



Lung injury, inflammatory mediators, and fibroblast activation in fibrosing alveolitis

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Scadding¹ defined fibrosing alveolitis as a disorder of the lower respiratory tract characterised by inflammation and fibrosis of the pulmonary interstitium and peripheral airspaces. This leads to derangement of the alveolar wall and loss of functional alveolar capillary units, resulting in a restrictive pattern in lung function tests.

The term has been used to describe a large number of pulmonary diseases, including those caused by inhaled dusts, such as asbestos, or the administration of drugs such as amiodarone, gold, busulphan, and bleomycin or those associated with collagen vascular diseases, such as rheumatoid arthritis and systemic sclerosis. In three quarters of cases, however, no causative agent or associated condition can be found and these are referred to as cryptogenic fibrosing alveolitis (Turner-Warwick *et al*²) to emphasise both the inflammatory and the fibrotic components of the disease. In the United States the condition is referred to as idiopathic pulmonary fibrosis. Its prevalence in the United Kingdom is five cases per 100 000 of the population³ and mortality is increasing, 1200-1400 people dying each year from the condition.⁴

Aetiology

Many studies, particularly in animals exposed to toxic agents such as bleomycin⁵ or asbestos,⁶ support the idea that direct injury to capillary

endothelial cells, shared basement membrane, and pneumocyte type 1 epithelial cells leads to interstitial fibrosis. Furthermore, in the adult respiratory distress syndrome acute lung injury results in similar damage and survivors often develop diffuse interstitial fibrosis.⁷

Injury is believed to be due either to inhalation of injurious agents or to blood borne agents. Many consider that viral infections play a part but this has never been proved. Only one study, of 13 patients, has found serum antibodies to Epstein-Barr virus but the meaning of this remains unclear.⁸ Occupational or environmental exposure could be relevant. Recent work has suggested that exposure to metal dust and wood fires may have a role.⁹ In general, the initiating agent or agents in fibrosing alveolitis remain unknown.

Epithelial and endothelial cell injury

Most current theories on the pathogenesis of pulmonary fibrosis are based on the hypothesis that there is initial damage to endothelial or epithelial cells or both. Inflammatory and immune cells then move into the interstitium and alveolar spaces from the circulation and release mediators that stimulate collagen production by fibroblasts.¹⁰ In fibrosing alveolitis and asbestosis there is ultrastructural evidence of both epithelial and endothelial cell injury from the earliest stages of disease.⁶ The injury and loss of these cells can affect the underlying tissues.

Epithelial control of fibroblast proliferation has been seen in tracheal grafts. If a tracheal graft is denuded of epithelium the lumen is gradually obliterated with fibrous tissue but this is prevented if the graft is re-epithelialised.¹¹ Experiments using cytotoxic agents have shown that prevention of re-epithelialisation similarly leads to fibrosis but this does not occur if epithelial regeneration is permitted to take place.¹² Thus repeated damage to alveolar epithelial cells may be an important mechanism in the development of fibrosing alveolitis. Repair of epithelial damage results in proliferation of type 2 pneumocytes, which is a prominent histological feature of cryptogenic fibrosing alveolitis (fig 1). A role for pulmonary epithelial cells in immunoregulation has also been described, with epithelial cells suppressing lymphocyte clonal expansion in the alveolar space.¹³ Thus damage to epithelial cells may alter the immunological environment and result in proliferation of lymphocytes, which is a feature of fibrosing alveolitis.

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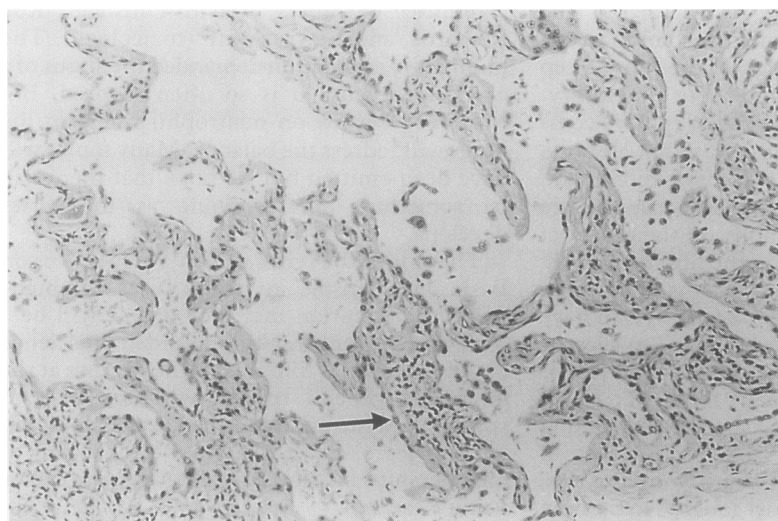


