL-1 production 5-20 fold. Both populations of lung fibroblasts are able to synthesize fibronectin and collagen (the major types produced I and III)(Derdak et al., 1992) however, the Thy1+ subset was observed to synthesize 2-3 fold more collagen. A review of the characterization of Thy1+ and Thy1- subpopulations can be found in Froncek et al., 1992.

Siminski et al. (1991) have examined fibroblasts derived from normal and fibrotic human lungs for Thy1 expression. Two populations of cells were identified with low Thy1+ cells exhibiting a greater proliferative capacity than those that expressed high levels of Thy1 antigen. Interestingly, the two subsets of cells were grown under the same culture conditions for the next 5 days and the cellular profiles of anti-human Thy1 surface expression repeated. The cells sorted initially as high and low binding Thy1+ cells retained their respective profiles after 24 h, but lost this differential expression after 5 days in culture.

1.7.5. Collagen Synthesis

Fibroblasts have also been shown to be heterogeneous with respect to the production of collagen. Mollenhauser et al. (1986) identified 3 distinct fibroblast subsets derived from rat lung and skin which displayed heterogeneity with respect to morphology, proliferative potential and collagen synthesis. Fibroblast cell types termed F1 produced little collagen and were highly proliferative, whereas F111 fibroblasts synthesized larger amounts of collagen and proliferated at a slower rate than the other two subtypes. F11 fibroblasts synthesized slightly more collagen than F1 fibroblasts. Interestingly, the relative proportions of F1, F11, and F111 cell types were found to change with the age of the animal, such that with increasing age a predominance of F111 cells was observed.

Goldring et al. (1990) observed that fibroblasts derived from neonatal skin exhibited marked heterogeneity with respect to the amount and pattern of collagen types produced. In addition, these clones of neonatal dermal fibroblasts were also observed to be heterogeneous in their response to specific hormones known to modulate collagen synthesis.

Botstein et al. (1982) observed that clones of neonatal foreskin fibroblasts exhibited at least a threefold difference in their collagen synthetic capacities. Moreover, the heterogeneity observed among the clones was conserved over multiple cell passages. Interestingly, when these clones were exposed to sera

from patients with Scleroderma, a selective outgrowth of fibroblasts which produced high levels of collagen occurred.

A number of investigators have also observed that dermal fibroblast populations derived from Scleroderma tissue differ in their collagen production. LeRoy et al. (1972) demonstrated enhanced collagen synthesis in skin fibroblasts obtained from patients with Scleroderma as compared to control subjects. Uitto et al. (1979) extended these studies and showed that skin fibroblasts derived from Scleroderma patients displayed an enhanced synthesis of both type I and III procollagens as compared to controls. Kahari et al. (1987) examined collagen synthesis in fibroblasts cultured from the skin of Scleroderma patients and observed a three fold increase in collagen gene transcription over control fibroblasts.

Studies in vivo have examined alterations in collagen synthesis by lung fibroblasts and support the idea that fibrosis is associated with an altered collagen synthesizing phenotype of tissue fibroblast. McDonald et al. (1986) examined lung biopsy specimens from patients with fibrotic disease and demonstrated by immunohistochemistry the presence of high collagen producing fibroblasts in fibrotic lung as compared to normal lung. Similarly, in a rat model of bleomycin induced lung fibrosis, Phan et al. (1985) demonstrated that fibroblasts isolated from bleomycin treated lungs synthesized elevated levels of collagen, with most of the increase owing to type I, when compared

to normal lung fibroblasts.

Finally, McCulloch et al. (1993) have examined fibroblasts from normal gingival connective tissues and from fibrotic lesions for their ability to phagocytose collagen-coated fluorescent beads. These authors demonstrated heterogeneity in the collagen phagocytic activity within the normal fibroblast population with as much as 88% of the fibroblast cell population being phagocytic. Interestingly, fibroblast cultures obtained from fibrotic lesions contained a smaller number of phagocytic cells and at no time were fibrotic fibroblast cell populations found to exhibit a high phagocytic activity.

1.8. RESEARCH PROPOSAL

The lung fibroblast represents the final pathway for the deposition of extracellular matrix elements under a variety of normal and disease processes. Although once believed to comprise a homogeneous cell population, increasing evidence has accumulated demonstrating significant differences within the phenotype and function of resident tissue fibroblasts. Fibroblasts have been found to be heterogeneous with respect to growth potentials, matrix production, responses to cellular mediators, and expression of cell surface proteins. Moreover, studies of fibroblasts derived from diseased tissue have demonstrated heterogeneity between healthy and diseased fibroblasts suggesting the involvement of particular fibroblast subsets in the pathogenesis

of diseases such as fibrosis.

It is clear that an increase in fibroblast number and an increase in connective tissue deposition are hallmarks of the fibrotic response of the lung. Moreover, it is now reasonable to assume that within normal lung subpopulations of fibroblasts coexist. Therefore, the possibility that inflammatory processes may select out or activate fibroblast subsets displaying phenotypic characteristics consistent with disease expresssion can be put forward as a mechanism for the pathogenesis of IPF and other connective tissue diseases. Once fibroblast heterogeneity has been established within the lung, a number of pathways leading to the emergence of distinct fibroblast subsets can be suggested. For example, proliferation studies have suggested the outgrowth of a "growth aggressive" fibroblast subpopulation within the fibrotic lung which could account for the increased numbers of fibroblasts and the increased ECM production observed during fibrosis. Moreover, the proliferative expansion of a particular subset of fibroblasts could occur as a result of prolonged exposure of subpopulations to mediators present during chronic inflammation resulting in the emergence of lung fibroblasts which are no longer responsive to factors which turn them off or down regulate their proliferation. In addition, clonal deletion or expansion of certain subpopulations could alter the phenotype of the fibroblast subsets present during fibrosis. For example, the emergence of fibroblasts in the lung which produce greater

amounts of PGE_2 could in effect downregulate the proliferation of surrounding fibroblast populations which are still responsive to the effects of PGE_2, thus skewing the phenotype of the fibroblast populations present during fibrosis. Fibroblast subsets could also accumulate in the lung either through direct migration, chemotaxis, or through the selective expansion of a fibroblast subpopulation expressing a specific surface receptor. Finally, due to the ongoing parenchymal injury occurring during the fibrotic response, the increased demand for lung repair could lead to the mobilization and increase of newly differentiated fibroblasts which rapidly proliferate and synthesize components of the ECM.

As stated previously, the contributions of short-lived inflammatory cells such as the neutrophil and alveolar macrophage to the fibrotic response have been well documented. However, treatment of patients with drugs known to modulate the effector functions of these cells has not appeared to significantly alter the overall course of disease expression in the majority of IPF patients. Since recent evidence has now accumulated demonstrating that fibroblasts may function as effector cells, the theme of this proposal is that long-lived structural cells such as the fibroblast may play a more direct role in the pathogenesis of inflammation and fibrosis. This proposal hypothesizes that during repeated episodes of inflammation, a subpopulation of fibroblasts emerge within the lung either by selection or differentiation, which express distinct phenotypes such

as enhanced proliferation, enhanced ECM production, and enhanced expression of inflammatory cytokines which ensure the continued presence of the chronic inflammatory response. Thus, once distinct subsets of fibroblasts have become predominant within the lung parenchyma, suppression of the inflammatory response alone may not be sufficient in itself to end the fibrotic process. The objectives of this study therefore were to further investigate the hypothesis of fibroblast heterogeneity in lung fibrosis, to characterize the phenotype of the 'fibrotic' lung fibroblast, and to identify mechanisms for the modulation of the emergence of the 'fibrotic' fibroblast phenotype. Both in vitro and in vivo approaches were utilized to address these issues.

Since previous work in our laboratory had documented that lung fibroblasts derived from areas of active fibrosis proliferated faster (similar to neonatal lung fibroblast lines) than normal adult lung fibroblast cell lines and since a series of papers from Lafyatis et al. (1989a, 1989b) indicated that fibroblast-cell lines derived from rheumatoid synovium were capable of sustaining clonal growth under anchorage-independent conditions, I hypothesized that lung fibroblast populations isolated from fibrotic lung tissue might also be capable of growth under anchorage-independent conditions, a characteristic generally associated with a "transformed" phenotype. To specifically address this issue, a soft agarose growth culture assay was established and the colony forming potential of adult fibrotic, neonatal, and

normal adult lung fibroblast cell lines was examined. The findings from this investigation are presented in the publication in Chapter two.

Having established an in vitro model which clearly depicts growth heterogeneity between fibrotic and normal lung fibroblast populations, I proceeded to investigate the modulation of this "growth aggressive" phenotype. Since neonatal lung fibroblast populations were also shown to be capable of growth under soft agarose conditions, it was suggested that the fibroblast present in fibrotic tissue might resemble a newly differentiated fibroblast. As such, experiments were performed in an attempt to modulate the differentiation of the fibrotic fibroblast to a more mature phenotype; one incapable of anchorage-independent growth. The results of this study are detailed in the publication in Chapter three.

Having demonstrated in vitro that the behaviour of anchorage-independent growth exhibited by the IPF fibroblast could be modulated following treatment with retinoids, known differentiating agents, and that retinoids have been shown capable of reducing collagen synthesis in scleroderma fibroblast cultures in vitro (Abergel et al., 1985), I examined the effect of the in vivo administration of all trans retinoic acid on the modulation of the fibrotic lung response in a rat model of bleomycin-induced fibrosis. The results of this study are detailed in the publication in Chapter four.

CHAPTER two

CHARACTERIZATION OF THE ANCHORAGE-INDEPENDENT PHENOTYPE OF LUNG FIBROBLASTS DERIVED FROM ADULT FIBROTIC LUNG TISSUE

The following article entitled "Anchorage-independent Colony Growth of Pulmonary Fibroblasts Derived from Fibrotic Human Lung Tissue" is published in The Journal of Clinical Investigation 93:1525-1532, 1994. This paper addresses the initial characterization of the anchorage-independent phenotype of the lung fibroblast derived from fibrotic lung tissue.

The work presented in this study was performed by the author of the thesis with the exception of electron microscopy (EM) studies in which help from EM technicians was employed. This research paper was written by the author of the thesis. Drs. Richards, Podor, and Gauldie provided supervision over the study resulting in multiple authorship.

Anchorage-independent Colony Growth of Pulmonary Fibroblasts Derived from Fibrotic Human Lung Tissue

Diane J. Torry, Carl D. Richards, Thomas J. Podor, and Jack Gauldie
Department of Pathology, Molecular Virology and Immunology Program, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Abstract

Fibroblast heterogeneity is known to exist in chronically inflamed tissue such as pulmonary fibrosis (IPF) and scleroderma. We have previously shown differences in proliferation rates in primary lines and cloned lines of fibroblasts derived from IPF tissue compared with normal lung. In this study, we report that cell lines derived from fibrotic tissue demonstrate anchorage-independent growth in soft agarose culture whereas normal lung fibroblast lines do not. We also show that fibroblast lines derived from neonatal lung tissue form colonies at about the same frequency as the fibrotic cells. Colonies from both fibrotic and neonatal lines were shown to be positive for vimentin, laminin, fibronectin, fibronectin receptor, β-actin, and tropomyosin by immunohistochemistry but were negative for desmin, keratin, Factor VIII, α-smooth muscle cell actin, and tenascin. Treatment with cytokines TGF-β and PDGF or with corticosteroid modified the colony-forming capacity of fibrotic and neonatal cell lines, however, none of these treatments induced normal lung cell lines to form colonies. The presence of cells in adult fibrotic tissue with growth characteristics similar to those exhibited by neonatal cells is further evidence of fibroblast heterogeneity and suggests newly differentiated fibroblasts may be prevalent in fibrotic tissue and contribute directly to the matrix disorder seen in this disease. (*J. Clin. Invest.* 1994. 93:1525–1532.) Key words: idiopathic pulmonary fibrosis • fibroblast heterogeneity • soft agarose growth • inflammation

Introduction

Pulmonary fibrosis is a chronic and devastating condition characterized by pathologic alterations of extracellular matrix elements ultimately affecting the functional integrity of the lung (1–6). Thought to be initiated by inflammatory reactions in the alveolar wall, fibrosis is most likely the end result of a dynamic repair cascade, which proceeds to chronicity by unknown mechanisms (7–9). In addition to well-recognized contributions made to the process by inflammatory cells, we and others have demonstrated that tissue structural cells, including fibroblasts, endothelial cells, and epithelial cells, are capable of releasing potent inflammatory and matrix modulating mediators (10, 11) and hence play a direct role, as effectors, in the pathogenesis of idiopathic pulmonary fibrosis (IPF).[1]

The existence of phenotypic heterogeneity within the resident fibroblast population of a given tissue has been widely described. We and others have previously reported that fibroblast lines established from histologically proven fibrotic tissue or chronically altered dermal tissue display differences in proliferative potential, matrix gene expression, cell surface marker expression, and cell-mediated responses (12–16). Moreover, we have also shown similar proliferative differences with primary lines established from neonatal lung tissue (17). However, whether these in vitro differences relate directly to the fibrotic process or contribute to the chronic inflammatory reaction is not yet clear.

Recently, a number of cytokines have been shown to have a profound impact on fibroblast characteristics, including growth factors such as PDGF and TGF-β. These factors modulate fibroblast proliferation and can mediate transformation of fibroblasts, inducing these cells to anchorage-independent growth (reviewed in references 18 and 19). Moreover, TGF-β and PDGF have been shown to be present in greatly enhanced amounts in lung biopsies from IPF patients (20–23), implying that cells found in IPF tissue may exhibit characteristics of growth consistent with chronic exposure to these cytokines.

To determine whether the various fibroblast lines we have established from adult and neonatal human lung tissue demonstrate abnormal growth characteristics, we established a soft agarose suspension culture assay and show here that primary fibroblast cell lines derived from human adult fibrotic lung tissue, but not those from normal adult lung tissue, form colonies under anchorage-independent growth conditions. Further, we show that primary fibroblast lines derived from neonatal lung tissue also exhibit the ability for colony formation under anchorage-independent conditions. These colonies demonstrate a pattern of synthesis of intra- and extracellular matrix (ECM) proteins typical of fibroblasts. Anchorage-independent growth characteristics are maintained by the fibrotic cells even after selection and clonal expansion of single colonies. The presence of these "transformed" phenotypes in fibrotic adult and neonatal cell lines suggests the process leading to fibrosis may involve de novo stromal cell differentiation, possibly under the influence of inflammatory mediators.

Methods

Reagents. Streptavidin-peroxidase immunohistological staining Kit was purchased (Histostain-SP Kit 95-6543 for use with mouse primary antibody; 95-6143 for use with rabbit primary antibody; Zymed Labs.

Address correspondence to Dr. Jack Gauldie, Department of Pathology, 2N16, McMaster University, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada.

Received for publication 9 September 1993 and in revised form 22 December 1993.

J. Clin. Invest.

0021-9738/94/04/1525/08 $2.00
Volume 93, April 1994, 1525–1532

1. *Abbreviations used in this paper.* AcA, acetic acid alcohol; DEX, dexamethasone; ECM, extracellular matrix; EM, electron micrograph; IPF, ideopathic pulmonary fibrosis; LMP, low-melting point.

Inc. San Francisco, CA). Hematoxylin solution Gill No. 2 was purchased (GHS-2-178; Sigma Chemical Co., St. Louis, MO). Agarose for use in the anchorage-independent growth assay was purchased (5510UB; Bethesda Research Laboratories, Gaithersburg, MD). All human sera used in growth assays were obtained from healthy normal volunteers using vacutainer sterile blood collection tubes with no additives (Becton Dickinson Canada Inc., Mississauga, Canada). Recombinant human PDGF was obtained (2038-01; Genzyme, Cambridge, MA), TGF-β was obtained (100-B; R & D Systems, Minneapolis, MN). Dexamethasone (DEX) was purchased (DIN 00664227; Sabex, Boucherville, Quebec, Canada).

Antibodies. Antisera to human antigens were purchased from Sigma Chemical Co.: α-actin (A-2668, polyclonal); α-tropomyosin (T-3651, poly) α-vimentin (V-6630, mAb); α-SMC actin (A-9047, mAb); α-laminin (L-6145, polyclonal); α-desmin (D-9159, mAb); α-cytokeratin (P-1548, mAb). Antisera to human antigens were purchased from Dako Corp. (Carpinteria, CA): α-tenascin (M-636, mAb); α-CD34 (M-824, mAb); α-fibronectin (A-245, polyclonal); α-Factor VIII (A-082, polyclonal); nonimmunized rabbit serum (X-902). Purified mouse myeloma IgG1 was purchased from Zymed Labs., Inc. (02-6100). Antiserum to human fibronectin receptor was purchased from Telios Pharmaceuticals, Inc. (San Diego, CA) (A-108, polyclonal).

Cell lines. We have previously established a number of human primary fibroblast lines. Fibroblast lines were established by outgrowth from explants as previously described (16, 17). Three cell lines were established from tissues taken at open lung biopsy for diagnosis of interstitial lung disease and from areas shown histologically to demonstrate features of active fibrosis. Four lines were established from histologically normal adult lung tissue removed as part of resection of tumor mass, and two lines were established from normal neonatal lung tissue removed at autopsy within 12 h of death from premature infants dying within the first 3 d of life from causes not related to pulmonary function. Cell lines were used within the first five passages only. Lung fibroblast lines were grown in 75-cm^2 tissue culture flasks (Corning Inc., Corning, NY) and maintained in RPMI supplemented with 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY) and 1% penicillin/streptomycin (Gibco Laboratories). The human fibrosarcoma cell line HT-1080 was obtained from American Type Culture Collection (Rockville, MD) (CCL 121). HT-1080 cells were maintained in MEM F-15 supplemented with 10% fetal bovine serum.

Anchorage-independent growth assay. An anchorage-independent growth assay similar to that previously described (24) was carried out in T-24 plates (Nunclon; InterMed Nunc, Roskilde, Denmark) with the following modifications. From a 2% stock agarose solution in PBS, a 0.8% agarose mixture was prepared and used to precoat 24-well plates. The agarose mixture was allowed to solidify at room temperature and the plates placed at 37°C until use. Subconfluent cultures of fibroblast cell lines (passage < 8) were washed twice in warm PBS, trypsinized, centrifuged, and resuspended to a concentration of 800,000 cells/ml. Cell viability was > 95% at the start of the assay as assessed by trypan blue exclusion. The colony-forming assay was optimized by varying percentages of normal human serum. For all experiments presented, a 0.4% agarose mixture containing 20% AB human serum was prepared, cooled to 37°C, and a fixed number of cells (1.5×10^4) added to this mixture. The agarose/cell mixture was then layered over the base layer, allowed to solidify, and 100 μl of media without FCS was added to each well in order to keep the cultures moist. The cultures were then incubated at 37°C/5% CO_2 and the wells examined daily for colony growth. We observed optimal colony growth 10–14 d after plating at which time colonies with a diameter > 45 μm were scored. We chose 45 μm to be representative of a colony, since in general, the size distribution of the colonies appearing in the agarose varied between 45 and 100 μm and only occasionally were there smaller clusters of cells within the agarose layer. A single-cell suspension was ensured (and confirmed by light microscopy) at the start of the assay by repeated passage of the cells through a 25-gauge needle. An inverted light microscope fitted with an eyepiece with a grid (Carl Zeiss, Inc., Thornwood, NY) was used to calculate the diameter of the colonies and to count total colonies in a well. Agarose blocks containing the cell colonies were harvested, fixed in acetic acid alcohol (AcA) for 24 h, paraffin embedded, and 4-μm sections cut onto APTEX slides for use in immunohistochemical studies. Two controls were included. (*a*) Normal human lung fibroblasts were grown to confluency, washed twice with warm PBS, trypsinized, centrifuged, and a cell pellet was obtained. A 2:1 ratio (vol/vol) of the agarose solution was added to the pellet and the mixture stirred to disperse the cells. The agarose/cell mixture was allowed to harden, fixed in AcA, and then processed as described above; (*b*) Human fibrosarcoma line HT-1080 was used as a positive control for colony formation. The cells were treated similar to the fibrotic lines.

Colony expansion. To pick and expand individual cell colonies, the above assay was repeated using low-melting point (LMP) agarose (5517UA; Bethesda Research Laboratories, Gaithersburg, MD) (25). Briefly, a base layer containing 1% LMP agarose was poured into each of 96 wells (Nunclon, InterMed Nunc, Roskilde, Denmark) and allowed to solidify at 4°C. A fixed number of cells (3×10^3 cells/well) was then added to the top layer (0.3% LMP agarose/20% human serum) and this mixture layered over the base layer. The top layer was allowed to solidify at room temperature and then the plates were wrapped in aluminum foil to avoid drying out and placed at 37°C/5% CO_2. In 7–10 d, those colonies with a diameter > 45 μm were removed from the agarose by use of a micropipette and inverted microscope and expanded in culture in RPMI supplemented with 10% FBS and 1% pen/strep.

Immunostaining of colonies. Immunoperoxidase staining of colonies was performed according to the manufacturer's instructions (Zymed Labs., Inc.) with minor modifications. Paraffin sections were dewaxed in xylene, passed through an ethanol series, and blocked for endogenous peroxidase activity with H_2O_2-methanol-HCl solution for 30 min at room temperature. Slides were then rinsed in 70% ethanol followed by distilled water, and then washed in Tris-buffered saline, pH 7.6 (TBS buffer). After incubation with nonimmune serum to block nonspecific binding, primary antibodies were applied to the sections and allowed to bind at room temperature for 1 h. The sections were then rinsed in TBS buffer, overlaid with a biotinylated secondary antibody, rinsed again in TBS buffer, and then incubated with a streptavidin-peroxidase conjugate. Sections were then incubated with a substrate-chromagen mixture (AEC; Zymed Labs., Inc.), counterstained with hematoxylin, and coverslipped.

Electron microscopy (EM) studies. Agarose blocks containing the fibroblast colonies were harvested between days 10 and 14, fixed overnight in 2% gluteraldehyde at 4°C, and then processed for transmission EM analysis.

Cytokine modulation of anchorage-independent growth. All soft agarose studies were performed as described above with the following modifications. Fibrotic, neonatal, and normal adult lung fibroblast lines were cultured in a 0.4% agarose top layer containing either PDGF (5 ng/ml), TGF-β (100 pg/ml), or dexamethasone (10^{-6} M) in the presence of 20% human serum. The cultures were then incubated at 37°C/5% CO_2 and colony growth (diameter, $\geq$ 45 μm) was assessed 10–14 d after plating. All experiments were performed in duplicate.

Results

Fibrotic adult and normal neonatal lung fibroblast cell lines exhibit colony formation under anchorage-independent conditions. The number of colonies appearing 10–14 d after plating is presented in Table I. Examination of three fibroblast cell lines derived from areas of fibrotic lung tissue reveals that IPF fibroblasts readily form colonies under soft agarose growth conditions. In contrast, fibroblast lines derived from normal adult lung tissue do not exhibit the capacity to grow under anchorage-independent conditions. As a group, IPF fibroblasts form colonies with a frequency of 0.54% compared with

Table I. Lung Cell Line Colonies

Tissue derivation	Cell line	Exp.	No. of colonies*	Percent CFU‡
Fibrotic	1	1	85.75±2.87	
		2	93.75±3.10	
	2	1	77.50±8.66	0.54
		2	88.00±13.95	
	3	1	89.00±5.48	
		2	79.50±11.45	
Normal	1	1	0.50±0.58	
		2	0.25±0.50	
	2	1	1.00±0.82	0.003
		2	0.25±0.50	
	3	1	0.00±0.00	
		2	0.00±0.00	
	4	1	1.25±0.96	
		2	1.00±1.41	
Neonatal	1	1	66.00±2.16	
		2	63.75±5.50	0.43
	2	1	65.25±1.89	
		2	71.00±2.31	
Tumor (HT-1080)		1	193.75±6.65	1.1
		2	157.75±20.15	

Data are number of colonies per well in semisolid agar suspension with a diameter >45 μm from 1.5×10^4 cells. * Values represent the mean±SD of the number of colonies per well in eight wells. All statistical analysis was performed with the aid of Minitab Data Analysis Software, Release 7.1® (1989; Minitab, Inc., State College, PA). ‡ Percent CFU was calculated by averaging the number of colonies obtained by each group (fibrotic, normal, etc.) and dividing by the number of input cells per well.

0.003% observed for normal adult lung fibroblasts. Our previous studies indicated that fibroblast lines derived from normal neonatal lung tissue proliferated faster than those from normal adult lung, and we examined whether these neonatal lines would also show abnormal growth in the assay. We show here that neonatal lung primary fibroblast lines exhibit colony formation under soft agarose growth conditions with a frequency of 0.43%, only slightly less frequent than the IPF lines.