Figure 1 Open lung biopsy specimen showing interstitial thickening with fibrosis. There is a chronic inflammatory infiltrate in the interstitium. Type 2 cell hyperplasia is present (arrow). (Haematoxylin and eosin.)

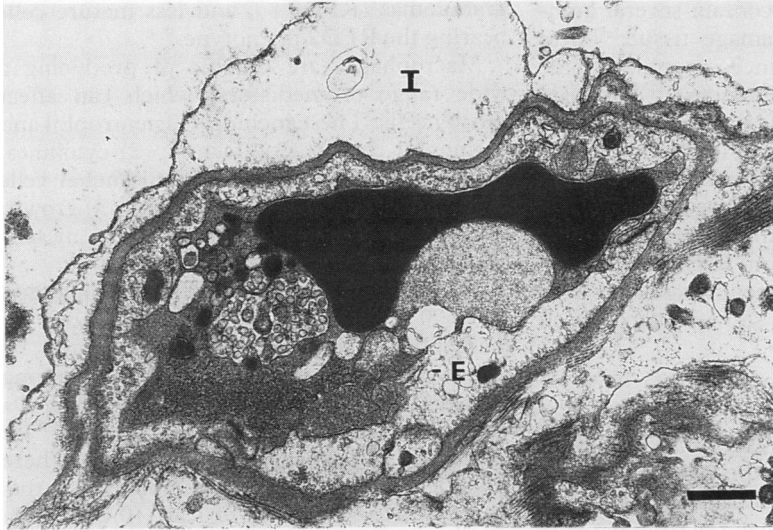


Figure 2 Electron lucent cytoplasmic granules with swelling in both endothelial (E) and type 1 (I) cells with reduction in the lumen of the alveolar capillary. There is also interstitial oedema. (Transmission electron microscopy.) Reproduced from Corrin *et al*⁶ by courtesy of the *Journal of Pathology*.

Recent ultrastructural studies suggest that in systemic sclerosis alveolar capillary endothelial cell injury precedes inflammation and fibrosis.¹⁴ Endothelial cells are also capable of releasing potent mediators, which may have a role in tissue damage.¹⁵ There is evidence from clinical studies and animal models that pulmonary vascular leakage of proteins occurs many days before lung fibrosis is detectable by biochemical or histological techniques.^{16,17} Two independent lines of evidence suggest that increased pulmonary vascular permeability occurs in fibrosing alveolitis. Firstly, ultrastructural studies have shown injured epithelial and endothelial cells with associated interstitial oedema in patients with early fibrosing alveolitis (fig 2).⁶ Secondly, clearance of radiolabelled low molecular weight solutes from lungs to blood is increased in such patients.¹⁸ Endothelium produces vasodilator substances, such as prostacyclin (PGI₂), in response to mast cell products, such as histamine and the leukotriene LTC₄.¹⁵ In addition, the discovery that vascular endothelium activates and produces many adhesion molecules for inflammatory cells¹⁵ has shown that it has a much more important role than was previously realised in the pathogenesis of inflammation.

Inflammatory cell infiltration

In fibrosing alveolitis inflammatory cells accumulate in the interstitium of the lung and alveolar airspaces. This "alveolitis" with subsequent fibrosis leads to the loss of normal alveolar architecture.¹⁰ This is an early feature of fibrosing alveolitis with expansion of the interstitium by lymphocyte and plasma cells (fig 2). In addition, many macrophages can be seen in the alveolar lumina. There are also increased numbers of neutrophils, eosinophils, and mast cells in bronchoalveolar lavage fluid, though fewer are seen in lung tissue of open lung biopsy specimens.¹⁹ Such cells can be identified at an early stage of the disease before fibrosis is

detected clinically.¹⁸ They are all capable of secreting mediators that can cause lung injury.

NEUTROPHILS

Infiltration of the lungs by neutrophils is an early event in animal models of lung injury induced by bleomycin and asbestos.⁵ Neutrophils migrate into tissue after adhering to endothelial cells by expressing cell surface adhesion molecules, particularly the integrin CD18/CD11b, which acts as a ligand for the intercellular adhesion molecule (ICAM)-1. In addition, endothelium produces endothelial leucocyte adhesion molecules (ELAM)-1, which serve to bind neutrophils, and this secretion is enhanced by the cytokines interferon γ , tumour necrosis factor (TNF)- α , and interleukin (IL)-1.²⁰ Neutrophils release a wide range of granule associated enzymes, particularly proteases and collagenases, which can degrade the connective tissue matrix.²¹ They also release highly reactive oxygen radicals such as superoxide anion, hydrogen peroxide, hydroxyl radicals, and hypohalous acids, which can cause epithelial cell injury.^{21,22} These oxygen radicals require myeloperoxidase, a granule protein found in neutrophils, and halide anions for their production. Glutathione, which is a powerful oxygen radical scavenger, is present in large amounts in the normal respiratory tract and protects the respiratory epithelium from damage.²³ There is a reduction in glutathione in the lower respiratory tract of patients with fibrosing alveolitis, which could exaggerate the effects of oxygen radicals.²⁴

Although few neutrophils are observed within the lung interstitium in fibrosing alveolitis, their number is increased in lavage fluid, suggesting that they rapidly traffic out of the capillaries into the airspaces. In patients at risk of developing fibrosing alveolitis, such as those with systemic sclerosis or rheumatoid arthritis, neutrophils are increased in lavage fluid in the absence of radiological evidence of lung disease, indicating subclinical alveolitis.^{18,25} Increases in neutrophil number in lavage specimens tend to be higher in patients who do not respond to steroids.^{26,27} Repeated lavage indicates that patients responding to high dose prednisolone have a significant decrease in neutrophils.²⁸ Raised concentrations of proteases and collagenases are seen in lavage fluid from patients with fibrosing alveolitis.²⁹ More recently, increased concentrations of myeloperoxidase have been found, supporting the theory that neutrophil mediated damage occurs in fibrosing alveolitis.³⁰ Thus neutrophil products, though not the initiating agents of lung injury, are likely to promote a vicious cycle of further damage.

EOSINOPHILS

Eosinophils are occasionally observed in the lung interstitium of patients with fibrosing alveolitis and may comprise up to 20% of the cells in lavage fluid.³¹ There is evidence that an increase in lavage eosinophils as well as neutrophils is associated with progressive disease before treatment and a poor response to

steroids.^{26,27} Eosinophils contain several granule proteins that can damage tissue. These include major basic protein, eosinophil cationic protein, and eosinophil peroxidase.³² There are raised concentrations of eosinophil cationic protein in lavage fluid samples from patients with fibrosing alveolitis and many of the eosinophils are degranulated.³³ This suggests that eosinophils, like neutrophils, may contribute to lung injury in this condition. In patients having cyclophosphamide treatment and low dose steroids, repeated lavage shows a decrease in eosinophils.²⁸

MAST CELLS

Several lines of evidence suggest that lung mast cells may play a part in the pathogenesis of pulmonary inflammation and fibrosis. Mast cells are found in lung biopsy material from patients with fibrosing alveolitis, particularly in areas of fibrosis.³⁴ Raised concentrations of histamine and tryptase have been observed in bronchoalveolar lavage fluid from patients with fibrosing alveolitis.^{35,36} The histamine concentrations show a significant correlation with percentage of eosinophils and neutrophils in lavage samples and with the higher grades of fibrosis in lung biopsy specimens from the same patients. In certain circumstances histamine causes proliferation of cultured fibroblasts in a dose dependent manner.³⁷ Mast cells are closely apposed to fibroblasts in normal lung and in fibrosing alveolitis.^{14,38} In vitro studies have also shown that mast cells and fibroblasts are functionally interdependent. Mast cells in culture cause multiplication of fibroblasts and increased collagen to accumulate in the extracellular matrix.³⁹ Heparin from mast cell granules is mitogenic for fibroblasts.⁴⁰ Transforming growth factor β produced by mast cells also stimulates lung fibroblast collagen production.⁴¹