For comparison with a fully transformed cell line, we also examined the pattern of anchorage-independent growth for the tumorigenic cell line HT-1080 (human fibrosarcoma cell line). HT-1080 yielded twice the number of colony-forming units (1.1% frequency) as compared with IPF lung fibroblast-derived colonies.

Morphological examination reveals a fibroblast-like nature to the colonies. Histological and EM examination of lung colonies was performed to determine morphological characteristics associated with colony growth. Over 30 colonies from each cell line were examined in detail. Fig. 1, *A* and *B*, illustrate a typical lung colony from a fibrotic cell line showing an inner multicellular centre surrounded by an extracellular granular type material, which stains intensely with hematoxylin and appears to be deposited into the agarose gel. Closer examination of each cell within the colony framework reveals the presence of large rounded nuclei, dispersed nuclear chromatin, and prominent nucleoli, all within an extensive cytoplasm.

The ultrastructural appearance of the colonies examined by EM analysis supports a fibroblast-like morphology for these cells (Fig. 2). The absence of intercellular junctions, basal lamina, or Weibel-Palade bodies all serve to exclude an epithelial or endothelial lineage to these cells (26). There does not appear to be myofibrils present, suggesting these are not myofibroblasts. Interestingly, EM analysis also demonstrates a number of ultrastructural features characteristic of proliferating fibroblasts, including the presence of rounded nuclei, prominent nucleoli, numerous cellular organelles with abundant elongated mitochondria, cytoplasmic processes, and the apparent absence of collagen fibres.

Immunohistochemical studies of fibrotic and neonatal lung colonies. Immunohistochemical studies were carried out on fixed colony sections derived from both fibrotic and neonatal lung lines in order to investigate the distribution of a variety of different cytoskeletal and ECM proteins characteristic of fibroblasts. Immunostaining of intermediate filaments and various actin isoforms reveals that both the fibrotic and neonatal lung colonies (> 30 examined) exhibit similar staining patterns for cytoskeletal and ECM proteins. Colonies stained positive for vimentin, laminin, fibronectin, and fibronectin receptor, exhibiting both cellular and extracellular localizations (Fig. 1, *C–F*). The colonies also demonstrated intense cytoplasmic and extracellular staining for β-actin and tropomyosin, indicating the structural organization of these proteins into stress fibers (27). Colonies stained negative for α smooth muscle cell actin, desmin, keratin, Factor VIII, and tenascin.

Control sections of adult human normal lung fibroblasts suspended, but not cultured, in agarose were similarly investigated by immunoperoxidase staining for expression of intermediate filaments and ECM organization. Normal lung fibroblasts stained positive for vimentin, laminin, β-actin, fibronectin, and fibronectin receptor, and stained negative for desmin, keratin, tenascin, and α smooth muscle cell actin.

Colony expansion and replating. To assess whether the capacity to form colonies develops as a result of the assay conditions or is an intrinsic property of a cell population, single colonies with a diameter > 45 μm were picked from low melt agarose. The cells were expanded to 5×10^6 (representing a further 22 population doublings from a single clone) and either reintroduced into the soft agarose culture system or carried for a further 2 passages and then reexamined in the colony assay. The ability of individual expanded clones to again form colonies was demonstrated, however, in general, the percentage of colony-forming units was observed to decrease with successive passage, from 0.38% with the parent line to 0.08% at a further two passages of the expanded colony (Table II).

Effect of PDGF, TGF-β, and corticosteroid on growth in soft agarose. To examine possible mediators of colony formation in the soft agarose conditions, PDGF, TGF-β, and DEX were tested for their ability to modulate anchorage-independent growth. Fig. 3 illustrates that the addition of PDGF or DEX greatly enhances soft agarose colony growth of fibroblasts derived from fibrotic lung tissue, but has little effect on neonatal fibroblast colony growth. TGF-β, in contrast, acts by partially inhibiting the ability of both fibrotic and neonatal lung fibroblasts to form colonies. On the contrary, the addition of PDGF, TGF-β, or corticosteroid did not induce any colony growth by normal adult lung fibroblasts.

Discussion

We have previously shown that cell lines isolated from fibrotic adult lung tissues compared with normal lung lines exhibit

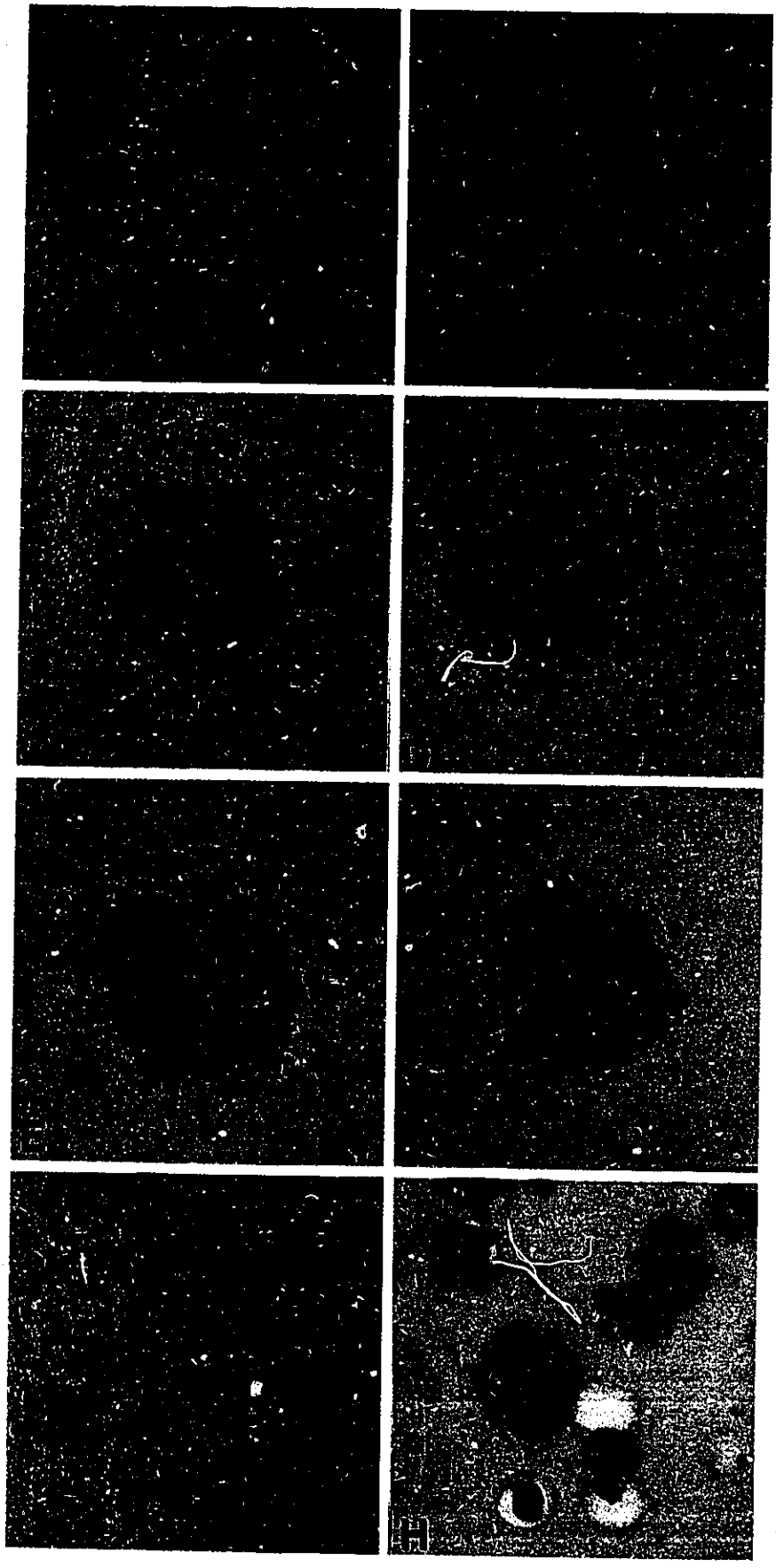


Figure 1. Morphology of anchorage-independent colony growth. (*A*) Appearance of fibrotic lung fibroblast-derived cell line cultured in soft agarose assay (day 14) as visualized under an inverted light microscope (original, ×63). (*B*) Representative hematoxylin and eosin–stained section of an adult fibrotic lung-derived colony (original, ×63). (*C–H*) Immunoperoxidase staining of AcA-fixed agarose sections containing lung colonies. Staining of adult fibrotic lung colonies for vimentin showing both an intracellular and extracellular deposition and the normal mouse serum control (*C–D*). Staining of adult fibrotic lung colony for the fibronectin (*F*) and its receptor (*E*). Immunoperoxidase staining of AcA-fixed normal adult lung fibroblasts in agarose stained for vimentin and the fibronectin receptor (*G–H*) (original, ×64).

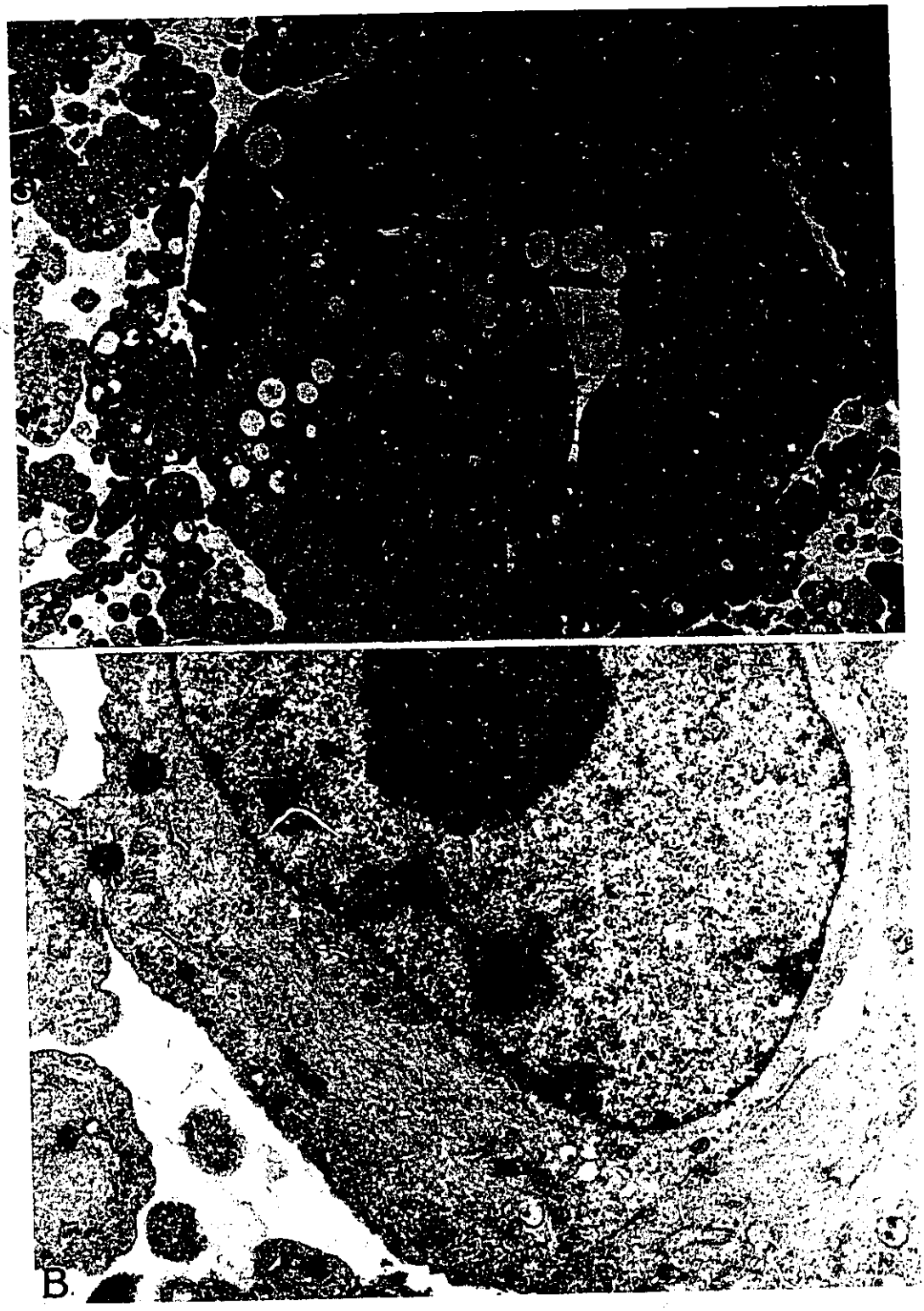


Figure 2. EM analysis. EM examination of a fibrotic lung-derived colony. (*A*) A micrograph of one fibrotic lung-derived colony showing the multicellular nature of the colony (original, ×1,900). (*B*) EM of a single cell within the colony. Note the large round nucleus, prominent nucleoli, and dispersed heterochromatin (original, ×9,100).

faster proliferative characteristics (16). In this study, using a soft agarose culture system, we have shown that fibroblasts derived from fibrotic lung tissue (IPF) exhibit colony growth under anchorage-independent conditions whereas fibroblasts derived from normal adult lung tissue do not. This is further evidence of heterogeneity within lung fibroblast populations and between healthy and diseased (IPF) fibroblasts. We have also previously shown that primary lines of neonatal lung fibroblasts proliferate faster than control fibroblast lines (17), and demonstrate here that neonatal lung cell lines also form colonies under anchorage-independent growth conditions (Table I). These results suggest that cells exhibiting the ability to grow under anchorage-independent conditions in fibrotic lung tissue may bear some resemblance to the differentiating embryonic

Table II. Results of Replating Experiment

Cell line	Exp.	No. of colonies*	Percent CFU‡
Fibrotic Cell line no. 1	1	85.75±2.87	0.58
	2	93.75±3.10	
Fibrotic Passage 4	1	55.50±8.43	0.38
	2	62.25±16.11	
Fibrotic Passage 6	1	12.75±8.42	0.08
	2	10.50±5.57	

Single colonies from a fibrotic cell line were picked from low-melt agarose, expanded in culture, and reintroduced into the soft agarose culture system. Colonies with a diameter >45 μm were counted and the results for one clone (no. 6) and its parent cell line depicted.
* Values represent the mean±SD of the number of colonies per well in eight wells. ‡ Percent CFU was calculated as per Table I.

fibroblast, a finding not at all surprising when one considers the sudden recruitment of actively dividing fibroblasts during wound healing and repair. Moreover, the ability of these cells to grow under anchorage-independent conditions, a characteristic generally associated with a transformed phenotype, suggests an aggressive nature to these cells consistent with the fibroproliferative nature of fibrosing disease. We cannot, however, relate proliferative capacity with anchorage-independent growth as we have no evidence directly linking the two characteristics.

The ability to form colonies under soft agarose growth assay conditions has also been documented for synoviocytes isolated from knee joints of patients with rheumatoid arthritis and from experimental animal models of rheumatoid arthritis, another chronic inflammatory connective tissue disease (24). It was proposed by these authors that these synoviocytes exhibit a "transformed-like" phenotype, although no evidence for transformation was demonstrated. Thus, long-lived structural cells may play a critical role in the pathogenesis of fibrosis, after repeated episodes of inflammation and repair, by means of the emergence of a group of phenotypically altered cells that possess characteristics pivotal to the continued presence of inflammation.

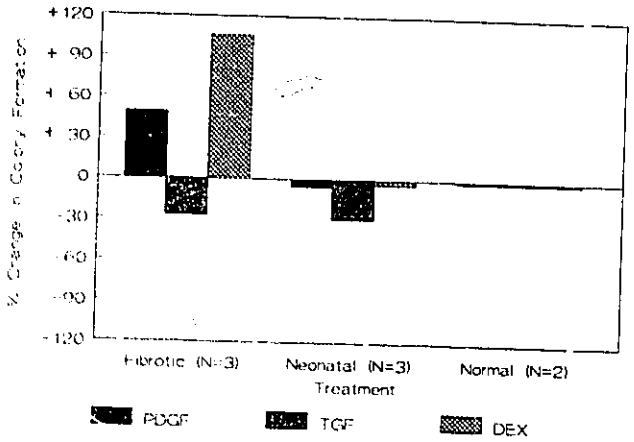


Figure 3. Modulation of growth in soft agarose by PDGF, TGF-β, and corticosteroid. Human fibroblast cell lines derived from fibrotic, neonatal, and normal adult lung tissue were cultured under soft agarose growth conditions in the presence of PDGF (5 ng/ml), TGF-β (100 pg/ml), of DEX (10^{-6} M) as described in Methods.

HT-1080 fibrosarcoma cells are transformed tumor cells presumably having clonal identity. Thus, all cells should be capable of growing as a colony in soft agarose. However, in the assay as performed, for HT-1080 cells, the colony-forming unit frequency is only 1.1% of plated cells, suggesting the assay may underestimate the potential for colony formation. The estimated frequency of colony-forming units for IPF lines (0.54%) may also underestimate this capacity.

The ability to exhibit anchorage-independent growth appears to be intrinsic to cells in the original population since colonies that are picked and expanded can again form colonies, although at a reduced rate when compared with the parent line (Table II). The fact that we observe a reduction after passage in culture of the colonies is consistent with previous reports (28, 29) demonstrating a similar decrease in proliferative capacity as clones of normal dermal fibroblasts are passaged. Moreover, a decrease in the colony-forming capacity of late passage synoviocytes as compared with early passage synoviocytes has also been documented (24). These data suggest a progressive shift in the proportion of immature fibroblasts, possibly those that proliferate faster, within the population capable of forming colonies to a more differentiated fibroblast that loses its ability to form colonies.

To better characterize the nature of the fibrotic fibroblast phenotype, we examined both fibrotic and neonatal lung colonies using conventional histological, immunohistological, and ultrastructural analysis. Upon histological examination, a dense granular-type material was seen to be deposited within the agarose directly surrounding the colony (Fig. 1 *B*). It is possible that this extracellular material may function, at least in part, to provide a source of stability and support to the growing colony. Both fibrotic and neonatal lung-derived colonies retained intermediate filament phenotypes consistent with fibroblast morphology as described by others (the presence of vimentin, and the absence of keratin and desmin) (30, 31). The immunolocalization of vimentin was found to be both intracellular and extracellular, indicating that this protein is being released into the matrix surrounding the colony, as a result of cell lysis (32).

In considering the source and nature of the cell that exhibits colony-forming activity in the primary line, two possible candidate cells in pulmonary tissue should be considered. The presence of a fibroblast-like cell in granulation tissue during healing has been described that shares structural and functional features of both smooth muscle cells and fibroblasts and that exhibits immunostaining to α-SMC actin (33, 34). Since myofibroblasts share immunohistochemical features with those of fibroblasts, that is, vimentin positive and desmin negative, and since myofibroblasts have been described and are implicated in the pathogenesis of IPF (35) and bronchial asthma (36), we examined our lung colonies for α-SMC actin expression. Neither the fibrotic-derived lung fibroblast colonies nor the neonatal-derived lung fibroblast colonies stained positive for α-SMC actin, suggesting the myofibroblast is not a component of the colony-forming unit.

A second cell that has possible relevance is the pericyte, which has morphology similar to the fibroblast and may provide a precursor population for newly differentiated fibroblasts and/or smooth muscle cells during wound repair (37, 38). Pericytes express an important matrix protein, tenascin, that is

known to be expressed during embryonic development, during tissue growth and reorganization, and in the stroma of some malignant tumors occurring in adulthood (39, 40). No tenascin-positive staining was observed in either the fibrotic or neonatal lung colonies.

The finding that colonies are negative for both α-SMC actin and tenascin would seem to rule out both the pericyte or myofibroblast as being present in the colony (39, 41), but does not necessarily rule out their role as precursors to the colony-forming cell. These aspects are currently under investigation in our laboratory.

Examination of lung colonies for the expression of other extracellular matrix proteins demonstrates that both fibrotic and neonatal lung colonies were positive for laminin expression (with most of the antibody localized in the cytoplasm) and that both types of colonies appear to synthesize and secrete large quantities of fibronectin (Fig. 1 *F*). Moreover, intense extracellular immunostaining was also demonstrated for the fibronectin receptor, suggesting the receptor is continually being expressed and removed from the cell surface, however, in the case of the colonies, this is held in the immediate area by the agarose matrix (Fig. 1 *E*). Normal lung fibroblasts also exhibited immunostaining to laminin, fibronectin, and the fibronectin receptor. In the case of the fibronectin receptor, localization was specific to the cytoplasm with no evidence of extracellular deposition into the surrounding agarose, however, these cells were not cultured in the agarose for any time. In the context of wound healing and repair, an increase in expression of the fibronectin receptor could promote the migration of selected clones of fibroblasts into areas of active injury, where under the appropriate signals they may continue to proliferate and lay down fibrous material (42, 43). Fibronectin receptor expression may also function to allow the growing colony to remain anchored to the extracellular fibronectin it has deposited.

To better understand the mechanisms surrounding the soft agarose growth response of the various lung fibroblast types (i.e., fibrotic, etc.), and determine susceptibility to cytokine exposure, PDGF, TGF-β, and DEX were tested for their ability to modulate colony formation. The addition of PDGF, which is a known stimulator of fibroblast proliferation, greatly enhanced (~ 50%) the number of colonies formed by fibrotic lung fibroblasts, but showed little effect on neonatal colony formation. TGF-β, which is known to mediate the transformation of fibroblasts, was found to partially inhibit the colony-forming abilities of both fibrotic and neonatal lung fibroblast cell lines. Similar findings have also been documented for rheumatoid arthritis synoviocytes, with PDGF simulating and TGF-β inhibiting colony formation (24, 44). The finding that PDGF and TGF-β are not able to induce colony formation in any of the normal adult lung fibroblast cell lines implies that the mechanisms surrounding colony formation in soft agarose are not simply due to susceptibility to growth factors present in the culture media, but likely the result of phenotypic differences inherent to the initial fibroblast subpopulation itself, as PDGF induces fibroblast proliferation under normal culture conditions (adherence) in these same cells (data not shown).

This study also demonstrates that glucocorticoid has a marked effect on fibrotic lung fibroblast colony formation, with DEX enhancing colony formation by nearly 100%. In contrast and similar to response to PDGF, DEX was shown to have little effect on neonatal lung fibroblast colonies, and does not induce colony formation by normal adult lung fibroblasts. The observation that treatment with steroid actually enhances the numbers of colony-forming cells in the fibrotic lung fibroblast population raises questions as to the exact role antiinflammatory drugs play in the treatment of fibrosing disease.

Our data demonstrate that fibroblasts obtained from chronically inflamed fibrotic lung tissue behave differently under anchorage-independent growth conditions than fibroblasts obtained from normal lung tissue. Further, this behavior is present in fibroblasts obtained from neonatal lung tissue. The similar behavior seen in fibrotic and neonatal cell lines suggest newly differentiated cells with aggressive growth characteristics populate fibrotic tissue contributing to the altered growth and matrix deposition. Whether these colony-forming cells are direct contributors to the process is as yet unclear, but clearly shows a phenotypic difference between normal and fibrotic lines in growth and cytokine response characteristics.

Lafyatis et al. (24) have previously shown that human synovial fibroblasts maintain colony growth in soft agarose. Synovial cells have been shown to possess a variety of markers indicative of an "aggressive phenotype," including PDGF, FGF, and TGF-β expression (45, 46). Moreover, the cytoskeletal and ECM components that we have observed in the lung colonies are also found in normal lung fibroblast cell lines. This suggests that the ability to grow in soft agarose may be due to a secreted product(s) or other membrane-bound protein important in growth and differentiation, and not expressed by quiescent normal lung fibroblasts. The future direction in characterizing the phenotype of the soft agarose-growing lung fibroblast may lie in the analysis of steady state levels of growth factors, cytokines, and their receptors known to be important in cell growth and differentiation. Moreover, further phenotyping of these lung colonies may help to address the role of subpopulations of fibroblasts in the propagation of chronic tissue inflammation.

Acknowledgments

This work was supported by Heart and Stroke Foundation of Ontario (HSFO) and the Medical Research Council of Canada and by Astra Pharma Canada. Dr. C. D. Richards is an Ontario Ministry of Health Scholar. Dr. T. Podor is an HSFO Scholar.

References

1. Basset, F., V. J. Ferrans, P. Soler, T. Takemura, Y. Fukuda, and R. G. Crystal. 1986. Intraluminal fibrosis in interstitial lung disorders. *Am. J. Pathol.* 122:443–461.

2. Katzenstein, A. L. 1985. Pathogenesis of "fibrosis" in interstitial pneumonia: an electron microscopy study. *Hum. Pathol.* 16:1015–1024.