Mast cells produce PGD₂, leukotriene TC₄, and the enzyme tryptase, which all have a role in promoting vascular leakage.⁴² Degranulated mast cells have been identified in other fibrotic conditions, such as scleroderma,⁴³ supporting the hypothesis that mast cells represent a link between activated endothelial cells and fibroblasts in the skin of patients with this condition. Taken together, these findings indicate that mast cells in the lung have the potential to promote inflammation and stimulate collagen deposition. Some workers, however, believe that mast cells may be a secondary phenomenon or have a minor role in interstitial fibrosis because pulmonary fibrosis can be induced with bleomycin in mice genetically deficient in mast cells.⁴⁴

MACROPHAGES

Lung biopsy specimens from patients with cryptogenic fibrosing alveolitis contain increased numbers of macrophages both in the alveolar spaces and in the lung interstitium. The macrophage population is mixed with mature (RFD7+) cells, those with markers normally found on interdigitating cells (RFD1+), macrophages seen in sarcoid

granulomas (RFD9+), and less mature cells bearing the RFD2 phenotype.^{45,46}

Macrophages are capable of producing a wide range of mediators, which can affect nearby cells. These include: (1) neutrophil and eosinophil chemotactic factors; (2) cytokines, which alter permeability of endothelial cells and adhesion molecule expression; (3) growth factor signals to fibroblasts and other mesenchymal cells.

The neutrophil chemotactic factors include a 400–600 Da factor, which is produced by macrophages that have been stimulated by immune complexes or lavage fluid containing immune complexes.⁴⁷ A second 10 000 Da moiety is produced when macrophages are stimulated by IgG or zymogen particles. There is evidence that IL-8 is a component of this moiety.⁴⁸ IL-8 is one of at least 10 8–10 kDa proteins that form the recently defined intercrine supergene family⁴⁹ and is a potent neutrophil chemoattractant and activator, which can be synthesised by many cells, including fibroblasts, epithelial cells, and macrophages.^{50,51}

There is some evidence that the cytokines TNF- α and IL-1 are produced by alveolar macrophages derived from patients with fibrosing alveolitis.^{52,53} TNF- α can have an auto-crine stimulatory effect on macrophages and can also influence other inflammatory cells, such as neutrophils and lymphocytes.⁵⁴ Further work needs to be done to clarify the expression and regulation of cytokines and adhesion molecules in fibrosing alveolitis.

Macrophages release several mediators that can stimulate lung fibroblast proliferation. Of these, fibronectin, platelet derived growth factor (PDGF), and insulin like growth factor 1 (IGF-1) have been implicated in the pathogenesis of fibrosing alveolitis and will be discussed in more detail below.

LYMPHOCYTES

Several lines of evidence point to a role for immunological mechanisms in the pathogenesis of fibrosing alveolitis. Most patients have polyclonal increases in serum immunoglobulin concentrations. Over 60% of patients have non-organ specific autoantibodies in their serum, particularly antinuclear antibodies or rheumatoid factor.⁵⁵ Fibrosing alveolitis is a frequent feature of such classical autoimmune disease as rheumatoid arthritis, systemic lupus erythematosus, and systemic sclerosis.² There is little evidence of immune complex deposition in the lung but increased concentrations of circulating immune complexes are reported and have also been noted in lavage fluid from patients with fibrosing alveolitis.^{56–58} These appear to correlate with the early stages of the disease and with a more favourable response to steroids.⁵⁷ Lavage fluid shows that lymphocytosis occurs in only 17% of patients with fibrosing alveolitis, but lavage lymphocytes show a significant correlation with immune complexes in both serum and lavage fluid.⁵⁹