3. Madri, J. A., and H. Furthmayr. 1980. Collagen polymorphism in the lung: an immunohistochemical study of pulmonary fibrosis. *Hum. Pathol.* 11:353–366.

4. Kavlinsky, J. B. 1982. Glycosaminoglycans in emphysematous and fibrotic hamster lungs. *Am. Rev. Respir. Dis.* 125:85–86.

5. Starcher, B. C., C. Kuhn, and J. E. Overton. 1978. Increased elastin and collagen content of the lungs of hamster receiving an intratracheal injection of bleomycin. *Am. Rev. Respir. Dis.* 117:299–305.

6. Bateman, E. D., M. Turner-Warwick, and B. C. Adelmann-Grill. 1981. Immunohistochemical study of collagen types in human foetal lung and fibrotic lung disease. *Thorax.* 36:645–653.

7. Dunnill, M. S. 1990. Pulmonary Fibrosis. *Histopathology (Oxf.)* 16:321–329.

8. Kuhn, C., J. Boldt, T. E. King, E. Crouch, T. Vartio, and J. A. McDonald. 1989. An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. *Am. Rev. Respir. Dis.* 140:1693–1703.

9. Crystal, R. G., P. B. Bitterman, S. I. Rennard, A. J. Hance, and B. A. Keogh. 1984. Interstitial lung diseases of unknown cause: disorders characterized by chronic inflammation of the lower respiratory tract. *N. Engl. J. Med.* 310:154–166.

10. Jordana, M., G. Cox, T. Ohtoshi, X. Zhou, J. Dolovich, J. Denburg, and J. Gauldie. 1991. The "TDR" concept in chronic airways inflammation: tissue driven response. *In* Clinical Impact of the Monitoring of Allergic Inflammation. P. Matson, S. Ahlstedt, P. Venge, and J. Thorell, editors. Academic Press Limited, London. 33–46.

11. Gauldie, J., M. Jordana, G. Cox, T. Ohtoshi, J. Dolovich, and J. Denburg. 1992. Fibroblasts and other structural cells in airway inflammation. *Am. Rev. Respir. Dis.* 145:S14–S17.

12. Uitto, J., E. A. Bauer, and A. Z. Eisen. 1979. Increased biosynthesis of triple-helical type I and type III procollagens associated with unaltered expression of collagenase by skin fibroblasts in culture. *J. Clin. Invest.* 64:921–930.

13. Raghu, G., Y. Chen, V. Rusch, and P. S. Rabinovitch. 1988. Differential proliferation of fibroblasts cultured from normal and fibrotic human lungs. *Am. Rev. Respir. Dis.* 138:703–708.

14. Bordin, S., R. C. Page, and A. S. Narayanan. 1984. Heterogeneity of normal human diploid fibroblasts: isolation and characterization of one phenotype. *Science (Wash. DC).* 223:171–173.

15. Derdak, S., D. P. Penney, P. Keng, M. E. Felch, D. Brown, and R. P. Phipps. 1992. Differential collagen and fibronectin production by Thy 1$^+$ and Thy 1$^-$ lung fibroblast subpopulations. *Am. J. Physiol.* 263:L283–L290.

16. Jordana, M., J. Schulman, C. McSharry, L. B. Irving, M. T. Newhouse, G. Jordana, and J. Gauldie. 1988. Heterogeneous proliferative characteristics of human adult lung fibroblast lines and clonally derived fibroblasts from control and fibrotic tissue. *Am. Rev. Respir. Dis.* 137:579–584.

17. Jordana, M., H. Kirpalani, and J. Gauldie. 1992. Heterogeneity of human lung fibroblast proliferation in relation to disease expression. *In* Pulmonary Fibroblast Heterogeneity. R. P. Phipps, editor. CRC Press, Boca Raton, Inc., FL. 229–249.

18. Kelley J. 1992. Transforming growth factor-β. *In* Cytokines in the Lung. J. Kelley, editor. Marcel Dekker, Inc., New York. 101–138.

19. Fabisiak, J. P., and J. Kelley. 1992. Platelet-derived growth factor. *In* Cytokines in the Lung. J. Kelley, editor. Marcel Dekker, Inc., New York. 3–37.

20. Antoniades, H. N., M. A. Bravo, R. E. Avila, T. Galanopoulos, J. Neville-Golden, M. Maxwell, and M. Selman. 1990. Platelet-derived growth factor in idiopathic pulmonary fibrosis. *J. Clin. Invest.* 80:1055–1064.

21. Martinet, Y., W. N. Rom, G. R. Grotendorst, G. R. Martin, and R. G. Crystal. 1987. Exaggerated spontaneous release of platelet-derived growth factor by alveolar macrophages from patients with idiopathic pulmonary fibrosis. *N. Engl. J. Med.* 317:202–209.

22. Khalil, N., R. N. O'Connor, H. W. Unruh, P. W. Warren, K. C. Flanders, A. Kemp, O. H. Bereznay, and A. H. Greenberg. 1991. Increased production and immunohistochemical localization of transforming growth factor in idiopathic pulmonary fibrosis. *Am. J. Respir. Cell Mol. Biol.* 5:155–162.

23. Broekelmann, T. J., A. H. Limper, T. V. Colby, and J. A. McDonald. 1991. Transforming growth factor β1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proc. Natl. Acad. Sci. USA.* 88:6642–6646.

24. Lafyatis, R., E. F. Remmers, A. B. Roberts, D. E. Yocum, M. B. Sporn, and R. L. Wilder. 1989. Anchorage-independent growth of synoviocytes from arthritic and normal joints: stimulation by Exogenous platelet-derived growth factor and inhibition by transforming growth factor-beta and retinoids. *J. Clin. Invest.* 83:1267–1276.

25. Hirte, H. W. 1993. A quantitative colony-forming bioassay for transforming growth factor-β using a colorimetric endpoint. *Proc. Am. Assoc. Cancer Res.* 34:1429 (Abstr.).

26. Rhodin, Johannes A. G. 1974. Connective Tissue. *In* Histology, a Text and Atlas. Oxford University Press, London. 140–171, 348.

27. Goldman, R. D., E. Lazarides, R. Pollack, and K. Weber. 1975. The distribution of actin in non-muscle cells: the use of actin antibody in the localization of actin within the microfilament bundles of mouse 3T3 cells. *Exp. Cell Res.* 90:333–344.

28. Kirkwood, T. B. L., and R. Holliday. 1975. Commitment to Senescence: a Model for the finite and infinite growth of diploid and transformed human fibroblasts in culture. *J. Theor. Biol.* 53:481–496.

29. Jones, R. B., and J. R. Smith. 1982. A stochastic model of cellular senescence. II. Concordance with experimental data. *J. Theor. Biol.* 96:443–460.

30. Franke, W. W., B. Appelhans, E. Schmid, C. Freudenstein, M. Osborn, and K. Weber. 1979. Identification and characterization of epithelial cells in mammalian tissues by immunofluorescence microscopy using antibodies to prekeratin. *Differentiation.* 15:7–25.

31. Franke, W. W., C. Grund, C. Kuhn, B. W. Jackson, and K. Illmensee. 1982. Formation of cytoskeletal elements during mouse embryogenesis III. Primary mesenchymal cells and the first appearance of vimentin filaments. *Differentiation.* 23:43–59.

32. Podor, T. J., J. I. Weitz, J. Hirsh, D. Seiffert, and D. J. Luskutoff. 1994. Vitronectin-mediated binding of type I plasminogen activator inhibitor to vimentin intermediate filaments in damaged endothelial cells: potential mechanism for regulating plasmin generation during the inflammatory process. *J. Clin. Invest.* In press.

33. Darby, I., O. Skalli, and G. Gabbiani. 1990. α-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab. Invest.* 63:21–29.

34. Gabbiani, G., G. B. Ryan, and G. Majno. 1971. Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia (Basel).* 27:549–550.

35. Kuhn, C., and J. A. McDonald. 1991. The roles of the myofibroblast in idiopathic pulmonary fibrosis: ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis. *Am. J. Pathol.* 138:1257–1265.

36. Roche, W. R., R. Beasley, J. H. Williams, and S. T. Holgate. 1989. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet.* ii:520–524.

37. Sims, D. E. 1991. Recent advances in pericyte biology: implications for health and disease. *Can. J. Cardiol.* 7:431–443.

38. Shepro, D., and N. M. L. Morel. 1993. Pericyte physiology. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 7:1031–1038.

39. Mackie, E. J., W. Halfter, and D. Liverani. 1988. Induction of tenascin in healing wounds. *J. Cell Biol.* 107:2757–2767.

40. Chiquet-Ehrismann, R., E. J. Mackie, C. A. Pearson, and T. Sakakura. 1986. Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell.* 47:131–139.

41. Skalli, O., M. F. Pelte, M. C. Peclet, G. Gabbiani, P. Gugliotta, G. Bussolati, M. Ravazzola, and L. Orci. 1989. α-smooth muscle actin, a differentiation marker of smooth muscle cells, is present in microfilamentous bundles of pericytes. *J. Histochem. Cytochem.* 37:315–321.

42. Clark, A. F. 1990. Fibronectin matrix deposition and fibronectin receptor expression in healing and normal skin. *J. Invest. Dermatol.* 94:128S–134S.

43. Grinnell, F., K. Toda, and A. Takashima. 1987. Activation of keratinocyte fibronectin receptor function during cutaneous wound healing. *J. Cell Sci. Suppl.* 8:199–209.

44. Wilder, R. L., R. Lafyatis, A. B. Roberts, J. P. Case, G. K. Kumkumian, H. Sano, M. B. Sporn, and E. F. Remmers. 1990. Transforming growth factor-β in Rheumatoid Arthritis. *Ann. NY Acad. Sci.* 593:197–207.

45. Goddard, D. H. 1992. Regulation of synovial cell growth by polypeptide growth factors. *DNA Cell Biol.* 11:259–263.

46. Remmers, E. F., J. Sano, R. Lafyatis, J. P. Case, G. K. Kumkumian, T. Hla, T. Maciag, and R. L. Wilder. 1991. Production of platelet derived growth factor β chain (PDGF-B/c-sis) mRNA and immunoreactive PDFG B-like polypeptide by rheumatoid synovium: coexpression with heparin binding acidic fibroblast growth factor-1. *J. Rheumatol.* 18:7–13.

CHAPTER three

MODULATION OF THE ANCHORAGE-INDEPENDENT PHENOTYPE BY RETINOID AND CORTICOSTEROID

The following article entitled "Modulation of the anchorage-independent phenotype of human lung fibroblasts obtained from fibrotic tissue following culture with retinoid and corticosteroid" has been accepted for publication in Experimental Lung Research, 1995. This research article addresses the role of growth and differentiating factors in the modulation of the anchorage-independent phenotype.

The work presented in this paper was performed by the author of the thesis with the help from lab technicians with proliferation assays. This paper was written by the author of the thesis, with the supervision from Drs. Richards, Podor, and Gauldie.

Modulation of the anchorage-independent phenotype of human lung fibroblasts obtained from fibrotic tissue following culture with retinoid and corticosteroid.

Diane J. Torry, Carl D. Richards, Thomas J. Podor and Jack Gauldie.

Department of Pathology, Molecular Virology and Immunology Program, Chedoke-McMaster Medical Centre, McMaster University, Hamilton, Ontario L8S 3Z5 Canada.

Running title: Retinoids modulate anchorage-independent growth of fibrotic lung fibroblasts

Correspondence: Jack Gauldie, Ph.D.
Department of Pathology
McMaster University
1200 Main Street West
Hamilton, Ontario, Canada L8N 3Z5
Tel: (905) 521-2100 Ext. 6332
Fax: (905) 577-0198

ABSTRACT

Fibroblast heterogeneity has been documented in fibrotic tissue from lung and skin. We have previously demonstrated differences in proliferative rates in fibroblasts derived from fibrotic lung tissue as compared to normal and that fibroblast lines derived from adult fibrotic lung tissue and neonatal normal lung tissue exhibit colony growth in soft agarose culture whereas fibroblast cell lines from normal adult lung tissue do not. The characteristic of anchorage-independent growth is consistent with the aggressive nature of the disease and with developmental lung growth. In this study, we have exposed fibrotic lung fibroblasts to growth and differentiating factors to determine whether the anchorage-independent phenotype can be modulated. Here we report that treatment of fibrotic lung fibroblasts with retinoic acid, known to modify matrix gene expression and induce differentiation, inhibits the cells ability to form colonies under soft agarose growth. Treatment with all-trans retinoic acid yielded the greatest effect inhibiting both IPF and neonatal lung fibroblast anchorage-independent growth approximately 90% at 10^{-6}M. Treatment of IPF fibroblasts with all-trans retinoic acid also inhibited corticosteroid induced colony growth. Modulation of the 'fibrotic' fibroblast phenotype through retinoid therapy may prove beneficial as a potential therapeutic strategy.

INTRODUCTION

The notion that fibroblasts within a single tissue do not comprise a homogenous cell population has received much attention of late. Several lines of evidence from a variety of diseases characterized by chronic inflammation and fibrosis support both morphological and functional heterogeneity within fibroblast populations. There are functional differences in the amounts and proportions of extracellular matrix proteins produced, responses to cellular mediators, proliferative potential and mediator release (1-8). Moreover, fibroblast subpopulations have also been described based on cell surface marker expression (9,10). We have previously shown that primary lung fibroblast cell lines derived from areas of fibrotic tissue proliferate faster than do control fibroblasts and that fibroblast clones derived from these primary lines exhibit similar enhanced rates of proliferation (5). We have extended these studies and have recently shown that IPF fibroblasts are capable of growing under anchorage-independent growth conditions, while fibroblast cell lines derived from normal adult lung tissue do not exhibit this ability (11). The presence of such cells which express aberrant growth characteristics in lines derived from fibrotic lung tissue again supports the notion of heterogeneity among fibroblast populations and between healthy versus diseased tissue. We also demonstrated that fibroblasts derived from neonatal lung tissue exhibit the

ability to grow under conditions of soft agarose growth (11), suggesting that the fibroblast present in IPF tissue may in fact resemble a newly differentiated fibroblast. Therefore, we have suggested that disease expression may result from the selection or prevalence of these growth aggressive clones which then synthesize and lay-down the structural components of fibrous tissue.

In an attempt to modulate the differentiation of the fibrotic fibroblast to what we believe is a more mature phenotype and hence one incapable of anchorage-independent growth, we examined the effect of various growth and differentiating factors as well as corticosteroid and various retinoids on fibrotic lung fibroblast soft agarose growth. Retinoids are a class of compounds important in the normal regulation of tissue development and differentiation and in vitro have been shown capable of regulating extracellular matrix production and inducing the differentiation of tumour cell lines (12-18).

We show here that the ability of IPF fibroblasts to grow under conditions of soft agarose is inhibited by treatment with various retinoids: retinol, retinal, all trans retinoic acid (AtRA), and 13-Cis retinoic acid (13-cis RA). AtRA demonstrated the greatest effect suppressing colony growth approximately 90% at 10^{-6}M. We also show that while dexamethasone (DEX)(10^{-6}M) essentially doubles the colony forming capabilities of fibrotic lung fibroblasts, this induction is totally inhibited by co-incident treatment with either AtRA or 13-cis RA.

MATERIALS AND METHODS

1. Reagents and Test Compounds. Dexamethasone was purchased from Sabex, Boucherville, Quebec, Canada (DIN 00664227). All trans retinoic acid (Cat No. R2625), 13-cis-retinoic acid (Cat No. R3255), retinol (Cat No. R7632) and retinal (Cat No. R2500) were purchased from Sigma Chemical Co., St. Louis, MO. Recombinant human platelet-derived growth factor $\beta\beta$ (PDGF) was obtained from R & D Systems, Minneapolis, MN (Cat. No. 100-B). Recombinant human fibroblast growth factor (FGF), basic (Cat. No. 341618) and transforming growth factor β_1 (TGFβ)(Cat. No. 619350) were purchased from Calbiochem Corp., La Jolla, CA. Recombinant human epidermal growth factor (EGF) was obtained from Genzyme, Cambridge, MA (Cat. No. 1213-00). Agarose for use in the anchorage-independent growth assay was purchased from Bethesda Research Laboratories, Gaithersburg, MD. Human sera used in AIG assay was collected from healthy volunteers using vacutainer sterile blood collection tubes with no additives (Becton Dickinson Canada Inc., Mississauga, Ontario, Canada).

2. Cell Lines. All human fibroblast cell lines for use in anchorage-independent growth assay were established by outgrowth from explants as previously described (4,5). All cell lines were used at passage < 10. Cell lines were grown in 75-cm^2 culture flasks (Corning Inc., Corning, NY) and maintained in

RPMI supplemented with 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY) and 1% penicillin / streptomycin (Gibco).

3. Anchorage-Independent Growth Assay (AIG). AIG assay was carried out as previously described with minor modifications (35). Briefly, a 0.8 % agarose mixture containing 10% fetal bovine serum was used to precoat 96 well plates (Nunclon, InterMed Nunc, Roskilde, Denmark). After allowing the bottom layer to solidify, a 0.4% agarose top layer containing 20% human serum (HS), 2 x 10^3 cells, various growth factor(s), dexamethasone or retinoid(s) was layered over the base layer. Following solidification of the top layer, 100 ul of media alone or media plus the various retinoid was added to each well. The plates were then wrapped in aluminum foil and placed at 37°C / 5% CO_2 for 10-14 days. Colonies with a diameter $\geq$ 45 μm were counted using an inverted light microscope fitted with a grided eyepiece (Carl Zeiss Inc., Thornwood, NY).

4. ^{3}H Thymidine (^{3}H-TdR) Incorporation. Human adult fibrotic lung fibroblasts were grown to confluency, trypsinized and resuspended in RPMI supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Two thousand cells were plated into the bottom of a 96 well plate and allowed to attach overnight. The following day, the cells were washed twice with warm PBS and the media changed to experimental conditions of varying concentrations (10^{-5}M

- 10^{-9}M) of retinol, retinal, AtRA, or 13-cis RA in 20% human serum $\pm$ dexamethasone. The cells were allowed to incubate for a further 72 hours at 37 C, 5% CO_2 and .5 uCi/well of ^{3}H-thymidine (20 Ci/mmol, NEN Corp., Boston, MA) added during the last 24 hours of incubation. The cells were then washed with PBS, lysed and cellular contents collected on a cell harvester.

5. Statistical Analysis. All statistical analyses were performed utilizing the Minitab Data Analysis software, Release 7.1 (1989, Minitab, Inc., State College, PA).

RESULTS

1. Effect of Retinoids on Cell Viability. The effect of retinoid (10^{-5}M to 10^{-9}M) and steroid (10^{-6}M) on cell viability was examined. Viability of lung fibroblast lines was assessed by trypan-blue exclusion and a concentration of 10^{-6}M chosen for subsequent studies since at this concentration, cell viability was $\geq$ 97% for all retinoids tested (data not shown).

2. The Effect of Retinoids on the Colony Forming Activity of Fibrotic Lung Fibroblast Cell Lines. The ability of fibroblast cell lines derived from adult fibrotic lung tissue to form colonies and the modulation of this growth by 4 natural retinoids (retinal, retinol, All-trans retinoic acid, and 13-cis retinoic acid) is presented in Figure 1. In the presence of 20% human serum, fibrotic fibroblast cell lines formed colonies at the predicted frequency (0.58 - 1.05 % (35)). However, in the presence of various retinoids, the frequency of this growth was significantly decreased (Cell line #1 ANOVA $F=55.81$, $p < 0.001$, Cell line #2 $F=53.08$, $p < 0.001$ and Cell line #3 $F=7.88$, $p=0.001$). All-trans retinoic acid (AtRA) had the greatest effect on colony formation, being much more active than retinol (vitamin A) and retinal at the same concentration, suppressing colony growth approximately 90% of control (Cell lines #1,2,3; $p < 0.0001$, $p=0.0007$, $p=0.0081$ respectively). The effect of retinoids on the colony forming ability of 3 neonatal lung derived fibroblast cell

lines was also examined. Again, all retinoids tested decreased the frequency of colony growth with all-trans retinoic acid being the most active ($p<0.001$, Data not shown).

3. The Effect of Retinoids on Corticosteroid Induced Colony Formation. We have shown previously that dexamethasone (DEX) (10^{-6}M) enhances the soft agarose colony growth of fibrotic lung fibroblast lines and we examined the effect of retinoids on this activity. In the presence of DEX (Figure 2), a significant increase in the the mean colony number was observed for all fibrotic lung fibroblast lines tested (Cell Line #1 $p=0.0014$ and cell lines #2 and 3 $p=0.0072$ and 0.0094 respectively). The co-incident addition of either retinol or retinal did not significantly alter these effects. However, the addition of AtRA was able to override the colony promoting activity of DEX, resulting in a marked decrease in the colony forming ability of fibrotic fibroblast cell lines (Cell line #1, 2 and 3; $p=0.0001$, $p=0.0023$, $p=0.0003$). 13-cis retinoic acid (13-cis RA) was also able to suppress DEX induced colony growth, but to a lesser extent than AtRA in 2 of 3 cell lines tested (Cell line #2 $p=0.0009$ and #3 $p=0.013$).

4. Modulation of colony growth by specific growth factors. In an attempt to identify specific factors potentially involved in the soft agarose growth

response of fibrotic lung fibroblasts, epidermal growth factor (EGF), transforming growth factor (TGFβ), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) were tested for their colony modulating effects. Figure 3 illustrates that EGF, PDGF, and FGF all function to enhance colony formation in soft agarose while TGFβ (demonstrated here and previously reported by our group (11)) was shown to partially inhibit soft agarose growth. The effects, however, were not as great as those seen with either DEX or AtRA.

5. The Effect of combinations of growth factors and retinoid on colony formation. In order to examine possible mechanisms involved in retinoid modulation of anchorage independent growth, EGF, TGFβ, FGF, and PDGF were added directly into the soft agarose growth assay in the presence of various retinoids. As shown in Figure 4, the ability of retinoids to suppress colony growth was not altered by the presence of specific growth factors. Interestingly, the pattern of modulation of colony growth observed in the presence of specific growth factor alone (ie PDGF $\uparrow$, TGFβ $\downarrow$) was maintained in the presence of retinoid, however the overall frequency of colony formation for each growth factor was reduced.

6. ^{3}H-TdR incorporation in the presence of corticosteroid and retinoid. The

results of a ^{3}H-TdR incorporation assay from one representative fibrotic lung fibroblast cell line is presented in Figure 5. The pattern of ^{3}H-TdR incorporation over 96 hours was not significantly altered in the presence of either retinol or retinal. A dose dependent decrease in ^{3}H-TdR incorporation was observed in the presence of AtRA and 13-cis, with a 33% drop in incorporation at 10^{-6}M as compared to human serum alone. Interestingly, in the presence of DEX alone, a similar decrease in thymidine incorporation is observed, although, in contrast to AtRA, we have demonstrated that treatment with DEX actually doubles colony number. In the presence of retinoid plus DEX, a decrease in fibroblast ^{3}H-TdR incorporation below that induced by DEX alone was observed for all retinoids tested.

DISCUSSION

We have suggested that chronic inflammatory connective tissue diseases, of which idiopathic pulmonary fibrosis (IPF) is an example, may result from enhanced presence of specific fibroblast subpopulations in the tissue which express an activated or altered phenotype consistent with disease expression. Moreover, since fibroblasts may function as effector cells, an activated fibroblast phenotype may perpetuate the disease process through the release of cytokines which could then modulate surrounding inflammatory cells (19,20). Hence, suppression of the inflammatory response alone without coincident modulation of the fibroblast phenotype may not be sufficient to arrest the fibrotic process.

The current study demonstrates that culture of fibrotic lung fibroblasts with retinoid reduces the ability of IPF fibroblasts to form colonies under conditions of anchorge-independent growth. The frequency of reduction of anchorage-independent growth varied among the retinoids tested, with AtRA manifesting the greatest effect (~ 90% inhibition) on the colony forming ability of IPF fibroblast cell lines. 13-cis-retinoic acid, the isomer of AtRA, showed a pattern of colony induced suppression similar to, but not identical to that of AtRA.

Since it is now widely accepted that nuclear retinoid receptors mediate the effect of retinoids, differences in the type of receptor present in lung

fibroblasts could account for the differing activities of the 4 retinoids tested (21,22). To date, 3 retinoic acid receptor isoforms have been detected in human bronchial fibroblasts (23) and mRNA for 2 retinoic acid receptor isoforms and the retinoic acid X receptor (RXRα) detected in normal human unstimulated interstitial lung fibroblasts (24) suggesting that lung fibroblasts may also be targets of retinoids.