T lymphocytes

In contrast to lavage fluid, where lymphocytes

are commonly not increased, numerous lymphocytes are present in the interstitial tissues of most patients with fibrosing alveolitis³⁴ (fig 2). T cells make up the majority of these cells, with variable numbers of T helper-inducer and T suppressor-cytotoxic phenotypes.³⁴⁻⁶⁰ These cells are activated as defined by immunostaining with antibodies to IL-2 and HLA-DR.³⁴⁻⁶⁰ There is also preliminary evidence in systemic sclerosis to suggest that a subset of T cells bear the CD45RO phenotype of committed memory type T cells within the lung interstitium and that the number of these cells is correlated with alveolar wall thickness.⁶¹ Soluble markers of T cell activation, such as IL-2 and soluble CD8, have also been identified in the serum of patients with fibrosing alveolitis. Activated T cells produce interferon γ , which is present in excess in lavage fluid from patients with fibrosing alveolitis.⁶² Interferon γ is known to have many functions. It is an activator of macrophages and T cells. It also stimulates endothelial cells to express ICAM-1 (the specific ligand for the T cell adhesion molecule LFA-1) and is a powerful inducer of HLA-DR expression on certain cells, including antigen presenting cells and lung epithelial cells. HLA-DR expression is found in macrophages, endothelial cells, and epithelial type 2 cells in fibrosing alveolitis.⁶⁰

In animal models of pulmonary fibrosis T lymphocytes have been found to influence collagen deposition. In the mouse model of bleomycin induced lung fibrosis Schrier and Phan⁶³ found that pretreatment with cyclophosphamide enhanced the fibrotic response, but that this could be reversed by an infusion of splenic T cells from normal mice, indicating that suppressor T cells may have a role in regulating pulmonary fibrosis. In keeping with this theory is the observation that an increased proportion of lymphocytes in lavage fluid from patients with fibrosing alveolitis appears to be associated with a more favourable prognosis.²⁷ Interactions between lung lymphocytes and fibroblasts are discussed further below.

B cells

Several studies have described lymphoid follicles in the lung interstitium of patients with fibrosing alveolitis. These lymphoid follicles consist of germinal centres surrounded by a mantle of B cells.⁶⁴ Studies of lavage fluid and peripheral blood also suggest that B cell stimulation has a role in the pathogenesis of fibrosing alveolitis as concentrations of immunoglobulin, especially IgG, are increased in both.⁵⁶⁻⁶⁵ Lavage cells from patients with fibrosing alveolitis secrete more B cell growth factor than controls.⁶⁶ These data provide convincing evidence that B cells are active in fibrosing alveolitis.

The mechanism for the recruitment of lymphocytes in fibrosing alveolitis remains unclear. One possibility is that activated lung fibroblasts play a part. Fibroblasts have been shown to produce large amounts of two inflammatory mediators IL-6 and IGF-1, which have potent effects on lymphocyte chemotaxis and mitosis.⁶⁷ Further studies are needed before firm conclusions about the role of lymphocytes in the pathogenesis of fibrosing alveolitis can be reached.

Activation of lung fibroblasts

There are several potential mechanisms for fibroblast activation in the lung⁶⁸⁻⁶⁹: firstly, an increase in fibroblast number, occurring as a result of either fibroblast proliferation or recruitment of fibroblasts from undifferentiated stem cells or by chemoattraction of cells from adjacent areas; secondly, increased collagen biosynthesis; and, thirdly, diminished collagen degradation, either through a reduction in the rate at which newly synthesised procollagens are degraded or by reduced enzymatic breakdown of collagens in the extracellular matrix. Each of these mechanisms will be discussed below.

FIBROBLAST PROLIFERATION

Evidence that fibroblast proliferation is an important mechanism in the pathogenesis of fibrosing alveolitis is based on two lines of evidence. Firstly, morphological studies have shown that fibroblast numbers are increased.⁷⁰ Secondly, alveolar macrophages from patients with fibrosing alveolitis spontaneously release fibroblast mitogens when cultured *in vitro*.⁷¹

Current theories of cell division propose that two different kinds of signal (called competence and progression factors) are required for mitosis to occur.⁷² These signals are thought to act at different stages of the cell cycle (fig 3).

Fibronectin is an important adhesive molecule in the extracellular matrix. It can also act as a chemoattractant for fibroblasts and as a competence factor, inducing cells in the G₁ phase of the cell cycle to enter G₁. Macrophages are known to infiltrate the lungs of patients with pulmonary fibrosis and spontaneously secrete more fibronectin than macrophages derived from healthy lung.⁷³⁻⁷⁴ Consequently, it has been proposed that macrophage derived fibronectin may have a role in the pathogenesis of fibrosing lung disease.