Our data is consistent with recent studies demonstrating the inhibition of anchorage-independent growth of human and rat arthritic synoviocytes by all-trans retinoic acid at concentrations of 10^{-6}M (25). In addition, the finding that neonatal lung fibroblast anchorage-independent growth is also inhibited by retinoid again suggests the modulation of a particular fibroblast phenotype. Palmer et al (26) have also previously demonstrated the inhibition of growth factor induced anchorage-independent growth of human skin neonatal fibroblasts by AtRA.

The effect of specific growth factors, known to mediate the soft agar growth of normal fibroblasts in culture was examined for their ability to modulate the colony growth of IPF fibroblasts in the presence of various retinoid (11,27-29). Again, an overall reduction in mean colony number was observed for all treatment conditions, with AtRA and 13-cis RA yielding the greatest effect. The fact that the addition of growth factors known to promote anchorage-independent growth were not able to override the colony modulating

effects of AtRA and 13-Cis RA suggests a change in the phenotype of our lung IPF fibroblasts such that they are no longer as responsive to these factors.

Since we recently demonstrated that glucocorticoids enhance the anchorage-independent growth of IPF fibroblasts, the action of DEX in combination with retinoid on the colony forming potential of these fibroblasts was examined. Our data indicate, that of the 4 retinoids tested, only AtRA and 13-cis RA were able to consistently counteract the effects of DEX at the concentrations utilized in the study. The finding that the presence of retinal or retinol did not oppose the effects of DEX again suggests that these compounds must first be converted to their active metabolites.

In order to address the possibility that the reduction of colony growth demonstrated in the presence of retinoid was due to some toxic activity of the retinoid which interfered with the metabolic capacity of the cell, we examined the pattern of ^{3}H-TdR incorporation of IPF fibroblast lines, in the presence of various retinoids. Our data indicates a dose-dependent decrease in fibroblast proliferation in the presence of AtRA and 13-cis RA with proliferation rates of IPF fibroblasts 67% of control. These results are consistent with our hypothesis since a decrease in cell growth rate would not be unexpected in a population of cells undergoing differentiation. Moreover, the fact that at a concentration of retinoid 10^{-6} M, cell viability was found to be $\geq$97% suggests that our IPF fibroblasts still remain a vital cell population following culture with

retinoid. In addition, others have shown that 10^{-5} M retinoid has little effect on total protein synthesis in lung fibroblasts (14). We also observed in the presence of DEX alone, a similar decrease in fibroblast proliferation, even though DEX is a potent enhancer of colony number. A decrease in fibroblast proliferation following treatment by AtRA and 13-cis RA has previously been reported (30,31). In addition a study by Demetriou et al (32) suggests that retinoic acid induces differentiation of murine 3T3 fibroblasts.

Zitnik et al. (24) have demonstrated in cultures of human lung fibroblasts that treatment with retinoic acid (10^{-6}M) is capable of modifying interleukin 1 induced gene expression of cytokine and that the inhibitory effects of AtRA appear independent of cytoxicity. Moreover, these authors report similar inhibitory activities following treatment with retinol and retinal (10^{-6}M), suggesting that lung fibroblasts are able to metabolize both retinol and retinal to their active compounds, a concept our findings would support. Similarly, we also demonstrate an effect of AtRA on fibroblast metabolism, however unlike Zitnik et al., retinol and retinal were not found as potent in our system. The finding that AtRA was twice as effective as either retinol or retinal in reducing colony number is consistent with the belief that in vivo, all-trans retinoic acid is the active metabolite of vitamin A (retinol) (33,34) and with studies demonstrating that particular retinoids function differently in their abilities to induce differentiation (35,36).

Several studies have suggested the importance of the extracellular matrix in normal cellular differentiation (37,38). In addition, changes to the extracellular matrix have been reported to affect fibroblast cell growth and fibroblast responsiveness to growth factors (39). A number of reports have suggested that AtRA may play a role in controlling extracellular matrix production (12-16) and several groups have reported the selective inhibition of collagen synthesis in fibroblasts following incubation wtih retinoid. Oikarinen et al (13) have reported in skin fibroblasts similar reductions in procollagen production and type I procollagen mRNA following treatment with AtRA (10^{-5}M), 13-Cis RA, retinol and retinal. No effect on fibronectin mRNA levels was evident. Krupsky and Goldstein (14) recently demonstrated that AtRA treatment of human lung fibroblasts results in the reduction of steady state mRNA levels of type I collagen and that this reduction is mediated by down regulating collagen gene transcription. In addition, matrix metalloproteinase gene expression has also been demonstrated to be repressed by AtRA treatment (15,16). Based on previous findings and the observations reported here, we suggest that retinoid treatment has an affect on fibroblast phenotype and that this effect is mediated possibly through a differentiation or matrix altering event.

Numerous investigators have documented that treatment of fibroblast lines established from keloid or scleroderma cultures with AtRA or 13-cis RA

greatly reduced collagen synthesis (40-41). Retinoids, specifically AtRA, have also proven to be effective in the treatment of keloid and hypertrophic scars and in treatment of patients afflicted with acute promyelocytic leukemia (42,43). Moreover, in animal models, it has been demonstrated that treatment of rats with all-trans retinoic acid significantly inhibits disease expression in a streptococcal cell wall-induced arthritis model (44). Since excessive accumulation of collagen is a hallmark of fibrotic disease, differentiation therapy (as suggested here by the use of all trans retinoic acid) as a therapeutic adjunct to anti-inflammatory treatment of fibrotic disorders may have great potential benefit. We are currently investigating this hypothesis in vivo, in a rat model of bleomycin induced lung fibrosis.

ACKNOWLEDGMENTS

The technical assistance of Jane Ann Schroeder is gratefully acknowledged. This work was supported by MRC Canada and by Astra Draco, Sweden. Carl D. Richards is an Ontario MOH Scholar. Thomas J. Podor is a HSFO Scholar.

REFERENCES

1. Bordin S, Page RC, and Narayanan AS: Heterogeneity of normal human diploid fibroblasts: isolation and characterization of one phenotype. Science 223:171-173, 1984.

2. Botstein GR, Sherer GK, and LeRoy EC: Fibroblast Selection in Scleroderma: an alternative model of fibrosis. Arthritis and Rheumatism. 25:189-195, 1982.

3. Goldstein RH and Fine A: Fibrotic Reactions in the lung: the activation of the lung fibroblast. Exp Lung Res. 11(4):245-261, 1986.

4. Jordana M., Kirpalani H, and Gauldie J: Heterogeneity of human lung fibroblast proliferation in relation to disease expression. In: Phipps, RP, editor, Pulmonary Fibroblast Heterogeneity. CRC Press, Boca Raton, Inc., FL. 1992, pp 229-249.

5. Jordana M, Schulman J, McSharry C, Irving LB, Newhouse MT, Jordana G, and Gauldie J: Heterogeneous proliferative characteristics of human adult lung fibroblast lines and clonally derived fibroblasts from control and fibrotic tissue. Am. Rev. Respir. Dis. 137:579-584, 1988.

6. Korn, JH, Torres D, and Downie, E: Clonal heterogeneity in the fibroblast response to mononuclear cell derived mediators. Arthritis and Rheumatism 27:174-179, 1984. 7. Raghu G, Chen Y, Rusch V and Rabinovitch PS: Differential proliferation of fibroblasts cultured from normal and fibrotic human lungs. Am Rev Respir Dis 138:703-708, 1988.

8. Uitto J, Bauer EA, and Eisen AZ: Increased biosynthesis of triple-helical type I and type III procollagens associated with unaltered expression of collagenase by skin fibroblasts in culture. J. Clin. Invest. 64:921-930, 1979.

9. Derdak, S, Dixon P, Watts H, Penney D, and Phipps R: CD4 Expression in Lung Fibroblasts. The Lancet 337:374A, 1991.

10. Derdak, S, Penney DP, Keng P, Felch ME, Brown D, and Phipps RP: Differential collagen and fibronectin production by Thy 1^+ and Thy 1^- lung fibroblast subpopulations. Am. J. Physiology 263:L283-L290, 1992.

11. Torry, DJ, CD Richards, TJ Podor, and Gauldie J: Anchorage-independent colony growth of pulmonary fibroblasts derived from fibrotic human lung tissue. J. Clin. Invest. 93:1525-1532, 1994.

12. Pearson D, and Sasse J: Differential Regulation of Biglycan and Decorin by retinoic acid in bovine chondrocytes. J. Biol. Chem. 267:25364-25370, 1992.

13. Oikarinen H, Oikarinen AI, Tan EML, Abergel RP, Meeker CA, Mon-Li C, Prockop DJ, and Uitto J: Modulation of Procollagen gene expression by retinoids. Inhibition of collagen production by retinoic acid accompanied by reduced type I procollagen messenger ribonucleic acid levels in human skin fibroblast cultures. J. Clin. Invest. 75:1545-1553, 1985.

14. Krupsky M, Fine A, Berk JL, and Goldstein RH: Retinoic acid-induced inhibition of type I collagen gene expression by human lung fibroblasts. Biochimica et Biophysica Acta 1219:335-341, 1994.

15. Nicholson RC, Mader S, Nagpal S, Leid M, Rochette-Egly C, and Chambon, P. 1990. Negative regulation of the rat stromelysin gene promotor by retinoic acid is mediated by an AP1 binding site. EMBO J 9:4443-4454, 1990.

16. Bauer EA, Seltzer, JL, Eisen AZ: Retinoic acid inhibition of collagenase and gelatinase expression in human skin fibroblast cultures, evidence for a dual mechanism. J. Invest. Dermatol. 81:162-169. 1983.

17. Breitman, TR, Selonick SE, and Collins SJ: Induction of differentiation of human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc. Natl. Acad. Sci. 77:2936-2940, 1980.

18. Strickland S, and Mahdavi V: The Induction of differentiation in Teratocarcinoma Stem Cells by Retinoic Acid. Cell 15:393-403, 1978.

19. Gauldie, J, Jordana M, Cox G, Ohtoshi T, Dolovich J and Denburg J: Fibroblasts and other structural cells in airway inflammation. Am. Rev. Respir. Dis. 145:S14-S17, 1992.

20. Jordana M, Cox G, Ohtoshi T, Zhou X, Dolovich J, Denburg J, and Gauldie J: The 'TDR' concept in chronic airways inflammation: tissue driven response. In: Matson P, Ahlstedt S, Venge P, and Thorell J, eds., Clinical Impact of the Monitoring of Allergic Inflammation. Academic Press Limited, London, 1991, pp 33-46.

21. Lehmann JM, Dawson MI, Hobbs PD, Husmann M, and Pfahl M: Identification of retinoids with nuclear receptor subtype-selective activities. Cancer Research 51:4804-4809, 1991.

22. Riaz-Ul-Haq, Pfahl M, and Chytil F: Retinoic acid affects the expression of nuclear retinoic acid receptors in tissues of retinol-deficient rats. Proc. Natl. Acad. Sci. 88:8272-8276, 1991.

23. Nervi C, Vollberg TM, George MD, Zelent A, Chambon P, and Jetten AM: Expression of Nuclear Retinoic Acid Receptors in Normal Tracheobronchial Cells and in Lung Carcinoma Cells. Exp. Cell Res. 195:163-170, 1991.

24. Zitnik RJ, Kotloff RM, Latifpour J, Zheng T, Whaiting NL, Schwalb J, and Elias JA: Retinoic Acid inhibition of IL-1 induced IL-6 production by human lung fibroblasts. J. Immunol. 152:1419-1427, 1994.

25. Lafyatis R, Remmers EF, Roberts AB, Yocum DE, Sporn MB, and Wilder RL: Anchorage-independent growth of synoviocytes from arthritic and normal joints. Stimulation by exogenous platelet-derived growth factor and inhibition by transforming growth factor-beta and retinoids. J. Clin. Invest. 83:1267-1276, 1989.

26. Palmer HJ, Maher VM, and McCormick JJ: Effect of retinoids on growth factor-induced anchorage independent growth of human fibroblasts. In Vitro Cellular & Developmental Biology. 25:1009-1015, 1989.

27. Palmer H, Maher VM, and McCormick JJ: Platelet-derived growth factor or basic fibroblast growth factor induce anchorage-independent growth of human fibroblasts. J. Cell. Physiology 137:588-592, 1988.

28. Rizzino A, and Ruff E: Fibroblast growth factor induces the soft agar growth of two non-transformed cell lines. In Vitro Cellular and Developmental Biology 22:749-755, 1986.

29. Rizzino A, Ruff E and Rizzino H: Induction and Modulation of-Anchorage-independent growth by platelet-derived growth factor, fibroblast growth factor, and transforming growth factor-β. Cancer Research 46:2816-2820, 1986.

30. Hein R, Mensing H, Muller PK, Braun-Falco O, and Krieg T: Effect of vitamin A and its derivatives on collagen production and chemotactic response of fibroblasts. Br. J. Dermat. III:37-44, 1984.

31. Lacroix A, Anderson GDL, and Lippman ME: Retinoids and cultured human fibroblasts. Effects on cell growth and presence of cellular retinoic acid-binding protein. Experimental Cell Research 130:339-344, 1980.

32. Demetriou AA, Levenson SM, Rettura G, and Seifter E: Vitamin A and retinoic acid: induced fibroblast differentiation in vitro. Surgery 98:931-934, 1985.

33. Chytil, F: The Lungs and Vitamin A. Am. J. Physiol 262:L517-L527, 1992.

34. Emerick RJ, Zile M, and DeLuca HF: Formation of Retinoic acid from Retinol in the Rat. Biochem. J. 102:606-611, 1967.

35. Bollag W, and Peck R: Modulation of Growth and Differentiation by Retinoids and Cytokines. 1991. In Saurat J-H editor, Retinoids: 10 Years On, Geneva Basel, Karger, 1991, pp 127-138.

36. Strickland S, Breitman TR, Frickel F, Nu rrenback A, Hadicke E, and Sporn MB: Structure-activity relationships of a new series of retinoidal benzoic acid derivatives as measured by induction of differentiation of murine F9 teratocarcinoma cells and human HL-60 promyelocytic leukemia cells. Cancer Research 43:5268-5272, 1983.

37. Menko AS, and Boettiger D: Occupation of the extracellular matrix receptor, Integrin, is a control point for myogenic differentiation. Cell 51:51-57, 1987.

38. Schuppan D, Somasundaram R, Dieterich W, Ehnis T, Bauer M: The extracellular matrix in cellular proliferation and differentiation. Annals NY Acad Sci 733:87-102, 1994.

39. Nakagawa S, Pawelek P, and Grinnell F: Extracellular matrix organization modulates fibroblast growth and growth factor responsiveness. Exp. Cell Res. 182:572-582, 1989.

40. Abergel RP, Meeker CA, Oikarinen H, Oikarinen AI and Uitto J: Retinoid Modulation of Connective Tissue Metabolism in Keloid Fibroblast Cultures. Arch Dermatol 121:632-635, 1985.

41. Ohta A and Uitto J: Procollagen gene expression by scleroderma fibroblasts in culture. Inhibition of collagen production and reduction of proα1(I) and proα1(III) collagen messenger RNA steady-state levels by retinoids. Arthritis and Rheumatism. 30:404-411, 1987.

42. Huang ME, Ye YI, Chen SR, Chai JR, Lu JX, Zhoa L, Gu LJ, Wang ZY: Use of all trans-retinoic acid in the treatment of acute promyelocytic leukemia. Blood 72:567-571, 1989.

43. Daly TJ and Weston WL: Retinoid effects on fibroblast proliferation and collagen synthesis in vitro and on fibrotic disease in vivo. J. Am. Acad. Dermatol. 15:900-902, 1986.

44. Haraoui B, Wilder RL, Allen JB, Sporn MB, Helfgott RK, and Brinckerhoff CE. Dose-dependent suppression by the synthetic retinoid, 4-hydroxyphenyl retinamide, of streptococcal cell wall-induced arthritis in rats. Int. J. Immunopharmacol. 7(6):903-916, 1985.

FIGURE LEGENDS

Figure 1. Modulation of anchorage-independent growth of lung fibroblasts by retinoids(s). Human adult lung fibrotic fibroblasts (n=3) were cultured under soft agarose growth conditions in the presence of absence or various retinoids (10^{-6}M). Colonies with a diameter > 45 um were scored. Data represents the mean number of colonies $\pm$ SD from 4 replicate wells.

Figure 2. The Effect of Corticosteroid on the soft agarose colony growth of fibrotic lung fibroblasts. Lung fibroblast cell lines from fibrotic lung were grown under conditions of anchorage-independent growth in the presence of DEX (10^{-6}M) or DEX plus retinoid (10^{-6}M). The results from 3 lung fibroblast cell lines are presented. Data represents the mean number of colonies $\pm$ standard deviation from 4 replicate wells.

Figure 3. The modulation of anchorage independent growth by specific growth factors. Fibrotic lung fibroblasts were assayed for soft agarose growth in the presence of human serum alone or serum containing either EGF (6 ng/ml), TGFβ (100 pg/ml), PDGF (5 ng/ml) or FGF (10 ng/ml). Colonies with a diameter > 45 μm were counted from 4 replicate wells. Colony counts were pooled and the data represents the mean number of colonies $\pm$ standard deviation from 3 fibrotic lung cell lines.

Figure 4. Effect of specific growth factors on the modulation of anchorage-independent growth by retinoids. Human fibrotic lung fibroblasts (n = 3) were assayed for growth under soft agarose conditions in the presence of retinoid (10^{-6}M) and the following growth factors: EGF (6 ng/ml), TGFβ (100 pg/ml), PDGF (5 ng/ml), FGF (10 ng/ml) and DEX (10^{-6}M). Data represent the mean number of colonies $\pm$ SD from 4 wells in each treatment group.

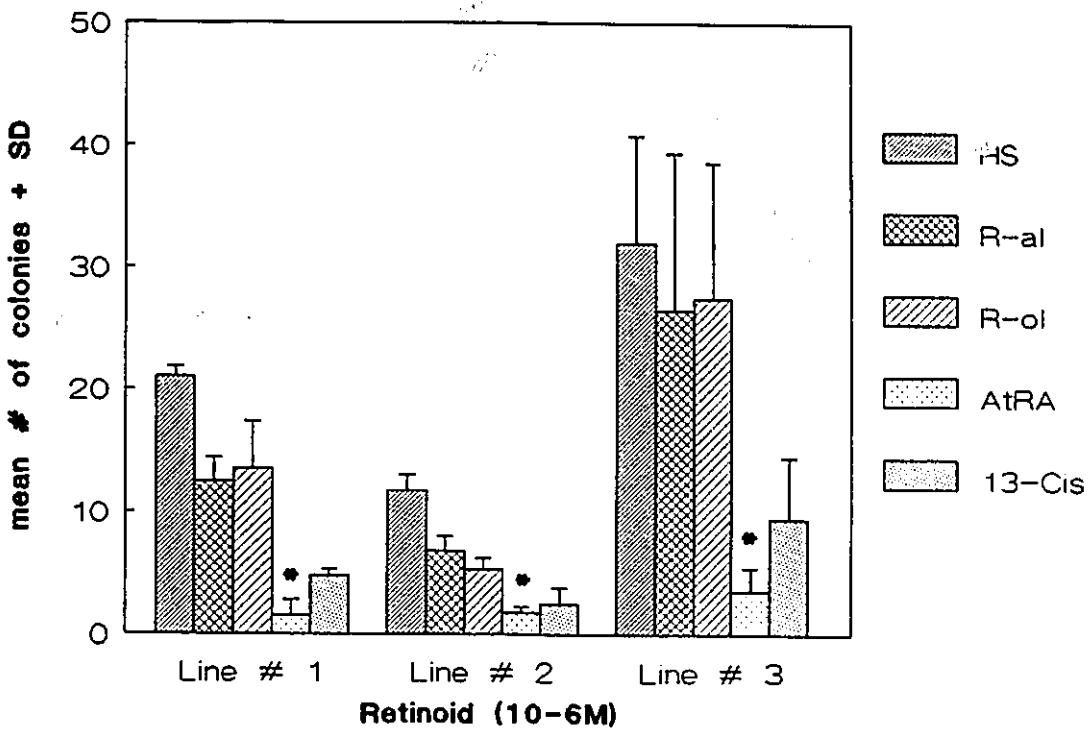
Figure 5. Influence of corticosteroid and retinoid on ^{3}H-TdR incorporation of fibrotic lung fibroblasts. Adult fibrotic lung fibroblasts were cultured for 72 hours in the presence of either retinoid, DEX plus retinoid (10^{-5}M - 10^{-9}M), DEX (10^{-6}M) alone, or 20% human serum. 3H-TdR was added to the system during the last 24 hours of culture. A representative profile from one fibrotic cell line is presented. The results are expressed as the mean cpm $\pm$ SD from 4 replicate wells.

Figure symbols:

○ - AtRA; + - 13-Cis RA; △ - R-ol; □ - R-al

● - AtRA + Dex; + - 13-Cis + Dex; ▲ - R-ol + Dex; ■ - R-al + Dex

Figure 1



* p < 0.01 as compared to HS control

Figure 2

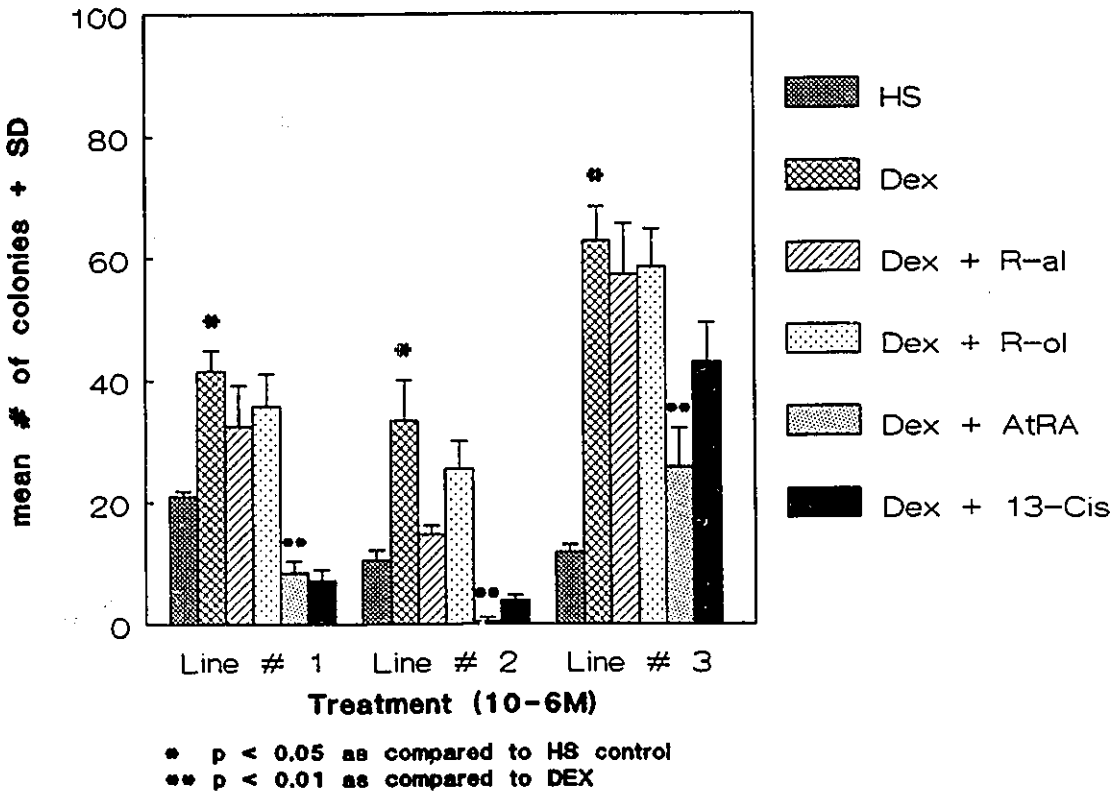
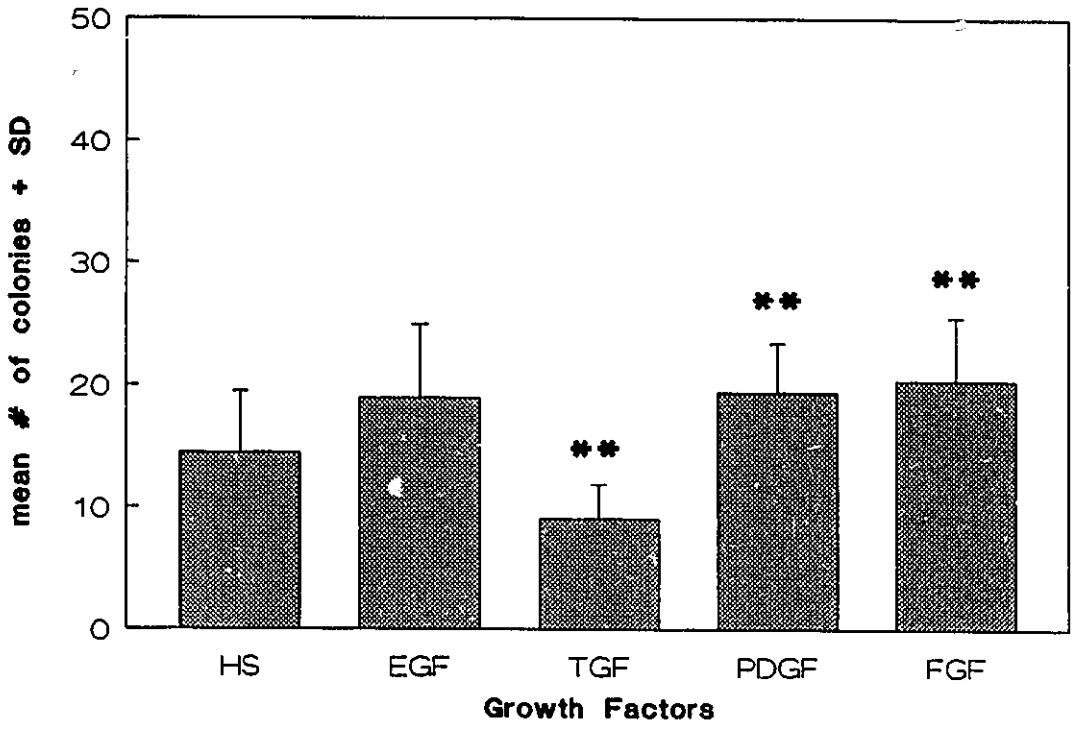

100
80
60
40
20
0
mean # of colonies + SD
Line # 1
Line # 2
Line # 3
Treatment (10-6M)
HS
Dex
Dex + R-al
Dex + R-ol
Dex + AtRA
Dex + 13-Cis
* p < 0.05 as compared to HS control
** p < 0.01 as compared to DEX

Figure 3



** p < 0.05 as compared to control

Figure 4

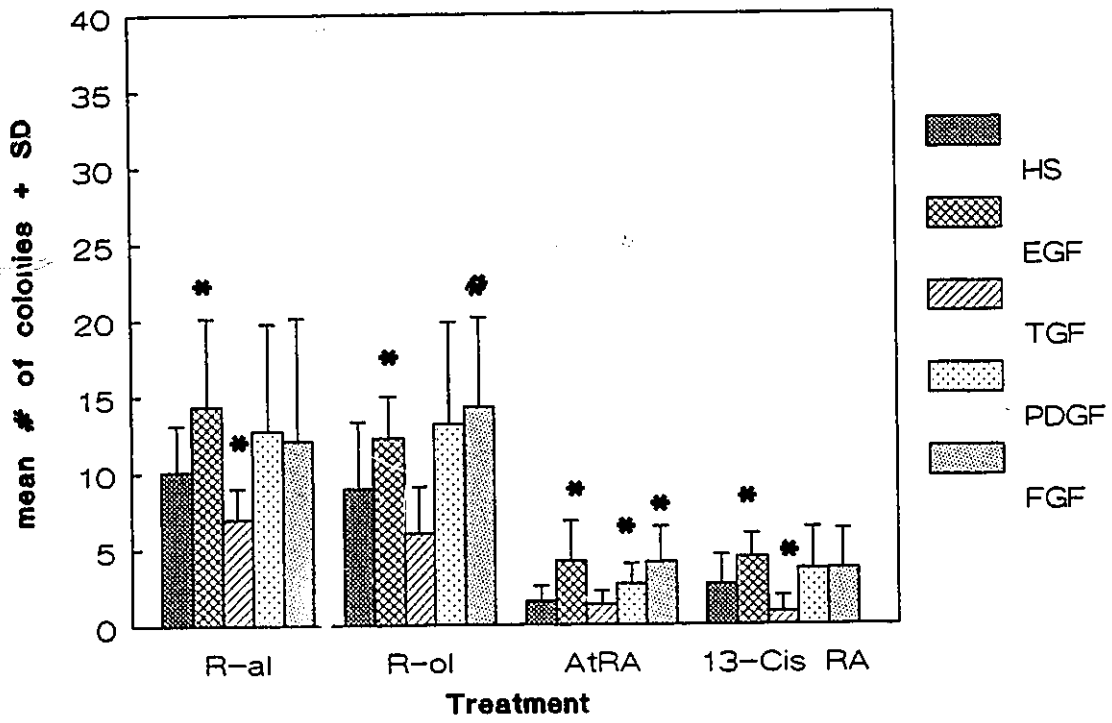
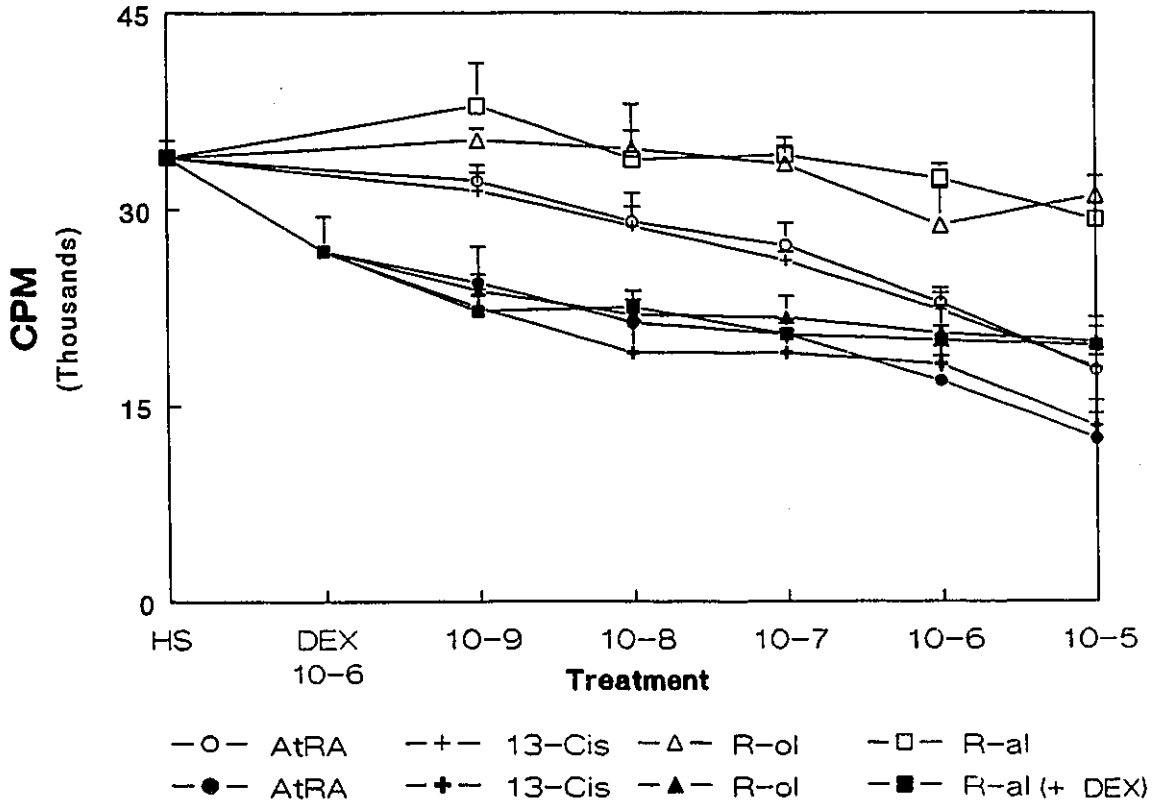

40
35
30
25
20
15
10
5
0
mean # of colonies + SD
R-al
R-ol
AtRA
13-Cis RA
Treatment
HS
EGF
TGF
PDGF
FGF

Figure 5


45
30
15
0
CPM
(Thousands)
HS
DEX
10-6
10-9
10-8
10-7
10-6
10-5
Treatment
—o— AtRA
—+— 13-Cis
—Δ— R-ol
—□— R-al
—●— AtRA
—✚— 13-Cis
—▲— R-ol
—■— R-al (+ DEX)

CHAPTER Four

MODULATION OF THE FIBROTIC LUNG RESPONSE FOLLOWING RETINOID THERAPY IN A RAT MODEL OF BLEOMYCIN INDUCED FIBROSIS

The following article entitled "The in-vivo administration of all-trans retinoic acid modulates the fibrotic outcome in a rat model of bleomycin induced pulmonary fibrosis" was submitted for publication in The American Journal of Physiology (Lung). This research article examines the effectiveness of retinoid as a potential therapy in the modulation of the fibrotic lung response induced following bleomycin instillation.

The work presented in this study was performed by the author of the thesis with the help from research technicians with animal handling and care. This research paper was written by the author of the thesis. Dr. Vincic assisted with histological assessments. Drs. Richards and Gauldie provided supervision.

The in-vivo administration of all-trans retinoic acid modulates the fibrotic outcome in a rat model of bleomycin induced pulmonary fibrosis.

Diane J. Torry, Lydia Vincic*, Carl D. Richards and Jack Gauldie.

Department of Pathology, Chedoke-McMaster Medical Centre, McMaster University and *St. Joseph's Hospital, Hamilton, Ontario, Canada.

Running title: Retinoic acid modulates bleomycin fibrosis

Correspondence: Jack Gauldie, Ph.D.

Department of Pathology

McMaster University

1200 Main Street West

Hamilton, Ontario, Canada L8N 3Z5

Tel: (905) 521-2100 Ext. 6332

Fax: (905) 577-0198

ABSTRACT

Fibroblast heterogeneity has been documented in fibrotic tissue. Therefore, we have suggested that idiopathic pulmonary fibrosis (IPF) (and other chronic inflammatory connective tissue diseases) may result from the overabundance of specific fibroblast subsets in the lung which express activated phenotypes consistent with disease expression. We have previously demonstrated that primary lines of fibroblasts derived from IPF tissue exhibited enhanced proliferative rates, are capable of anchorage-independent growth, and that the colony forming ability of IPF fibroblasts could be inhibited with retinoid, known differentiating agents. Similar findings were also demonstrable in fibroblast lines derived from neonatal lung suggesting the modulation/differentiation of a particular fibroblast phenotype toward a more mature adult phenotype; one incapable of anchorage-independent growth. In this study, we report the in vivo administration of all trans retinoic acid (AtRA) significantly decreased the number of fibrotic lesions in a rat model of bleomycin induced lung fibrosis. Moreover, the daily adminstration of AtRA dramatically reduced the pattern of collagen deposition in the lung compared to the bleomycin control tissue.

Index terms: fibroblast heterogeneity, retinoic acid, pulmonary fibrosis, inflammation, collagen.

ABBREVIATIONS

AtRA	All-trans retinoic acid
DEX	Dexamethasone
IPF	Idiopathic pulmonary fibrosis

INTRODUCTION

The development of pulmonary fibrosis may be the end result of exposure of the lung to a variety of known etiological agents including inorganic dusts and chemicals such as asbestos and silica, infectious agents and therapeutic agents such as radiation. However, approximately 2/3 of the cases diagnosed are of unknown etiology and are termed idiopathic pulmonary fibrosis (IPF)(2,6-8)

Pulmonary fibrosis is characterized by fibroblast hyperplasia and excessive connective tissue deposition resulting in decreased lung volumes and compliance with impairment of gas exchange (18,19,23,28). In addition, an increase in the proportion of fibroblasts actively synthesizing collagen has been documented in the lungs of patients with pulmonary fibrosis (8).

The process of fibrosis is believed to be initiated by inflammatory reactions in the alveoli and alveolar wall which for reasons not yet clear proceed to chronicity (2,6,8). While inflammatory/immune cells clearly contribute to the fibrotic process, we and others believe that tissue structural cells, particularly the interstitial fibroblast, may play a more direct role as effector cells in the pathogenesis of IPF through the release of potent inflammatory and matrix modulating mediators (11,15).

Moreover, evidence clearly exists of morphological and functional

heterogeneity within fibroblast populations isolated from chronically inflamed tissues. Fibroblast populations with enhanced proliferative rates, differences in ECM production, differences in release and response to cellular mediators, and differences in cell surface marker expression have all been documented (3,10,13,16,17,21,29,35). In addition, we have previously demonstrated that fibroblasts derived from human IPF tissues and human neonatal lung exhibit the ability to grow as colonies under conditions of soft agarose culture, whereas fibroblasts obtained from normal lung do not form colonies (32). We further demonstrated in vitro that the colony forming activity of the IPF fibroblast could be decreased by treatment of cell lines with retinoids, known differentiating agents (33).

The use of retinoids in vivo, has proven to be effective in the treatment of rat streptococcal cell wall induced arthritis (12) and in treatment of patients afflicted with keloid and hypertrophic scars (7,14). In this study, a rat model of bleomycin induced pulmonary fibrosis was employed to evaluate the use of retinoid or a combination of retinoid and steroid as potential therapies. The data presented here demonstrate that the daily oral administration of all trans retinoic acid beginning 10 days post-bleomycin exposure dramatically reduces the number of lesions and alters the pattern of collagen deposition in the fibrotic lung.

MATERIALS AND METHODS

1. Animals.

Pathogen free adult male Lewis rats weighing 180-250 g were obtained from Charles River Laboratories Canada (St.-Constant, Quebec) and housed under specific pathogen free conditions in our central animal facility. Animals had free access to water and commercial rat food. All manipulations to the animals were carried out under a laminar flow hood.

2. Chemicals and Reagents.

All trans retinoic acid (Sigma Chemical Company, St. Louis, MO R-2625) was dissolved in arachis oil and stored protected from light in 1 ml aliquots at -20°C. Dexamethasone was obtained from Sabex Boucherville, Quebec (DIN 00664227). Bleomycin (Blenoxane) was purchased from Bristol Laboratories of Canada, Montreal, Quebec (DIN 00258482). Ketamine (Ketalean) was purchased from MTC Pharmaceuticals, Cambridge, Ontario (DIN 00612316). Xylazine (Rompun) was purchased from Chemagro Limited, Etobicoke, Ontario (DIN 00408972).

3. Bleomycin Model.

Specific pathogen free rats, weighing between 245 - 260 g at the start of the experiment, were anesthetized under a mixture of ketamine hydrochloride and

xylazine given intraperitoneal. At Day 0, the intratracheal injection of bleomycin was accomplished via tracheostomy. Bleomycin dissolved in sterile PBS was administered at a concentration of 1.5 U / 250 g body weight. At Day 10 and again beginning Day 14, animals (n=3) received either i) an oral dose of AtRA via gastric intubation at a concentration of 1 mg / 250 g rat to achieve blood levels of the drug approximately 10^{-6}M (30), ii) a subcutaneous injection of DEX in sterile PBS (25 ug / 250 g rat), or iii) a combination of AtRA / DEX (at the above concentrations) daily until Day 28. On day 28, the animals were sacrificed by exsanguination and lungs excised. One lung was fixed by perfusion with 10% neutral buffered saline and then paraffin embedded for histological study. A piece of the right lung was obtained for cell culture and the remainder of the lung placed directly in GITC solution for RNA extraction.

4. Total RNA Preparation.

Total tissue RNA was extracted from the lung using a slightly modified single-step method (5). Lung tissue was placed directly into a solution of guanidinium buffer (4 M guanidinium isothiocyanate, 17mM sodium lauroyl sarcosine, 25mM sodium citrate, 0.1 M 2-mercaptoethanol), homogenized and then stored at - 70°C for future processing. RNA was extracted with 2M sodium acetate pH 4.0, water saturated phenol and chloroform, and then precipitated at -20°C

overnight in isopropanol. For northern gel hybridization, 20 ug of total RNA was electrophoresed on a 1.0% agarose formaldehyde gel, transferred onto nylon membrane in 0.025M phosphate buffer pH 6.5 (Biotrans, Pall Biosupport Corporation, East Hills, NY) and ultraviolet fixed for 2 minutes. The blot was hybridized with a ^{32}P-γ-ATP labeled rat α_1(I) procollagen oligonucleotide antisense probe which was synthesized based on the sequence quoted by Zhang et al. (37), d(AGG-GCC-AGT-CTC-AGC-ACG-GTC-ACC-CTT-GGC).

5. Lung histology.

4 μm rat lung sections were cut from paraffin blocks and serial sections stained with either hematoxylin and eosin (H and E) or Miller's Elastic Fibre Stain (24). The slides were examined by light microscopy and graded to assess levels of fibrosis. All histological assessments were performed by the same pathologist without knowledge of the treatment conditions.

6. ^{3}H-Thymidine (^{3}H-TdR) Incorporation.

Primary rat cell lines were established from rat lung tissue utilizing methodology as previously described (16). For assay, rat lung fibroblasts were grown to confluency, trypsinized and resuspended in RPMI media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco Laboratories, Grand Island, NY). Two thousand cells were plated into the bottom of a 96

well plate and allowed to attach overnight. The following day, the cells were washed with PBS, and fresh media added. The cells were allowed to incubate for a further 72 hrs at 37°C, 5% CO_2 and 0.5 μCi/well of ^{3}H-thymidine (20 Ci/mmol, NEN Corporation, Boston, MA) added for the last 24 hours of culture. The cells were then washed, lysed and cellular contents collected on a cell harvester.

7. Total Leukocyte Counts and Differential Cellular Analysis.

Total leukocyte counts were performed on peripheral blood samples obtained at Day 28 using a standard hemocytometer. Differential cell counts were performed on blood smears stained with a modified Wright-Giemsa Stain (Hema-tek Stain Pack, Miles Inc Diagnostics, Elkhard Indiana).

8. Statisticai Analysis. All statistical analyses were performed utilizing the Minitab Data Analysis Software, Release 7.1 (1989, Minitab, Inc., State College, PA).

RESULTS

1. Histopathologic Features.

The fibrotic 'control' animals showed histologic findings consistent with bleomycin induced pulmonary fibrosis (25,31). There was evidence of interstitial widening and obliteration of bronchioles and airspaces. The lesions appeared to be cellular in nature containing inflammatory cells, smooth muscle cells and fibroblast-like cells and were often centered around bronchioles or bronchi. From the corresponding Miller matrix stained section we observed (i) areas of intense collagen staining and (ii) areas of staining in the obliterated airspaces indicating cellular fibroblastic lesions undergoing organization. The appearance of foamy histocytes indicated areas of obstructive pneumonia (Figure 1(a)-(c)).

The DEX and AtRA/DEX treatment groups beginning either day 10 or 14 post bleomycin treatment exhibited similar histology to bleomycin controls (data not shown). Again, widening of the interstitium was observed as well as obliteration of bronchioles and airways. Examination of lung sections stained with H and E revealed similar numbers of lesions as compared to bleomycin control. However, upon examination of the corresponding sections developed with Miller stain, the type of lesion was found to differ. In both the DEX and AtRA/DEX groups, less cellular fibroblastic processes were observed in the lesions. At the periphery of lesions a darker stain was observed indicating more

dense collagen in these scars as compared to bleomcyin only controls. These findings are typical of more organized (older) lesions. Also, the lesions appeared to be 'smaller' or contracted. The presence of fibrous plugs was also observed.

2. Differences in the AtRA Treatment Groups Day 10 vs Day 14.

In the animals beginning treatment with AtRA Day 10 post-bleomycin (AtRA 10), there appeared significantly fewer numbers of lesions with decreased intensity of collagen staining as compared to the fibrotic control and the other treatment groups (Figure 1(d) - (f)).

In the animals receiving AtRA beginning Day 14 (AtRA 14) post-bleomycin treatment both the H and E and Miller stains appeared indistinguishable from bleomycin controls (Figure 1(g) - (i)). Interstitial and airways fibrosis was observed. The numbers and type of lesions present were similar to controls i.e. the lesions appeared to be organizing (more cellular with very little collagen in periphery of lesion).

3. mRNA Analysis.

In order to examine if the changes observed in lung histology were associated with changes in the levels of mRNA for procollagen α_1(I), total lung RNA was isolated and analyzed by Northern blot (Figure 2). In accordance with

previous reports the intratracheal instillation of bleomycin resulted in increased collagen mRNA levels (Fig 2, Lane 2)(20,26). Analysis of lung RNA from AtRA, DEX, and AtRA/DEX treated rats beginning day 10 post-bleomycin treatment revealed similar procollagen α_1(I) mRNA band intensities which were indistinguishable from the bleomycin control (Fig 2, lanes 3-5).

4. Changes in the proliferative rates of lung fibroblast lines derived from bleomycin control and AtRA treated rats.

The results of a ^{3}H-TdR incorporation assay utilizing primary rat fibroblast cell lines derived from treated and untreated rat lung are presented in Figure 3. The pattern of ^{3}H-TdR incorporation over a 96 hour time interval revealed: (i) rat fibroblast cell lines derived from fibrotic lung tissue proliferated slower than normal lung fibroblast cell lines (Figure 3a) and, (ii) treatment with AtRA beginning Day 10 post bleomycin exposure resulted in derivation of cell lines with significant increases in the mean proliferative rate above fibrotic controls (Figure 3b), tending towards a more adult fibroblast behaviour.

DISCUSSION

To date, the mainstay of treatment for patients with pulmonary fibrosis has been aimed at modulating the inflammatory process. However, the treatment of patients with corticosteroid appears most effective in patients with early stage disease i.e. those exhibiting little scarring (4,34). Since most patients present clinically in the later stages of disease when the fibrotic component in the lung is well established, treatment with antiinflammatory agents may not be an effective drug strategy.

We have previously demonstrated that human lung fibroblasts derived from inflamed lung and those derived from normal neonatal lung: (i) exhibit quicker proliferative rates than normal adult lung fibroblasts (16,17), (ii) exhibit the ability to form colonies under conditions of anchorage-independent growth (32) and (iii) that the ability to form colonies under anchorage-independent growth can be modulated with retinoid (33). The characteristic of anchorage-independent growth is consistent with the aggressive nature of fibroproliferative disease and with developmental lung growth. The observation that fibrotic lung fibroblast cell lines exhibit similar growth characteristics to neonatal lung fibroblasts suggests the prevalance of newly differentiated fibroblasts in fibrotic lung tissue exhibiting growth characteristics such as would be found in the developing lung, or a lung undergoing tissue injury and repair. Moreover, the observation that retinoid treatment inhibits colony growth

implies the modulation of the fibrotic phenotype may involve a differentiation event. It was on this premise that we examined the effect of retinoid in a rat model of bleomycin induced fibrosis. Treatments began Day 10 or Day 14 post-bleomycin injection as we wanted to treat animals during the acute inflammatory stage (Day 10) and at a time point where for the most part, the inflammatory changes had subsided and fibrosis was developing (Day 14). Since our in vitro data utilized 10^{-6}M AtRA and other in vitro studies have used 10^{-7} to 10^{-6}M AtRA to induce cell differentiation, a dose of 4 mg / kg rat was administered in order to achieve maximal blood levels of the drug at approximately 10^{-6}M (30). Moreover, overt toxicity was not observed following the daily administration of AtRA for a period of 14-18 days.

The histological findings reported here clearly demonstrate that daily treatments with AtRA (4 mg/kg) beginning Day 10 was able to modulate the fibrotic outcome in the lung following bleomycin exposure. A dramatic decrease in the number of lesions present in the lung was noted in all animals treated this way. Moreover, the collagen content of the AtRA 10 lung, as assessed by the use of Miller stain, was determined to be dramatically reduced as compared to the fibrotic control rat lung. Interestingly, in the animals treated from 10 days with AtRA increases in lymphoid cells were observed in blood vessels compared to other treatment groups. As such we decided to assess the numbers and types of white blood cells circulating in the peripheral

blood. Mean total leukocyte counts (n = 3) for all treatment groups were similar (5.1 - 5.5 x 10^6 cells/ml), however marginal differences were observed in the white blood cell profiles from AtRA treated animals. Specifically, AtRA treated rats (n = 3) displayed a significant increase in mean lymphocyte blood counts (81.33 $\pm$ 4.62 vs 63.0 $\pm$ 3.61; AtRA vs Fibrotic, $p < 0.05$), and a decrease in neutrophil and monocyte counts as compared to fibrotic animals (11.33 $\pm$ 1.53 vs 20.0 $\pm$ 1.00, 5.67 $\pm$ 3.06 vs 16.67 $\pm$ 2.68 respectively).