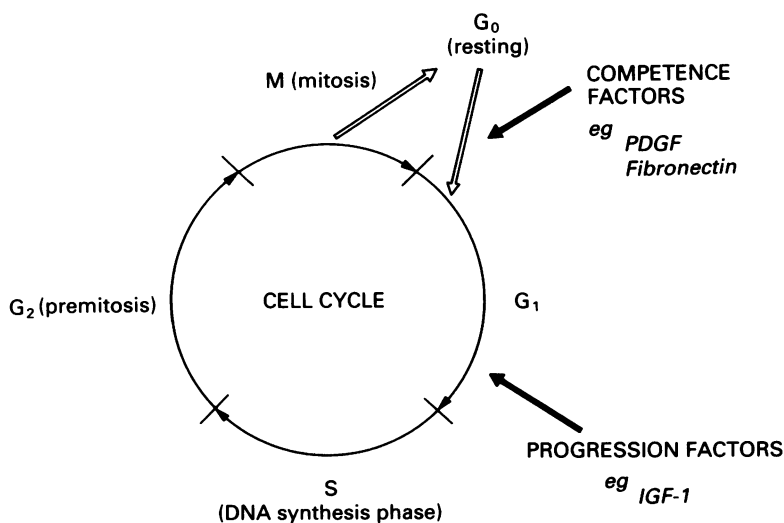


Figure 3 Competence and progression factors influencing fibroblast mitosis. PDGF—platelet derived growth factor.

The relevance of these observations is uncertain, however, because preparations of fibronectin derived from macrophage conditioned media may contain other growth factor contaminants.⁷⁵ A further consideration is that fibronectin in the lung may be derived from other sources. For example, lung fibroblasts are themselves capable of secreting considerable quantities of fibronectin when cultured *in vitro*.⁷⁶ Moreover, fibronectin is present in the circulation⁷⁷ and substantial concentrations could enter into the lung interstitium after endothelial cell injury.

Platelet derived growth factor is a 32 kDa heterodimeric peptide that can exist as different isoforms (designated AA, AB, and BB). It also acts as a competence factor for fibroblast proliferation and is a potent chemoattractant for mesenchymal cells *in vitro*.⁷⁸ It was originally isolated from platelets but is now found to be produced by several cell types, including macrophages. Alveolar macrophages from patients with fibrosing alveolitis spontaneously release platelet derived growth factor and express messenger RNA (mRNA) for platelet derived growth factor when cultured *in vitro*.⁷⁹ Similar findings have been reported for macrophages from rats exposed to asbestos but these experiments also indicated that platelet derived growth factor is associated with macrophage derived α_2 macroglobulin and other less well characterised binding proteins, which raises questions about its bioavailability *in vivo*.⁸⁰

A recent study of lung tissue from patients with fibrosing alveolitis indicated that tissue macrophages produce platelet derived growth factor like molecules and express mRNA transcripts of platelet derived growth factor genes.⁸¹ Other investigators, however, using similar techniques found that macrophages produced relatively little platelet derived growth factor by comparison with type II alveolar epithelial cells, suggesting that regenerating alveolar epithelium may be an important source of this mediator.⁸² Insulin like growth factor 1 (IGF-1), also called somatomedin C, is a 70 amino acid peptide with a molecular weight of 7.6 kDa. IGF-1 acts as a potent progression factor for fibroblasts, stimulating competence primed cells to complete G₁ and progress to the S phase (DNA synthesis phase) of the cell cycle (fig 3). It is also capable of stimulating collagen production by human lung fibroblasts.⁸³ Alveolar macrophages from patients with fibrosing alveolitis and asbestosis have been shown to spontaneously release a progression type growth factor for human lung fibroblasts.⁷¹ A component of this has since been purified and characterised as a 25 kDa molecule with IGF-1 like activity.⁷⁴ Like fibronectin, IGF-1 occurs in high concentration in the blood, where it is bound to large carrier proteins. This circulating IGF-1 is thought to be synthesised mainly in the liver.⁸⁴ Furthermore, IGF-1 like molecules are synthesised *in vitro* by several other cell lines, including lung fibroblasts.⁸⁵

Attempts to quantify production rates of IGF-1 like molecules by macrophages in cell culture have met with difficulties because of the

presence of binding proteins,⁸⁵ but the amount produced by alveolar macrophages may be small compared with that from other potential sources.