Treatment of rats beginning Day 14 post-bleomycin with AtRA did not appear to have any appreciable effects on the overall outcome of the fibrotic lung response. These results suggest that treatment with AtRA might only affect cells present during a small window of time (i.e. between Day 10-14). Since our hypothesis suggests the modulation of the fibrotic 'anchorage-independent' phenotype through the differentiation of the fibrotic fibroblast, it is possible that AtRA treatment in vivo might not be affecting the fibroblast in the manner we thought unless the cells present in the lung parenchyma at the later time point are completely differentiated and not affected by retinoid. We would therefore expect to see an effect at Day 10 because at this time point we would interfere with the differentiation events taking place. The idea of newly differentiating cell populations emerging in the lung between 7-14 days post bleomycin injection is supported by a recent paper by Zhang et al. (36) who demonstrate that newly reactive/differentiated myofibroblast/fibroblast

populations are seen during this time and contribute to increased lung TGFβ_1 expression during active fibrosis following bleomycin.

In order to assess whether the histological changes observed following retinoid treatment were associated with changes in collagen gene expression, whole lung RNA was isolated and northern blot analysis performed. Figure 2 shows a dramatic increase in the amount of α_1(I) procollagen mRNA detected in the fibrotic lung as compared to control. However, treatment with AtRA did not appear to have any effect on the modulation of this expression. The discrepancy between reduced collagen protein in the AtRA 10 treated lung and procollagen mRNA levels suggests possibly that changes in the collagen protein distribution of the AtRA 10 treated lung might involve other cell types present at the inflammatory site, or alternatively that AtRA might be affecting collagen synthesis at the translational level, not the transcriptional level (9). The reduction in the accumulation of collagen in the lung of animals treated from Day 10 onwards with AtRA might also involve enhanced collagen degradation.

In the present study, we have demonstrated that treatment with corticosteroid did not lessen or ameloriate the fibrotic lung response at the histological level, nor decrease levels of α_1(I) procollagen gene expression beyond fibrotic control levels. These results suggest therefore that it may not be the modulation of the initial inflammatory event itself that is important. Interestingly, AtRA/DEX 10 treatments yielded similar results to those of DEX

alone, implying that the AtRA dependent events occurring Day 10 post-bleo are steroid sensitive.

The growth characteristics of primary lung fibroblast lines derived from Day 28 treated and untreated rats were examined. Figure 3a illustrates that fibrotic rat lung fibroblast lines exhibited decreased growth rates as compared to normal fibroblast lines. These results are consistent with studies by Absher et. al. (1) and Raghu et. al. (29) who examined the proliferative potential of fibroblasts derived from early versus late stage fibrosis and reported that lung fibroblasts derived from end stage fibrotic tissue displayed decreased proliferative rates as compared to fibroblasts obtained from early disease or normals. Interestingly, fibroblasts derived from AtRA 10 treated rats displayed a significantly greater proliferative rate than fibrotic lung fibroblasts (Figure 3b). However, the fact that the proliferative rates of AtRA 10 fibroblasts never reach those of normal fibroblasts implies that the proliferative capacity of the cell and the arrest of fibrosis are not necessarily related.

In conclusion, the findings presented here clearly demonstrate changes in the histology of the fibrotic lung following retinoid treatment. Since events occurring around Day 10, but not from Day 14 appear AtRA sensitive, the theory that AtRA is working through a differentiation pathway in vivo may not hold true. Whether these histologic changes associated with retinoid treatment in vivo correlate with positive changes in pulmonary function remain to be

determined. Nonetheless, it would appear that treatment of fibrosis with agents aimed at modulation of structural cell behaviour may ameliorate the fibrotic outcome and serves as a template for future therapy in IPF.

ACKNOWLEDGEMENTS

The technical assistance of Jane Ann Schroeder is gratefully acknowledged. This work was supported by MRC Canada and by Astra Draco, Sweden. Carl D. Richards is an Ontario MOH Scholar.

REFERENCES

1. Absher, M., J. Hildebran, L. Trombley, J. Woodcock-Mitchell, and J. Marsh. Characteristics of cultured lung fibroblasts from bleomycin-treated rats: comparisons with in vitro exposed normal fibroblasts. Am. Rev. Respir. Dis. 129:125-129, 1984.

2. Basset, F., V.J. Ferrans, P. Soler, T. Takemura, Y. Fukuda, and R.G. Crystal. Intraluminal fibrosis in interstitial lung disorders. Am. J. Pathol. 122:443-461, 1986.

3. Botstein, G.R., G.K. Sherer, and E.C. LeRoy. Fibroblast selection in Scleroderma: an alternative model of fibrosis. Arthritis and Rheumatism. 25:189-195, 1982.

4. Carrington C.B., E.A. Gaenster, R.E. Coutu, M.X. FitzGerald, and R.G. Gupta. Natural history and treated course of usual and desquamative interstitial pneumonia. N. Eng. J. Med. 298:801-809, 1978.

5. Chomczynski, P. and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159, 1987.

6. Crystal, R.G., P.B. Bitterman, S.I. Rennard, A.J. Hance, and B.A. Keogh. Interstitial lung diseases of unknown causes: disorders characterized by chronic inflammation of the lower respiratory tract. N. Eng. J. Med. 310:154-166, 1984.

7. Daly, T.J. and W.L. Weston. Retinoid effects on fibroblast proliferation and collagen synthesis in vitro and on fibrotic disease in vivo. J. Am. Acad. Dermatol. 15:900-902, 1986.

8. Dunnill, M.S. Pulmonary Fibrosis. Histopathology. 16:321-329, 1990.

9. Focht R.J., and S.L. Adams. Tissue specificity of type I collagen gene expression is determined at both transcriptional and post-transcriptional levels. Mol. Cell. Biol. 4:1843-1852, 1984.

10. Fries, K.M., T. Blieden, R.J. Looney, G.D. Sempowski, M.R. Silvera, R.A. Willis, and R.P. Phipps. Evidence of fibroblast heterogeneity and the role of fibroblast subpopulations in fibrosis. Clin. Immunol. and Immunopath. 72:283-292, 1994.

11. Gauldie J., M. Jordana, G. Cox, T. Ohtoshi, J. Dolovich, and J. Denburg. Fibroblasts and other structural cells in airway inflammation. Am Rev Respir Dis 145:S14-S17, 1992.

12. Haraoui, B., R.L. Wilder, J.B. Allen, M.B. Sporn, R.K. Helfgott and C.E. Brinckerhoff. Dose-dependent suppresssion by the synthetic retinoid, 4-hydroxyphenyl retinamide, of streptococcal cell wall-induced arthritis in rats. Int J Immunopharmacol 7(6):903-916, 1985.

13. Heufelder, A.E., T.J. Smith, C.A. Gorman and R.S. Bahn. Increased induction of HLA-DR by interferon gamma in cultured fibroblasts derived from patients with Graves' Ophthalmopathy and Pretibial Dermopathy. J. Clin. Endocrinol. Metab. 73:307-313, 1991.

14. Janssen de Limpens, A.M.P. The local treatment of hypertrophic scars and keloids with topical retinoic acid. Br. J. Dermatology 103:319-323, 1980.

15. Jordana, M., G. Cox, T. Ohtoshi, Z. Xing, J. Dolovich, J. Denburg, and J. Gauldie. The 'TDR' concept in chronic airways inflammation: tissue driven response. In Clinical Impact of the Monitoring of Allergic Inflammation. P. Matson, S. Ahlstedt, P. Venge, and J. Thorell, editors. Academic Press Limited, London, p 33-46, 1991.

16. Jordana, M., J. Schulman, C. McSharry, L.B. Irving, M.T. Newhouse, G. Jordana and J. Gauldie. Heterogeneous proliferative characteristics of human adult lung fibroblast lines and clonally derived fibroblasts from control and fibrotic tissue. Am. Rev. Respir. Dis. 137:579-584, 1988.

17. Jordana, M., H. Kirpalani, and J. Gauldie. Heterogeneity of human lung fibroblast proliferation in relation to disease expression. In Pulmonary Fibroblast Heterogeneity. R.P. Phipps, editor, CRC Press, Boca Raton, Inc., FL. 229-249, 1992.

18. Katzenstein, A.L. Pathogenesis of "fibrosis" in interstitial pneumonia: an electron microscopy study. Hum. Pathol. 16:1015-1024, 1985.

19. Karlinksy, J.B. Glycosaminoglycans in emphysematous and fibrotic hamster lungs. Am. Rev. Respir. Dis. 117:299-305, 1982.

20. Kelley, J., L. Chrin, S. Shull, D.W. Rowe, and K.R. Cutroneo. Bleomycin selectively elevates mRNA levels for procollagen and fibronectin following acute lung injury. Biochem Biophys Res Commun 131:836-843, 1985.

21. Korn, J.H., R.S. Thrall, D.C. Wilbur, B.E. Kream, and T.H. Piela-Smith. Fibroblast Heterogeneity: Clonal selection of fibroblasts as a model for fibrotic disease. In: RP Phipps (Ed). Pulmonary Fibroblast Heterogeneity. CRC Press, Boca Raton, Inc., FL. 1992. pp 119-133.

22. Kuhn, C., J. Boldt, T.E. King, E. Crouch, T. Vartio., and J.A. MacDonald. An immunohistochemical study of architectural remodelling and connective tissue synthesis in pulmonary fibrosis. Am. Rev. Respir. Dis. 14:1693-1703, 1989.

23. Madri, J.A., and H. Furthmayr. Collagen polymorphism in the lung: an immunohistochemical study of pulmonary fibrosis. Hum. Pathol. 11:353-366, 1980.

24. Miller, P.J. An elastin stain. Medical Laboratory Technology. 28:148-149, 1971.

25. Phan, S.H., R.S. Thrall, and P.A. Ward. Bleomycin-induced pulmonary fibrosis in rats: biochemical demonstration of increased rate of collagen synthesis. Am Rev Respir Dis 121:501-506, 1980.

26. Raghow, R., S. Lurie, J.M. Seyer, A.H. Kang. Profiles of steady state levels of messenger RNAs coding for type I procollagen, elasin, and fibronectin in hamster lungs undergoing bleomycin-induced interstitial pulmonary fibrosis. J. Clin. Invest. 76:1733-1739, 1985.

27. Raghu, G. Idiopathic pulmonary fibrosis. A rational clinical approach. Chest 92:148-154, 1987.

28. Raghu, G., L.J. Striker, L.D. Hudson, and G.E. Striker. Extracellular matrix in normal and fibrotic human lungs. Am. Rev. Respir. Dis. 131:281-289, 1985.

29. Raghu, G., Y. Chen, V. Rusch, and P.S. Rabinovitch. Differential proliferation of fibroblasts cultured from normal and fibrotic human lungs. Am Rev Respir Dis. 138:703-708, 1988.

30. Shelley, R.S., H.W. Jun, J.C. Price, and D.E. Cadwallader. Blood level studies of all-trans and 13-cis-retinoic acids in rats using different formulations. J. Pharmaceutical Sciences. 71:904-907, 1982.

31. Thrall R.S., J.R. McCormick, R.M. Jack, R.A. McReynolds and P.A. Ward. Bleomycin-induced pulmonary fibrosis in the rat. Am. J. Pathol. 95:117-127, 1979.

32. Torry, D.J., C.D. Richards, T.J. Podor, and J. Gauldie. Anchorage-independent colony growth of pulmonary fibroblasts derived from fibrotic human lung tissue. J. Clin. Invest. 93:1525-1532, 1994.

33. Torry, D.J., C.D. Richards, T.J. Podor, and J. Gauldie. Modulation of the anchorage-independent phenotype of human lung fibroblasts obtained from fibrotic tissue following culture with retinoid and corticosteroid. Exp Lung Res (in press), 1995.

34. Tukiainen, P., E. Taskinen, P. Holsti, O. Korhola, and M. Valle. Prognosis of cryptogenic fibrosing alveolitis. Thorax 38:349-355, 1983.

35. Uitto, J., E.A. Bauer, A.Z. Eisen. Scleroderma: increased biosynthesis of triple-helical type I and type III procollagens associated with unaltered expression of collagenase by skin fibroblasts in culture. J. Clin. Invest. 64:921-930, 1979..

36. Zhang K., K.C. Flanders and S.H. Phan. Cellular localization of transforming growth factor β expression in bleomycin-induced pulmonary fibrosis. Am. J. Physiol (in press), 1995.

37. Zhang, K., M. Gharaee-Kermani, B. McGarry and S.H. Phan. In situ hybridization analysis of rat lung α_1(I) and α_2(II) collagen gene expression in Pulmonary Fibrosis induced by endotracheal bleomycin injection. Lab. Invest. 70:192-202, 1994.

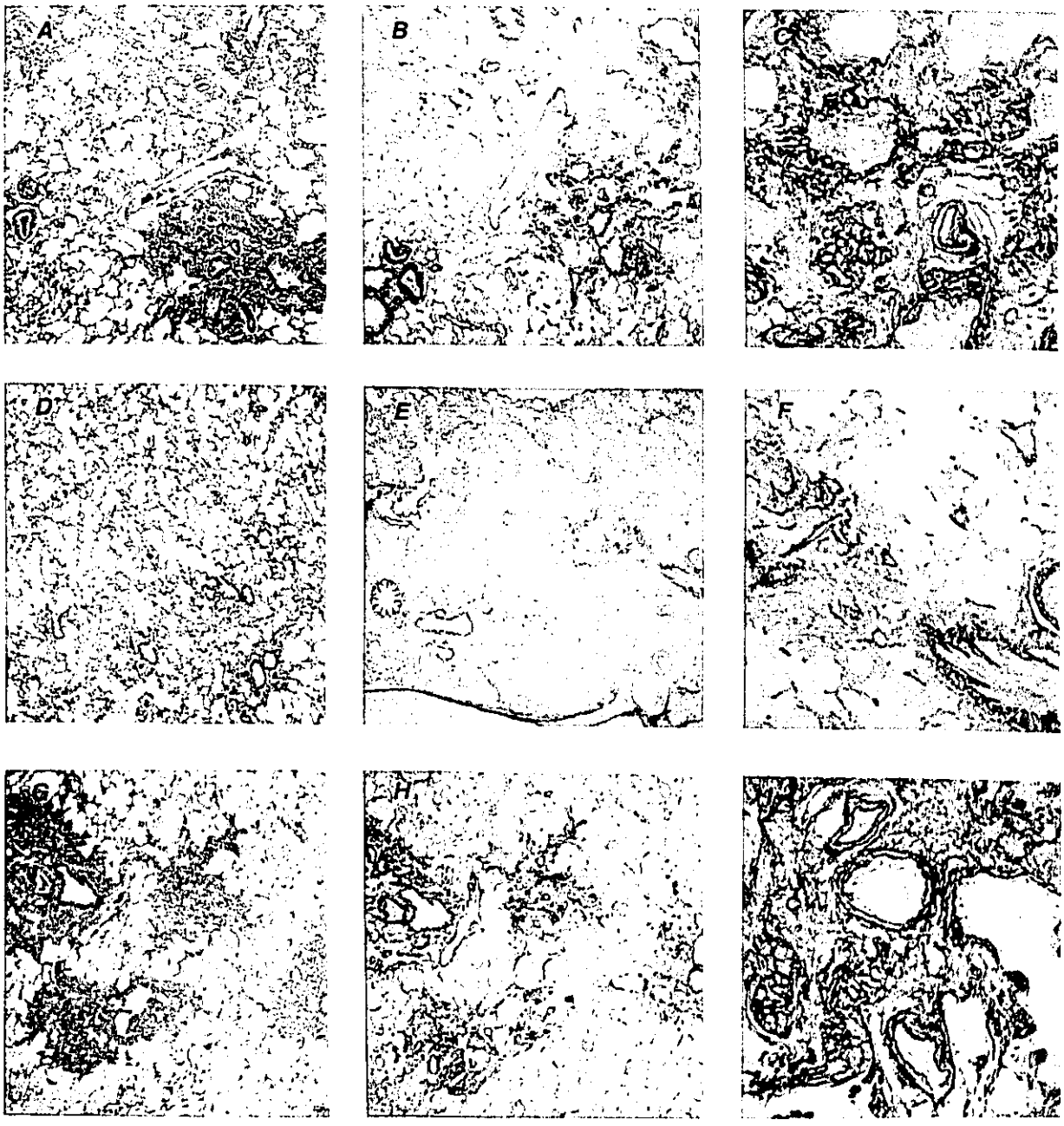
FIGURE LEGENDS

Figure 1. Histology of Lung Sections. Histological sections of rat pulmonary tissue stained with Miller's Elastic Fibre Stain. The elastic fibres and mast cell granules stain black. The connective tissues and bone stain red. Muscle, red blood cells, nuclei, cytoplasm and other tissue components stain yellow. 1(a) - (c), Bleomycin control; 1(d) - (f), Bleomycin + AtRA 10; 1(g) - (i), Bleo + AtRA 14. Figures 1(a), (d), and (g) H and E sections, magnification x 31.25 original. Figures 1(b), (e), and (h), Miller stained sections, magnification x 31.25 original. Figures 1(c), (f), and (i), Miller stained sections, magnification x 125 original.

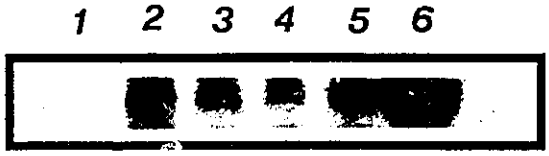
Figure 2. Northern Blot Analysis. Figure 2a. Total rat lung RNA (20μg) was fractionated by electrophoresis on 1% agarose gel, blotted to nylon membrane, and hybridized with ^{32}P-γ-ATP procollagen α_1(I) oligonucleotide. Normal rat lung RNA (Lane 1), fibrotic (Bleomycin) control (Lane 2), AtRA (Lane 3), DEX (Lane 4), AtRA/DEX (Lane 5), interleukin 1 stimulated rat lung fibroblast total RNA (Lane 6). Each lane represents pooled RNA extracted from 3 animals (except Lanes 1 and 6 which represent 1 animal). All RNA was extracted from lung tissue at Day 28 with treatments beginning at Day 10 post-bleomycin administration. Figure 2b. Quantitative analysis of the blot using a light scanning densitometer (GS Transmitter/Reflectance Scanning Densitometer,

Hoefer Scientific Instruments, San Francisco).

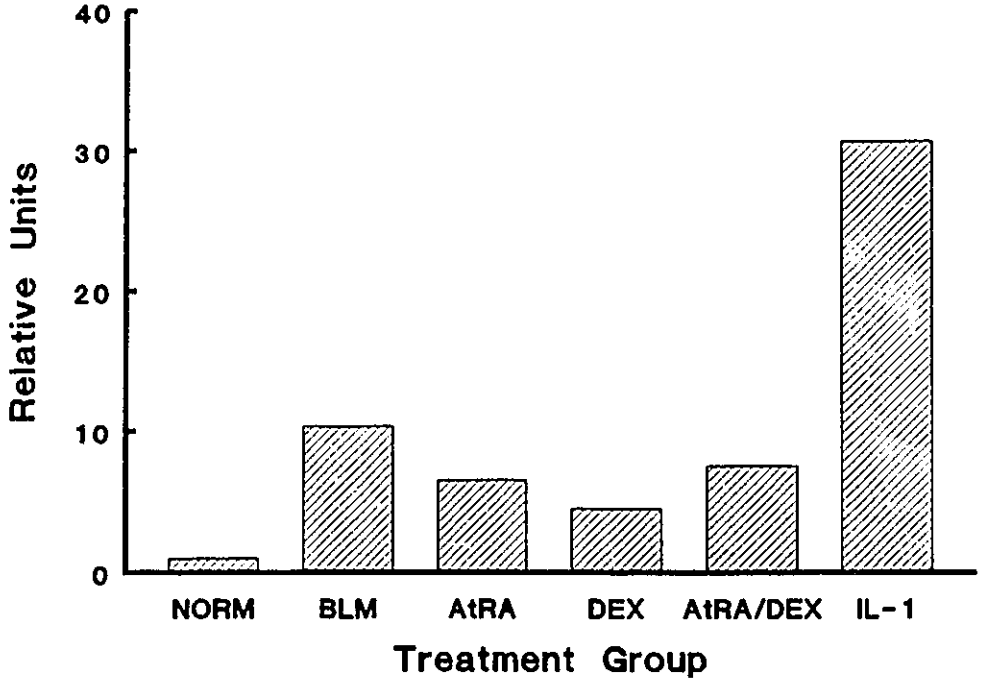
Figure 3. The effect of in vivo administration of retinoid and corticosteroid on the proliferation of rat lung fibroblasts. Primary rat lung fibroblast lines derived from either bleomycin treated rats or animals treated at 10 days and onwards with bleomycin plus AtRA (4 mg/kg rat), DEX (25 μg/250 gm rat) or a combination of AtRA/DEX were plated at a density of 2000 cells per well and maintained for 72 hours in culture media. ^{3}H-TdR was added to the system during the last 24 hours of culture. The results are expressed as the mean cpm $\pm$ SD from 8 replicate wells. Cells were used within the first 5 passages. Figure 3b represents the above data expanded to clearly depict the significant differences among bleomycin treatment groups.

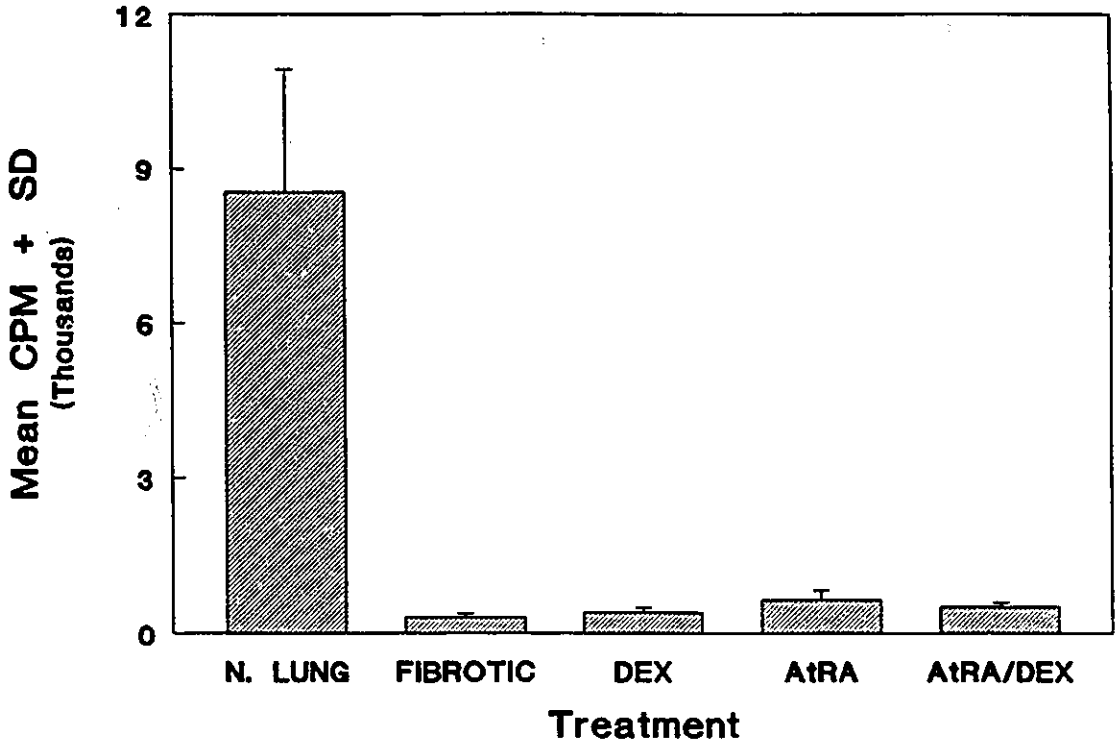

A
B
C
D
E
F
G
H

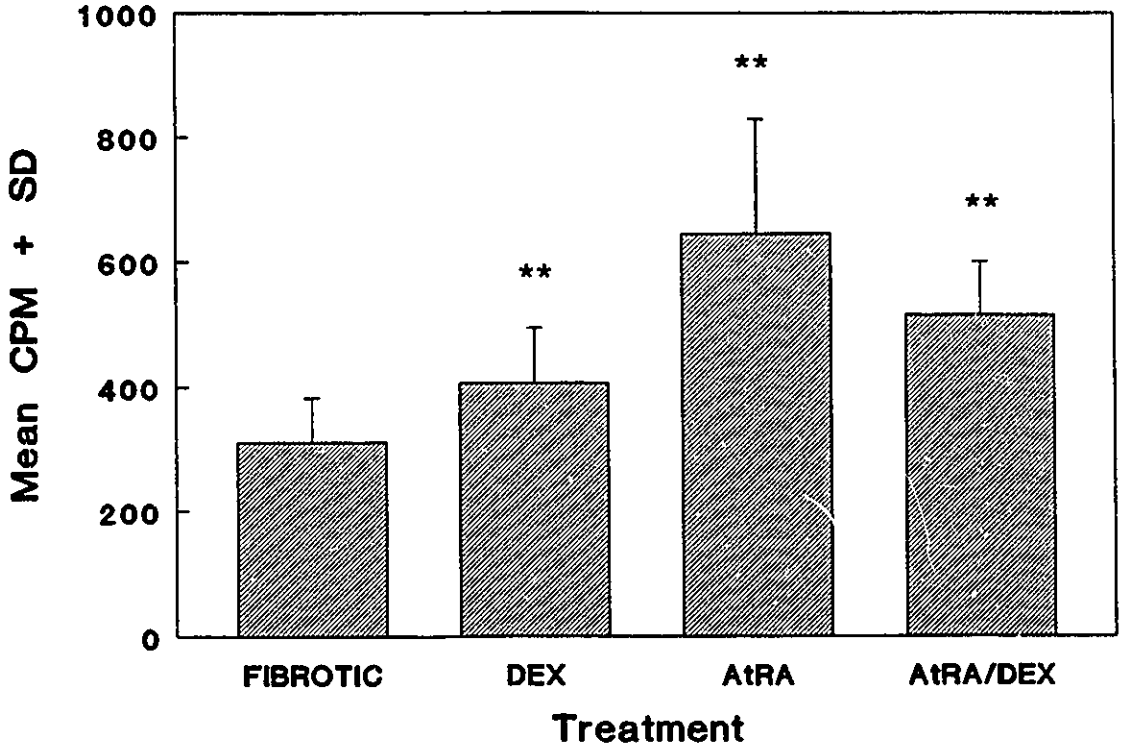
A


1 2 3 4 5 6

B


40
30
20
10
0
Relative Units
NORM
BLM
AtRA
DEX
AtRA/DEX
IL-1
Treatment Group


12
9
6
3
0
Mean CPM + SD
(Thousands)
N. LUNG
FIBROTIC
DEX
AtRA
AtRA/DEX
Treatment


1000
800
600
400
200
0
Mean CPM + SD
**
**
**
FIBROTIC
DEX
AtRA
AtRA/DEX
Treatment

** $p < 0.05$

CHAPTER Five

SUMMARY

The lung fibroblast is a differentiated mesenchymal cell responsible for the synthesis of the major ECM proteins which determine the structure and influence the function of the lung. Fibroblasts, once believed to comprise a homogeneous cell population, have more recently been demonstrated to consist of subpopulations with unique phenotypes and functions. Moreover, data from studies comparing fibroblasts from normal and diseased tissue support the idea of fibroblast subsets expressing disease specific phenotypes.

Fibroblast hyperplasia and the overproduction of ECM proteins are hallmarks of a variety of fibrotic diseases which affect various anatomical sites. To date, the molecular mechanisms underlying the pathology of fibrotic disease remain to be determined. This thesis therefore deals with investigating the hypothesis of fibroblast heterogeneity in idiopathic pulmonary fibrosis and the potential contribution of various fibroblast subsets to the pathological process in the lung.

Previous studies have focused on whether the growth characteristics of the lung fibroblast are permanently altered in lung fibrosis. Evidence to date documents that primary lines of fibroblasts derived from IPF tissue proliferate faster than normal lung fibroblasts (Jordana et al., 1988, Raghu et al., 1988) and that clones of fibroblasts established from fibrotic lines also exhibit enhanced proliferative rates (Jordana et al., 1988). Therefore, the existence

of fibroblast subpopulations in IPF tissue exhibiting accelerated growth rates would be consistent with the fibroproliferative nature of fibrotic disease. Interestingly, a series of papers by Lafyatis et al. (1989a, 1989b) have indicated that fibroblast-like cells obtained from rheumatoid synovium are capable of clonal growth in soft agarose culture. The ability to growth under conditions of anchorage independent growth is a characteristic generally associated with a transformed phenotype suggesting an aggressive nature to these cells. Lafyatis et al. (1989) have called these cells "transformed" because of this finding.

In the preceeding chapters, a series of investigations were performed to further examine growth heterogeneity among fibroblast populations derived from healthy and diseased lung tissue utilizing a colony forming assay and to determine whether these phenotypic activities could be modified by growth and differentiating agents. Having shown that agents such as retinoic acid and steroid could elicit phenotype modulation, a rat model of bleomycin induced pulmonary fibrosis was used to study the effect of these agents on the modulation of the fibrotic lung response in vivo.

In the first study, the data demonstrate that fibroblasts derived from IPF tissue are capable of anchorage-independent growth in soft agarose cultures, whereas fibroblasts derived from normal lung do not exhibit this ability. These findings provide further evidence of heterogeneity among fibroblast populations

and between fibroblasts derived from normal and fibrotic tissue. Moreover, these data support the hypothesis that fibroblasts derived from fibrotic tissue exhibit a more "aggressive" growth potential than those from normal tissue, as suggested previously by studies of Jordana et al. (1988) and Raghu et al. (1988). The characteristic of anchorage-independent growth displayed by IPF fibroblasts was maintained in vitro over a number of passages and colonies of fibroblasts that were picked and expanded again formed colonies when subjected to soft agarose culture, thus defining a stable phenotype. The maintenance of an anchorage-independent phenotype by fibroblast clones in vitro suggests that the "growth aggressive" phenotype is a permanent aspect of the primary fibroblast line and may ultimately be indicative of the various behaviours of fibroblast subsets in vivo.

The data from these studies further demonstrate that the characteristic of anchorage-independent growth exhibited by fibrotic fibroblasts is also observed in fibroblast lines derived from neonatal lung tissue suggesting the presence and contribution of a newly differentiated young fibroblast in the pathogenesis of IPF. The implication of these findings is that the development of fibrosis may involve a differentiating event which results in the over-accumulation of young fibroblast populations in the lung expressing behaviours in keeping with a growing and developing lung. The presence of such a population of cells in the fibrotic lung would not be at all surprising when one

considers the requirement of actively dividing fibroblasts during episodes of injury and repair. The ability of neonatal and fibrotic lung fibroblasts to exhibit similar proliferative behaviours has also been evidenced by Jordana et al., (1992). These studies revealed the enhanced proliferative behaviours of fibroblasts derived from chronically inflamed lung and neonatal lung as compared to normal adult lung fibroblasts. It is conceivable then that the fibrotic process might involve (1) the inability of the tissue to regulate the behaviour of the newly differentiated fibroblast or (2) the inability to change its behaviour to one more in keeping with the adult phenotype (ie one incapable of anchorage-independent growth).

That the resolution of IPF might involve the modification of the differentiation state of the fibrotic fibroblast was the axiom behind the second set of investigations undertaken in this thesis. Here, we considered the behaviour of anchorage-independent growth exhibited by IPF fibroblasts to be a behaviour consistent with an IPF phenotype. This is an assumption undoubtedly, however if true, agents that could modify this behaviour may ultimately play a role in reversing or arresting the fibrotic outcome in vivo. In this study, a number of growth and differentiating factors were examined for their capacity to modify the anchorage-independent phenotype of lung fibroblast populations. Consistent with our hypothesis, the treatment of IPF fibroblasts with one particular from of retinoic acid, all trans-retinoic acid

(AtRA), inhibited the ability of the fibroblast to form colonies under soft agarose growth conditions. In addition, retinoid treatment of neonatal lung fibroblasts similarly decreased their frequency of colony formation and growth. These findings again support the notion of an association between fibroblast behaviour in fibrosis and neonatal fibroblast behaviour and suggest the modulation of a particular fibroblast phenotype. Moreover, that the addition of growth factors known to promote the anchorage-independent growth of fibroblasts were not able to reverse the inhibitory effects of retinoid on colony formation implies a change to the initial phenotype of the cell. Normal adult lung fibroblast populations also exposed to colony enhancing growth factors and corticosteroid (under the same experimental conditions as the IPF fibroblast lines) could not be induced to form colonies indicating that the outcome observed in experiments with the IPF fibroblast lines was likely due to the selection of a specific fibroblast phenotype from a heterogenous cell population rather than simply the induction of a particular phenotype. Such a subset of fibroblasts could be selected in vivo as a result of chronic exposure to inflammatory mediators as would likely occur during repeated episodes of inflammation and repair as the disease is suspected to develop over a number of months to years.

Based on the previous two studies, we believed that the ability of IPF fibroblasts to form colonies under soft agarose growth conditions was a

consistent and disease specific phenotype. Moreover, we believed that we were able to successfully modulate this behaviour in vitro utilizing differentiation agents. Since a number of studies have reported that (1) retinoid treatment reduces collagen synthesis in fibroblast lines established from keloid or scleroderma cultures (Abergel et al., 1985, Ohta et al., 1987) and (2) retinoid therapy in vivo is effective in treatment of keloid and hypertrophic scars (Daly et al., 1986) and in inhibiting rat streptococcal cell wall induced arthritis (Haraoui et al., 1985) we assessed the effect of AtRA in a rat model of bleomycin induced lung fibrosis. The results presented from this study clearly demonstrate that the daily treatment of rats with all trans retinoic acid results in a significant decrease in the number of fibrotic lesions and in the alteration of the pattern of collagen deposition in the lung following bleomycin injection. This effect was time dependent in that retinoid treatments beginning Day 14 were not sufficient to alter the fibrotic events occurring in the lung implying that the theory of AtRA working through a differentiation pathway in vitro may not hold true in vivo unless the population of cells present at the later time point are completely differentiated and hence nonresponsive to retinoid therapy. The concomitant treatment of animals beginning Day 10/14 with retinoid and corticosteroid yielded results similar to those exhibited by steroid treated animals alone suggesting that the AtRA dependent events occuring early in the inflammatory process were steroid sensitive.

Changes in collagen gene expression were examined at the mRNA level for all groups and an apparent discrepancy between collagen distribution and procollagen mRNA levels was documented in the responsive animals treated with AtRA from day 10. That the histological demonstration of collagen and procollagen message levels did not correlate suggests that AtRA might be affecting collagen synthesis (1) at the translational level (Focht et al., 1984), (2) through the upregulation of collagen degradative enzymes or (3) indirectly through the involvement of other cell types present during the inflammatory process. It should be noted however that that the collagen content/distribution of the lung was determined via histological assessment. The relative synthesis of collagen by lung fibroblasts as measured via radioactive hydroxyproline incorporation relative to free intracellular radioactive proline as suggested by Oikarinen et al. (1989) may have yielded a different understanding as to the actions of AtRA on collagen synthesis.

The ability of cells to grow under soft agarose growth conditions suggests an "aggressive" nature to the IPF fibroblast consistent with the proliferative aspect of fibrosing disease. However, no evidence exists linking the proliferative capacity of the fibroblast or the characteristic of anchorage-independent growth exhibited by IPF fibroblasts directly to disease progress. The findings presented from our <u>in vivo</u> investigations imply that events occurring at or around Day 10 (during the acute inflammatory stage) but not by

Day 14 (when fibrosis is becoming established) after bleomycin administration are AtRA dependent. Although these results are encouraging they do not necessarily correlate with our in vitro data reporting retinoid modulation of colony formation in fibrotic lung fibroblasts. In addition, that the in vitro findings of inhibition of steroid induced colony growth by retinoid also did not translate to a detectable effect in vivo implies a tenuous relationship between the anchorage-independent phenotype and fibrosis.

In summary, the fibroblast is a charismatic cell type, acting as a target for mediators released by inflammatory-immune cells and as a potent effector cell itself. The notion of fibroblast heterogeneity and its investigation is of great interest in fibrotic disease owing to the potential contribution of various subsets to disease progression. By understanding the role of various fibroblast subpopulations in disease, the potential manipulation of fibroblasts subsets expressing distinct functions could prove therapeutic.

While there exists a large body of data suggesting the role of fibroblast subsets in IPF and fibrotic disease, a number of questions still remain. Foremost, how the lung becomes populated with actively proliferating fibroblasts remains to be determined as does the source or stem cell of the proliferating fibroblast itself. Secondly, whether their prevalence is a selection or differentiating event is unknown. Finally, the effectiveness of retinoid or similar drugs in vivo to modulate the fibrotic lung response and alter the

function of the lung warrants further investigation.

REFERENCES

Abergel RP, Meeker CA, Oikarinen H, Oikarinen AI, and Uitto J. 1985. Retinoid modulation of connective tissue metabolism in keloid fibroblast cultures. Arch Dermatol 121:632-635.

Absher M, Hildebran J, Trombley L, Woodcock-Mitchell J, and Marsh J. 1984. Characteristics of cultured lung fibroblasts from bleomycin-treated rats: Comparisons with in vitro exposed normal fibroblasts. Am Rev Respir Dis 129:125-129.

Adams SL. 1989. Collagen gene expression. Am J Respir Cell Mol Biol 1:161-168.

Adler KB, Craighead JE, Vallyathan NV, and Evans JN. 1981. Actin-containing cells in human pulmonary fibrosis. Am J Pathol 102:427-437.

Azzarone B, and Macieira-Coelho A. 1982. Heterogeneity of the kinetics of proliferation within human skin fibroblastic cell populations. J Cell Sci 57:177-187.

Bachwich PR, Chensue SW, Larrick JW, and Kunkel SL. 1986. Tumor necrosis factor stimulates interleukin-1 and prostaglandin E_2 production in resting macrophages. Biochem Biophys Res Commun 136:94-101.

Basset F, Ferrans VJ, Soler P, Takemura T, Fukuda Y, and Crystal RG. 1986. Intraluminal fibrosis in interstitial lung disorders. Am J Pathol 122:443-461.

Bauer EA, Cooper TW, Huang JS, Altman J, and Deuel TF. 1985. Stimulation of in vitro human skin collagenase expression by platelet-derived growth factor. Proc Natl Acad Sci 82:4132-4136.

Berk JL, Fine A, and Goldstein RH. 1992. Pulmonary lipid interstitial cells. In: RP Phipps (Ed.). Pulmonary Fibroblast Heterogeneity. CRC Press, Boca Raton, Inc., FL. pp. 55-77.

Beutler B, and Cerami A. 1988. The history, properties, and biological effects of cachectin. Biochem J 27:7575-7582.

Bienkowski RS, and Gotkin MG. 1995. Control of collagen deposition in mammalian lung. Proc Soc Exp Biol Med 209:118-140.

Bitterman PB, Rennard SI, Adelberg S and Crystal RG. 1983. Role of fibronectin as a growth factor for fibroblasts. J Cell Biol 97:1925-1932.

Bitterman PB, Rennard SI, Hunninghake GW, and Crystal RG. 1982. Human alveolar macrophage growth factor for fibroblasts: regulation and partial characterization. J Clin Invest 70:806-822.

Bordin S, Page RC, and Narayanan AS. 1984. Heterogeneity of normal human diploid fibroblasts: Isolation and characterization of one phenotype. Science 223:171-173.

Botstein GR. Sherer GK, LeRoy EC. 1982. Fibroblast selection in scleroderma: An alternative model of fibrosis. Arthritis Rheum 25:189-195.

Bradley KH, Kawanami O, Ferrans VJ, and Crystal RG. 1980. The fibroblasts of human lung alveolar structures: a differentiated cell with a major role in lung structure and function. Methods Cell Biol 21A:37-64.

Breen E, Falco VM, Absher M, and Cutroneo KR. 1990. Subpopulations of rat lung fibroblasts with different amounts of type I and type III collagen mRNAs. J Biol Chem 265:6286-6290.

Brody JS, and Kaplan NB. 1983. Proliferation of alveolar interstitial cells during postnatal lung growth: Evidence for two distinct populations of pulmonary fibroblasts. Am Rev Respir Dis 127:763-770.

Broekelmann TJ, Limper AH, Colby TV, and McDonald JA. 1991. Transforming growth factor β1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. Proc Natl Acad Sci 88:6642-6646.

Caniggia I, Tanswell K, and Post M. 1992. Temporal and spatial differences in glycosaminoglycan synthesis by fetal lung fibroblasts. Exp Cell Res 202:252-258.

Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, and Williamson B. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci 72:3666-3670.

Castor CW, Prince RK, and Dorstewitz EL. 1962. Characteristics of Human "Fibroblasts" Cultivated in Vitro from Different Anatomical Sites. Lab Invest 11:703-713.

Chamley-Campbell J, Campbell GR, and Ross R. 1979. The smooth muscle cell in culture. Physiol Rev 59:1-61.

Chen B, Polunovsky V, White J, Blazar B, Nakhleh R, Jessurun J, Peterson M, and Bitterman P. 1992. Mesenchymal cells isolated after acute lung injury manifest an enhanced proliferative phenotype. J Clin Invest 90:1778-1785.

Clark JG, Madtes DK, and Raghu G. 1993. Effects of platelet-derived growth factor isoforms on human lung fibroblast proliferation and procollagen gene expression. Exp Lung Res 19:327-344.

Clark JG, Kuhn C, McDonald JA, and Mecham RP. 1983. Lung Connective tissue. Int Rev Conn Tiss Res 10:249-331.

Clemmons DR and Van Wyk JJ. 1985. Evidence for a functional role of endogenously produced somatomedinlike peptides in the regulation of DNA synthesis in cultured human fibroblasts and porcine smooth muscle cells. J Clin Invest 75:1914-1918.

Clemmons DR. 1983. Age-dependent production of a competent factor by human fibroblasts. J Cell Physiol 114:61-67.

Conrad GW, Hart GW, and Chen Y. 1977. Differences in vitro between fibroblast-like cells from cornea, heart, and skin of embryonic chicks. J Cell Sci 26:119-137.

Crystal RG. 1974. Lung collagen: definition, diversity and development. Federation Proc 33:2248-2255.

Crystal RG, Fulmer JD, Baum BJ et al. 1978. Cells, collagen and idiopathic pulmonary fibrosis. Lung 155:199-224.

Crystal RG, Bitterman PB, Rennard SI, Hance AJ, and Keogh BA. 1984. Interstitial lung diseases of unknown cause. Disorders characterized by chronic inflammation of the lower respiratory tract. N Engl J Med 310:154-166.

Crystal RG, Fulmer JD, Roberts WC et al 1976. Idiopathic pulmonary fibrosis: clinical, histological, radiographic, physiologic, scintigraphic, cytologic and biochemical aspects. Ann Intern Med 85:769-788.

D'Amore PA. 1990. Modes of FGF release in vivo and in vitro. Cancer Metastasis Rev 9:227-238.

Daly TJ, and Weston WL. 1986. Retinoid effects on fibroblast proliferation and collagen synthesis in vitro and on fibrotic disease in vivo. J Am Acad Dermatol 15:900-902.

Darby G, Skalli O, and Gabbiani G. 1990. α-smooth muscle cell actin is transiently expressed by myofibroblasts during experimental wound healing. Lab Invest 63:21-29.

Daughaday WH. 1989. Fetal lung fibroblasts secrete and respond to insulin-like growth factors. Am J Respir Cell Mol Biol 1:11-12.

Dayer JM, Beutler B, and Cerami A. 1985. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E_2 production by human synovial cells and dermal fibroblasts. J Exp Med 162:2163-2168.

De Larco JE, and Todaro GJ. 1978. Growth factors from murine sarcoma virus-transformed cells. Proc Natl Acad Sci 75:4001-4005.

Derdak S, Penney DP, Keng P, Felch ME, Brown D, and Phipps RP. 1992. Differential collagen and fibronectin production by Thy 1+ and Thy 1- lung fibroblast subpopulations. Am J Physiol 263:L283-L290.

Deuel TF, and Huang JS. 1984. Platelet-derived growth factor: Structure, function, and roles in normal and transformed cells. J Clin Invest 74:669-676.

Dinarello CA. Interleukin-1 and its biologically related cytokines. 1989. Adv Immunol 44:153-205.

Duncan MR, and Berman B. 1989. Differential regulation of collagen, glycosaminoglycan, fibronectin, and collagenase activity production in cultured human adult dermal fibroblasts by interleukin 1-alpha and beta and tumor necrosis factor-alpha and beta. J Invest Derm 92:699-706.

Edwards DR, Murphy G, Reynolds JJ, Whitham SE, Docherty AJP, Angel P and Heath JK. 1987. Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. EMBO J 6:1899-1904.

Elias JA, Rossman MD, and Phillips PD. 1987. Phenotypic variability among-density-fractionated human lung fibroblasts. Am Rev Respir Dis 135:57-61.

Elias JA, Gustilo K, Baeder W, and Freundlich B. 1987b. Syngergistic stimulation of fibroblast prostaglandin production by recombinant interleukin 1 and tumor necrosis factor. J Immunol 138:3812-3816.

Elias JA, Krol RC et al. 1988. Regulation of human lung fibroblast glycosaminoglycan production by recombinant interferon, tumor necrosis factor and lymphotoxin. J Clin Invest 82:325-333.

Elias JA, Reynolds MM, Kotloff RM, and Kern JA. 1989. Fibroblast interleukin 1 beta: synergistic stimulation by recombinant interleukin 1 and tumor necrosis factor and posttranscriptional regulation. Proc Natl Acad Sci 86:6171-6175.

Fabisiak JP, Absher M, Evans JN, and Kelley J. 1992. Spontaneous production of PDGF A-chain homodimer by rat lung fibroblasts in vitro. Am J Physiol 263:L185-L193.

Focht RJ, and Adams SL. 1984. Tissue specificity of type I collagen gene expression is determined at both transcriptional and post-transcriptional levels. Mol. Cell. Biol. 4:1843-1852.

Froncek MJ, Derdak S, Felch ME et al. 1992. Cellular and molecular characterization of Thy-1 + and Thy-1- murine lung fibroblasts. In: RP Phipps (Ed.). Pulmonary Fibroblast Heterogeneity. CRC Press, Boca Raton Inc., FL pp 137-198.

Fulmer JD, Bienkowski RS, Cowan MJ, Breul SD, Bradley KM, Ferratis VJ, Roberts WC, and Crystal RG. 1980. Collagen concentration and rates of synthesis in idiopathic pulmonary fibrosis. Am Rev Respir Dis 122:289-301.

Gabbiani G, Ryan GB, and Majno G. 1971. Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. Experientia 27:549-550.

Goldring MB, and SM Krane. 1987. Modulation by recombinant interleukin 1 of synthesis of types I and III collagens and associated procollagen mRNA levels in cultured human cells. J Biol Chem 262:16724-16729.

Goldring SR, Stephenson ML, Downie E, Krane SM, and Korn JH. 1990. Heterogeneity in hormone responses and patterns of collagen synthesis in cloned dermal fibroblasts. J. Clin Invest 85:798-803.

Goldring MB, and Krane SM. 1987. Modulation by recombinant interleukin 1 of synthesis of types I and III collagens and associated procollagen mRNA levels in cultured human cells. J Biol Chem 262:16724-16729.

Goldstein RH, Poliks CF, Pilch PF, Smith BD, and Fine A. 1989. Stimulation of collagen formation by insulin and insulin-like growth factor I in cultures of human lung fibroblasts. Endocrinol 124:964-970.

Grotendorst G, Seppa HEJ, Kleinman HK, and Martin G. 1982. Attachment of smooth muscle cells and their migration toward platelet-drived growth factor. Proc Natl Acad Sci 78:3669-3672.

Hance AJ and Crystal RG. 1975. The connective tissue of lung. Am Rev Respir Dis 112:657-709.

Hance AJ, Bradley K, and Crystal RG. 1976. Lung Collagen Heterogeneity. Synthesis of type I and type III collagen by rabbit and human lung cells in culture. J Clin Invest 57;102-111.

Haraoui B, Wilder RL, Allen JB, Sporn MB, Helfgott RK, and Brinckerhoff CE. 1985. Dose-dependent suppression by the synthetic retinoid, 4-hydroxyphenyl retinamide, of streptococcal cell wall-induced arthritis in rats. Int J Immunopharmacol 7:903-916.

Harper RA, and Grove G. 1979. Human skin fibroblasts derived from papillary and reticular dermis: Differences in growth potential in vitro. Science 204:526-527.

Harrison NK, Cambrey AD, Myers AR, Southcott AM, Black CM, Du Bois RM, Laurent GJ, and McAnulty RJ. 1994. Insulin-like growth factor-I is partially responsible for fibroblast proliferation induced by bronchoalveolar lavage fluid from patients with systemic sclerosis. Clinical Science 86:141-148.

Harrison NK, Argent AC, McAnulty RJ, Black CM, Corrin B, and Laurent GJ. 1991. Collagen synthesis and degradation by systemic sclerosis lung fibroblasts. Responses to transforming growth factor-beta. Chest 99:71S-72S.

Hassell TM, and Stanek EJ III. 1983. Evidence that healthy gingiva contains functionally heterogeneous fibroblast subpopulations. Arch Oral Biol 28:617-625.

Hayflick L, and Moorhead PS. 1961. The serial cultivation of human diploid cell strains. Exp Cell Res 25:585-621.

Heldin CH, Backstrom G, Ostman A, Hammacher A, Ronnstrand L, Rubin K, Nister M, and Westermark B. 1988. Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. EMBO J 7:1387-1393.

Hill DJ, Strain AJ, Elstow SF, Swenne I, and RDG Milner. 1986. Bi-functional action of transforming growth factor-beta on DNA synthesis in early passage human fetal fibroblasts. J Cell Physiol 128:322-328.

Hoyt DG, and Lazo JS. 1988. Alterations in pulmonary mRNA encoding procollagens, fibronectin and transforming growth factor-β precede bleomycin-induced pulmonary fibrosis in mice. J Pharmacol Exp Ther 246:765-771.

Ignotz RA, Endo R, and Massague J. 1987. Regulation of fibronectin and type I collagen mRNA levels by transforming growth factor-β. J Biol Chem 262:6443-6446.

Ignotz RA, and Massague J. 1986. Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem 261:4337-4345.

Jones RB, and Smith JR. 1982. A stochastic model of cellular senescence. II. Concordance with experimental data. J Theor Biol 96:443-460.

Jordana M, Schulman J, McSharry C, Irving LB, Newhouse MT, Jordana G, and Gauldie J. 1988. Heterogeneous proliferative characteristics of human adult lung fibroblast lines and clonally derived fibroblasts from control and fibrotic tissue. Am Rev Resp Dis 137:579-584.

Jordana M, Kirpalani H, and Gauldie J. 1992. Heterogeneity of human lung fibroblast proliferation in relation to disease expression. In: RP Phipps (Ed). Pulmonary Fibroblast Heterogeneity. CRC Press, Boca Raton, Inc., FL. pp. 229-249.

Juliano RL and Haskill S. 1993. Signal transduction from the extracellular matrix. J Cell Biol 120:577-585.

Kahari VM, Multimaki P, and Vuorio E. 1987. Elevated proα2(I) collagen mRNA levels in cultured scleroderma fibroblasts result from an increased transcription rate of the corresponding gene. FEBS Letts 215:331-334.

Kapanci Y, Assimacopoulos A, Irle C, Zwahlen A, and Gabbiani G. 1974. "Contractile Interstitial Cells" in pulmonary alveolar septa: a possible regulator of ventilation/perfusion ratio? Ultrastructural, Immunofluorescence, and In Vitro Studies. J Cell Biol 60:375-392.

Kaplan NB, Grant MM, and Brody JS. 1985. The lipid interstitial cell of the pulmonary alveolus: Age and species differences. Am Rev Respir Dis 132:1307-1312.

Katzenstein Al. 1985. Pathogenesis of "fibrosis" in interstitial pneumonia: an electron microscopic study. Hum Pathol 16:1015-1024.

Karlinsky JB. 1982. Glycosaminoglycans in emphysematous and fibrotic hamster lungs. Am Rev Respir Dis 125:85-88.

Kelley J. 1990. Cytokines of the lung. Am Rev Respir Dis 141:765-788.

Kelley J. Transforming growth factor-β. 1992. In: J Kelley (Ed.). Cytokine of the lung. New York, New York. Marcel Dekker, pp 101-137.

Khalil N, Bereznay O, Sporn M, and Greenberg AH. 1989. Macrophage production of transforming growth factor β and fibroblast collagen synthesis in chronic pulmonary inflammation. J Exp Med 170:727-737.

Khalil N, O'Connor RN, Unruh HW, Warren PW, Flanders KC, Kemp A, Bereznay OH, and Greenberg AH. 1991. Increased production and immunohistochemical localization of transforming growth factor-β in idiopathic pulmonary fibrosis. Am J Respir Cell Mol Biol 5:155-162.

Kirk JME, Heard BE, Kerr I et al., 1984. Quantification of type I and III collagen in biopsy lung samples from patients with cryptogenic fibrosing alveolitis. Coll Rel Res 4:169-182.

Kirkwood TBL, and Holliday R. 1975. Commitment to senescence: A model for the finite and infinite growth of diploid and transformed human fibroblasts in culture. J Theor Biol 53:481-496.

Ko SD, Page RC, and Narayanan AS. 1977. Fibroblast heterogeneity and prostaglandin regulation of subpopulations. Proc Natl Acad Sci 74:3429-3432.

Kondo H, Kasuga H, and Noumura T. 1985. The heterogeneity of human fibroblasts as determined from the effects of hydrocortisone on cell growth and specific dexamethasone binding. Exp Cell Res 158:342-348.

Korn JH. 1985. Substrain heterogeneity in prostaglandin E_2 synthesis of human dermal fibroblasts: Differences in prostaglandin E_2 synthetic capacity of substrains are not stimulus-restricted. Arthritis Rheum 28:315-322.

Korn JH, Torres D, and Downie E. 1983. Fibroblast prostaglandin E_2 synthesis: Persistence of an abnormal phenotype after short-term exposure to mononuclear cell products. J. Clin Invest. 71:1240-1246.

Korn JH, Torres D, and Downie E. 1984. Clonal heterogeneity in the fibroblast response to mononuclear cell derived mediators. Arthritis Rheum 27:174-179.

Krane SM, Dayer JM, Simon LS, and Byrne MS. 1985. Mononuclear cell-conditioned medium containing mononuclear cell factor (MCF), homologous with interleukin 1, stimulates collagen and fibronectin synthesis by adherent rheumatoid synovial cells: effects of prostaglandin E2 and indomethacin. Coll Rel Res 5:99-117.

Kuhn C, and McDonald JA. 1991. The roles of the myofibroblast in idiopathic pulmonary fibrosis. Ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis. Am J Pathol 138:1257-1265.

Kuhn C, Boldt J, King TE, Crouch E, Vartio T, and McDonald JA. 1989. An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. Am Rev Respir Dis 140:1693-1703.

Lafyatis R, Remmers EF, Roberts AB, Yocum DE, Sporn MB, and Wilder RL. 1989. Anchorage-independent growth of synoviocytes from arthritic and normal joints. Stimulation by exogenous platelet-derived growth factor and inhibition by transforming growth factor-beta and retinoids. J Clin Invest 83:1267-1276..

Lafyatis R, Thompson NL, Remmers EF, Flanders KC, Roche NS, Kim SJ et al. 1989. Transforming growth factor-beta production by synovial tissues from rheumatoid patients and streptococcal cell wall arthritic rats. Studies on secretion by synovial fibroblast-like cells and immunohistologic localization. J Immunol 143:1142-1148.

Laurent GJ, McAnulty RJ. 1983. Protein metabolism during bleomycin-induced pulmonary fibrosis in rabbits. In vivo evidence for collagen accumulation because of increased synthesis and decreased degradation of the newly synthesized collagen. Am Rev Respir Dis 128:82-88.

Le J, and Vilcek J. 1987. Tumor necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. Lab Invest 56:234-248.

LeRoy EC. 1972. Connective tissue synthesis by scleroderma skin fibroblasts in cell culture. J Exp Med 135:1351-1362.

Lobb R, Sasse J, Sullivan R, Shing Y, D'Amore P, Jacobs J, and Klagsbrun M. 1986. Purification and characterization of heparin-binding endothelial cell growth factors. J Biol Chem 261:1924-1928.

Madri JA, and Furthmayr H. 1980. Collagen polymorphism in the lung. An immunohistochemical study of pulmonary fibrosis. Hum Pathol 1980 11:353-366.

Majno G, Gabbiani G, Hirschel BJ, Ryan GB, and Statkov PR. 1971. Contraction of granulation tissue in vitro: similarity to smooth muscle. Science 173:548-550.

Maksvytis HJ, Vaccaro C, and Brody JS. 1981. Isolation and characterization of the lipid-containing interstitial cell from the developing rat lung. Lab Invest 45:248-259.

Maksvytis HJ, Niles RM, Simanovsky L, Minassian IA, Richardson LL, Hamosh M, Hamosh P, and Brody JS. 1984. In vitro characteristics of the lipid-filled interstitial cell associated with postnatal lung growth: Evidence for fibroblast heterogeneity. J Cell Physiol 118:113-123.

Martin GM, Sprague CA, Norwood TH, and Pendergrass WR. 1974. Clonal selection, attenuation and differentiation in an in vitro model of hyperplasia. Am J Pathol 74:137-154.

Martinet Y, Rom WN, Grotendorst GR, Martin GR, and Crystal RG. 1987. Exaggerated spontaneous release of platelet-derived growth factor by alveolar macrophages from patients with idiopathic pulmonary fibrosis. N Engl J Med 317:202-209.

Matrisian LM. 1994. Matrix metalloproteinase gene expression. Ann NY Acad Sci 732:42-50.

Matsuura H and Hakomori S. 1982. The oncofetal domain of fibronectin defined by monoclonal antibody FDC-6: its presence in fibronectins from fetal and tumor tissues and its absence in those from normal adult tissues and plasma. Proc Natl Acad Sci 83:6517-6521.

Mauviel A, Daireaux M, Redini F, Galera p, Loyau G, and Pujol JP. 1988. Tumor necrosis factor inhibits collagen and fibronectin synthesis in human dermal fibroblasts. FEBS Lett 236:47-52.

Maxwell DB, Grotendorst CA, Grotendorst GR, and LeRoy EC. 1987. Fibroblast heterogeneity in Scleroderma: C1q studies. J Rheumatol 14:756-759.

McCartney-Francis N, Mizel D, Wong H, Wahl L, and Wahl S. 1990. TGF-beta regulates production of growth factors and TGF-beta by human peripheral blood monocytes. Growth Factors 4:27-35.

McCulloch CAG, and Knowles GC. 1993. Deficiencies in collagen phagocytosis by human fibroblasts in vitro: a mechanism for fibrosis? J Cell Physiol 155:461-471.

McDonald JA, Broekelmann TJ, Matheke ML, Crouch E, Koo M, and Kuhn C III. 1986. A monoclonal antibody to the carboxyterminal domain of procollagen type 1 -visualizes collagen-synthesizing fibroblasts. Detection of an altered fibroblast phenotype in lungs of patients with pulmonary fibrosis. J Clin Invest 78:1237-1244.

Mio T, Nagai S, Kitaichi M, Kawatani A, and Izumi T. 1992. Proliferative characteristics of fibroblast lines derived from open lung biopsy specimens of patients with IPF (UIP). Chest 102:832-837.

Mitsui Y, and Schneider EL. 1976. Characterization of fractionated human diploid fibroblast cell populations. Exp Cell Res 103:23-30.

Mollenhauser J, and Bayreuther K. 1986. Donor-age-related changes in the morphology, growth potential, and collagen biosynthesis in rat fibroblast subpopulations in vitro. Differentiation 32:165-172.

Nagaoka I, Trapnell BC, and Crystal RG. 1990. Upregulation of platelet-derived growth factor-A and -B gene expression in alveolar macrophages of individuals with idiopathic pulmonary fibrosis. J Clin Invest 85:2023-2027.

Nakano S and Ts'O PO. 1981. Cellular differentiation and neoplasia: characterization of subpopulations of cells that have neoplasia-related growth properties in Syrian hamster embryo cell cultures. Proc Natl Acad Sci 78:4995-4999.

Nathan C, and Sporn M. 1991. Cytokines in context. J Cell Bio 113:981-986.

Nawroth PP, Bank I, Handley D, Cassimeris J, Chess L, and Stern D. 1986. Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. J Exp Med 163:1363-1375.

Ndumbe PM, and Levinsky RJ. 1985. Differences in surface antigen expression of human fibroblasts cultured from bone marrow compared with those obtained from skin and embryo lung tissue. Immunology 56:189-194.

Ohta A. and Uitto J. 1987. Procollagen gene expression by scleroderma fibroblasts in culture. Inhibition of collagen production and reduction of proα1(I) and proα1(III) collagen messenger RNA steady-state levels by retinoids. Arthritis Rheum 30:404-411.

Oikarinen A, Vuorio E, and Vuorio T. 1989. Comparison of the effects of dexamethasone and 13-cis retinoic acid on connective tissue biosynthesis in human skin fibroblasts. Arch Dermatol Res 281:273-278.

Overall CM, Wrang JL, and Sodek J. 1989. Independent regulation of collagenase, 72 kDa progelatinase and metalloproteinase inhibitor expression in human fibroblasts by transforming growth factor-beta. J Biol Chem 264:1860-1869.

Phan SH, Varani J, and Smith D. 1985. Rat lung fibroblast collagen metabolism in bleomycin-induced pulmonary fibrosis. J Clin Invest 76:241-247.

Phipps RP, Penney DP, Keng P, Quill H, Paxhia A, Derdak S, and Felch ME. 1989. Characterization of two major populations of lung fibroblasts: Distinguishing morphology and discordant display of Thy 1 and Class II MHC. Am J Respir Cell Mol Biol 1:65-74.

Piguet PF, Grau GE, and Vassalli P. 1990. Subcutaneous perfusion of tumor necrosis factor induces local proliferation of fibroblasts, capillaries, and epidermal cells, or massive tissue necrosis. Am J Pathol 136:103-110.

Plisko A, and Gilchrest BA. 1983. Growth factor responsiveness of cultured human fibroblasts declines with age. J Gerontol 38:513-518.

Postlethwaite AE, Keeski-Oja J, Moses HL, and Kang AH. 1987. Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor β. J Exp Med 165:251-256.

Postlethwaite AE, Lachman LB, Mainardi CL, and Kang AH. 1983. Interleukin 1 stimulation of collagenase production by cultured fibroblasts. J Exp Med 157:801-806.

Postlethwaite AE, Raghow R, Stricklin GP, Poppleton H, Seyer JM, and Kang AH. 1988. Modulation of fibroblast functions by interleukin 1: Increased steady-state accumulation of type I procollagen messenger RNAs and stimulation of other functions but not chemotaxis by human recombinant interleukin 1α and β. J Cell Biol 106:311-318.

Postlethwaite AE, Keski-Oja J, Balian G, and Kang AH. 1981. Induction of fibroblast chemotaxis by fibronectin. Localization of the chemotactic region to a 140,000 molecular weight non-gelatin-binding fragment. J Exp Med 153:494-499.

Raghu G, Chen Y, Rusch V, and Rabinovitch PS. 1988. Differential proliferation of fibroblasts cultured from normal and fibrotic human lungs. Am Rev Respir Dis 138:703-708.

Raghu G, and Kavanagh TJ. 1991. The human lung fibroblast: a multifaceted target and effector cell. In: MS Lama and R Barrios (Eds). Interstitial Pulmonary Diseases: Selected Topics. CRC Press, Boca Raton, FL. pp 1-34.

Raghu G. 1987. Idiopathic Pulmonary Fibrosis. A rational Clinical Approach. Chest 92:148-154.

Raghu G, Masta S, Meyers D, and Narayanan AS. 1989. Collagen synthesis by normal and fibrotic human lung fibroblasts and the effect of transforming growth factor-β. Am Rev Respir Dis 140:95-100.

Raines EW, Dower SK, and Ross R. 1989. Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells is due to PDGF-AA. Science 243:393-396.

Rennard SI, and Ferrans VJ et al. 1982. Lung Connective Tissue. In: H. Witschi (Ed.). Mechanisms in Respiratory Toxicology. CRC Press. Boca Raton, FL. pp. 115-153.

Rennard SI, Hunninghake GW, Bitterman PB, and Crystal RG. 1981. Production of fibronectin by the human alveolar macrophage: mechanism for the recruitment of fibroblasts to sites of tissue injury in interstitial lung diseases. Proc Natl Acad Sci 78:7147-7151.

Rennard SI, and Crystal. 1981. Fibronectin in human bronchopulmonary lavage fluid. Elevation in patients with interstitial lung disease. J Clin Invest 69:113-122.

Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, and Fauci AS. 1986. Transforming growth factor β: Rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci 83:4167-4171.

Rodemann HP, and Muller GA. 1990. Abnormal growth and clonal proliferation of fibroblasts derived from kidneys with interstitial fibrosis. Proc Exp Biol Med 195:57-63.

Roche WR. 1995. Fibroblasts and extracellular matrix in bronchial asthma. In: WW Busse and SI Holgate (Eds.). Asthma and Rhinitis. Blackwell Scientific, Boston, pp 554-562.

Rolfe MW, Kunkel SL, Standiford TJ et al., 1991. Pulmonary fibroblast expression of interleukin-8: a model for alveolar macrophage-derived cytokine networking

Rom WN, Basset P, Fells GA, Nukiwa T, Trapnell BC, and RG Crystal. 1988. Alveolar macrophages releasse an insulin-like growth factor I-type molecule. J Clin Invest 82:1685-1693.

Romaris M, Heredia A, Molist A, and Bassols A. 1991. Differential effect of transforming growth factor beta on proteoglycan synthesis in human embryonic lung fibroblasts. Biochim Biophys Acta 1093:229-233.

Ross R, Glomset J, Kariya B, and Harker L. 1974. A platelet-derived serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. Proc Natl Acad Sci 71:1207-1210.

Ryan GB, Cliff WJ, Gabbiani G, Irle C, Montandon D, Statkov PR, and Majno G. 1974. Myofibroblasts in human granulation tissue. Hum Pathol 5:55-67.

Schmidt JA, Mizel SB, Cohen D, and Green IRA. 1982. Interleukin 1, a potential regulator of fibroblast proliferation. J Immunol 128:2177-2182.

Schmitt-Graff A, Desmouliere A, and Gabbiani G. 1994. Heterogeneity of myofibroblast phenotypic features: an example of fibroblastic cell plasticity. Virchows Archiv 425:3-24.

Schneider EL, Mitsui Y, AU S, and Shorr SS. 1977. Tissue-specific differences in cultured human diploid fibroblasts. Exp Cell Res 108:1-6.

Schor SL, Schor AM, Howell A, and Crowther D. 1987. Hypothesis: Persistent expression of fetal phenotypic characteristics by fibroblasts is associated with an increased susceptibility to neoplastic disease. Exp Cell Biol 55:11-17.

Schubert D. 1992. Collaborative interactions between growth factors and the extracellular matrix. Trends Cell Bio 2:63-66.

Schwartz SM, Reidy MR, and Clowes A. 1985. Kinetics of atherosclerosis: A stem cell model. Ann NY Acad Sci 454:292-296.

Scott S, Pandolfi F, and Kurnick JT. 1990. Fibroblasts mediate T cell survival: a proposed mechanism for retention of primed T cells. J Exp Med 172:1873-1876.

Selman M and Montana M et al. 1986. Concentration, biosynthesis and degradation of collagen in idiopathic pulmonary fibrosis. Thorax 41:355-359.

Senior RM, Griffin GL, and Mecham RP. 1982. Chemotactic responses of fibroblasts to tropoelastin and elastin-derived peptides. J Clin Invest 70:614-618.

Seppa H, Grotendorst G, Seppa S, Schiffmann E, and Martin GR. 1982. Platelet-derived growth factor is chemotactic for fibroblasts. J Cell Biol 92:584-588.

Shaw RJ, Benedict SH, Clark RAF, and King TE. 1991. Pathogenesis of pulmonary fibrosis in interstitial lung disease: Alveolar macrophage PDGF(B) gene activation and up-regulation by interferon gamma. Am Rev Respir Dis 143:167-173.

Silver RM, Miller KS, Kinsella MB, Smith EA, and Schabel SI. 1990. Evaluation and management of Scleroderma lung disease using bronchoalveolar lavage. Am J Med 88:470-476.

Siminski J, Kavanagh T, and Raghu G. 1991. Subpopulations of human lung fibroblasts based on thymocyte-1 surface antigen expression. Am Rev Respir Dis 1991 143:A518.

Sims JE, March CJ, Cosman D, Widmer MB, MacDonald HR, McMahan CJ, Grubin CE, Wignall JM, Jackson JL, Call SM, Friend D, Alpert AR, Gillis S, Urdal DL, and Dower SK. 1988. cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. Science 241:585-589.

Smith TJ, Bahn RS, Gorman CA, and Cheavens M. 1991. Stimulation of glycosaminoglycan accumulation by interferon gamma in cultured human retroocular fibroblasts. J Clin Endocrinol and Metabolism 72:1169-1171.

Smith JR and Hayflick L. 1974. Variation in the life-span of clones derived from human diploid cell strains. J Cell Biol 62:48-53.

Smith TJ, Bahn RS, and Gorman CA. 1989. Hormonal regulation of hyaluronate synthesis in cultured human fibroblasts: evidence for differences between retroocular and dermal fibroblasts. J Clin Endocrinol Metab 69:1019-1023.

Sprugel KH, McPherson JM, Clowes AW, and Ross R. 1987. Effects of growth factors in vivo. I. Cell ingrowth into porous subcutaneous chambers. Am J Pathol 129:601-613.

Stiles AD, Moats-Staats BM, and Retsch-Bogart GZ. 1993. Insulin-like growth factors. In: J Kelley (Ed). Cytokines of the lung. Marcel Dekker, Inc., New York, NY 77-99.

Streiter RM, Phan SM, Showell HJ, Remick DG, Lynch JP, Genord M, Raiford C, Eskandari M, Marks RM, and Kunkel SF. 1989a. Monokine-induced neutrophil chemotactic factor gene expression in human fibroblasts. J Biol Chem 264:10621-10626.

Strieter RM, Wiggins R, Phan SH et al., 1989b. Monocyte chemotactic protein gene expression by cytokine-treated human fibroblasts and endothelial cells. Biochem Biophys Res Commun 162:694-700)

Sugarman BJ, Aggarwal BB, Hasss PE, Figari IS, Palladino MA, and Shepard BM. 1985. Recombinant human tumor necrosis factor-α: Effects on proliferation of normal and transformed cells in vitro. Science Was DC 230:943-945.

Thornton SC, Por SB, Walsh BJ, Penny R, and Breit SN. 1990. Interaction of immune and connective tissue cells: 1. The effect of lymphokines and monokines on fibroblast growth. J Leuk Biol 47:312-320.

Tremblay GM, Nonaka M, Sarnstrand B, Dolovich J, Gauldie J, and Jordana M. 1994. Myofibroblast differentiation in nasal polyposis: down regulation by topical steroids. Am J Respir Crit Care Med 149:A632

Ulich, TR. 1993. Tumor Necrosis Factor. In: J Kelley (Ed). Cytokines of the lung. Marcel Dekker, Inc., New York, NY pp. 307-332.

Uitto J, Bauer EA, and Eisen AZ. 1979. Increased biosynthesis of triple-helical type I and type III procollagens associated with unaltered expression of collagenase by skin fibroblasts in culture. J Clin Invest 64:921-930.

Vancheri C, Gauldie J, Bienenstock J, Cox G, Scicchitano R, Stanisz A, and Jordana M. 1989. Human lung fibroblast-derived granulocyte-macrophage colony stimulating factor (GM-CSF) mediates eosinophil survival in vitro. Am J Respir Cell Mol Biol 1:267-268.

van-der-Rest M, and Garrone R. 1991. Collagen family of proteins. FASEB J 5:2814-2823.

Varga J, Rosenbloom J, and Jimenez SA. 1987. Transforming growth factor β (TGFβ) causes a persistent increase in steady-state amounts of type I and type III collagen and fibronectin mRNAs in normal human dermal fibroblasts. Biochem 247:597-604.

Vignaud JM, Allam M, Martinet N, Pech M, Plenat F, and Martinet Y. 1991. Presence of platelet-derived growth factor in normal and fibrotic lung is specifically associated with interstitial macrophages, while both interstitial macrophages and alveolar epithelial cells express the c-sis proto-oncogene. Am J Respir Cell Mol Biol 5:531-538.

Vilcek J, Palombella VJ, Henriksen-DeStefano D, Swenson C, Feinman R, Hirai M, and Tsujimoto M. 1986. Fibroblast growth enhancing activity of tumor necrosis factor and its relationship to other polypeptide growth factors. J Exp Med 163:632-643.

Voccaro C, and Brody JS. 1978. Ultrastructure of developing alveoli: The role of the interstitial fibroblast. Anat Rec 192:467-480.

Wahl SM, Hunt DA, Wakefield LM, McCartney-Francis N, Wahl LM, Roberts, and MB Sporn. 1987. Transforming growth factor type β induces monocyte chemotaxis and growth factor production. Proc Natl Acad Sci 84:5788-5792.

Weissler JC. 1989. Southwestern Internal Medicine Conference: Idiopathic Pulmonary Fibrosis: Cellular and molecular pathogenesis. Am J Med Sci 297:91-104.

Wolosewick JJ, and Porter KR. 1976. Observations on the morphological heterogeneity of WI-38 cells. Am J Anat 149:197-226.

Xing Z, Jordana M, Braciak T, Ohtoshi T, and Gauldie J. 1993. Lipopolysaccharide induces expression of granulocyte/macrophage colony-stimulating factor, interleukin-8, and interleukin-6 in human nasal, but not lung, fibroblasts: Evidence for heterogeneity within the respiratory tract. Am J Respir Cell Mol Biol 9:255-263.