The ease by which alveolar macrophages can be obtained by the technique of broncho-alveolar lavage may have led to an exaggerated view of the importance of this cell in the pathogenesis of pulmonary fibrosis. We do not know whether the alveolar macrophages are capable of producing sufficient quantities of growth factors (both in terms of absolute quantities and because their bioavailability may be affected by the presence of binding proteins) to activate interstitial fibroblasts *in vivo*.

For a balanced view of the pathogenesis of fibrosing alveolitis we must consider the possibility that mediators from sources other than the alveolar macrophage will affect lung fibroblasts. Selman and coworkers⁸⁶ showed that T lymphocytes from the lungs of patients with cryptogenic fibrosing alveolitis and extrinsic allergic alveolitis released a mediator that inhibited fibroblast proliferation. By contrast, circulating T lymphocytes from the same patients and from controls released a mediator that caused modest stimulation of fibroblast proliferation, confirming a similar observation from an earlier study.⁸⁷ These findings indicate differences in behaviour between lung and blood lymphocytes and illustrate the danger of drawing generalised conclusions about lung lymphocytes from studies of cells derived from the circulation. They also support the observations discussed earlier that lymphocytes have an antifibrotic effect in animal models of pulmonary fibrosis.

Attempts to isolate mediators from purified preparations of individual cell types cultured *in vitro* may also give a misleading impression of their importance as activators of lung fibroblasts *in vivo*. Cantin and coworkers⁸⁸ determined the overall effect of lavage concentrates on human fibroblasts. This approach permits the combined effects of mediators present *in vivo* to be assessed but it does not permit their source to be identified. They found that lavage fluid from patients with fibrosing alveolitis stimulated significantly more uptake of thymidine by fibroblasts than did controls, suggesting that it contained fibroblast growth factors. Furthermore, this stimulatory activity was correlated with indices of lung collagen production *in vivo*.

Alternative hypotheses have been proposed to explain the observed increase in fibroblast numbers in patients with pulmonary fibrosis. Jordana and colleagues⁸⁹ showed that fibroblasts derived from fibrotic lung tissue proliferate significantly faster than controls when cultured *in vitro*. They observed a pronounced heterogeneity in the proliferative characteristics of lung fibroblasts, with certain clone derived cell lines showing a "fast replicating" phenotype, and proposed that the conditions of injury and repair prevailing in the lower respiratory tract might select out fast replicating fibroblast clones. In a similar study Raghu and coworkers⁹⁰ found variability in the

rate of fibroblast proliferation. Fibroblasts derived from lung biopsy specimens that showed histological evidence of early disease had greater proliferative potential than those from areas with more established fibrosis.

Collagen production by lung fibroblasts

Regulation of collagen production by lung fibroblasts may occur at the level of procollagen gene transcription, translation of procollagen mRNA or sites of post-translation modifications to procollagen molecules. There are now known to be over 25 different procollagen genes, located on several different chromosomes. The elements regulating transcription of the interstitial procollagen types I, II, III and V, which comprise over 95% of all lung collagen, are thought to be generally similar.

TRANSCRIPTION AND TRANSLATION OF PROCOLLAGEN GENES

The mechanisms of transcription and translation of the procollagen type I ($\alpha 1$) gene are outlined in fig 4. DNA is transcribed by the enzyme RNA polymerase and the resulting complementary sequence undergoes further processing within the nucleus, during which introns are excised and exons respliced. The product is a 6 kb mRNA, which leaves the nucleus to be translated on membrane bound polysomes. The resultant polypeptide chain then undergoes extensive post-translational modifications catalysed by a series of hydroxylating and glycosylating enzymes.⁶⁸

The hydroxylation of proline is vital for the ultimate tertiary structure and function of the collagen molecule, and is therefore a potentially vulnerable point in the collagen biosynthetic pathway that could be open to therapeutic manipulation. For example, the use of proline analogues, which result in underhydroxylation of α chains, has been shown to reduce the

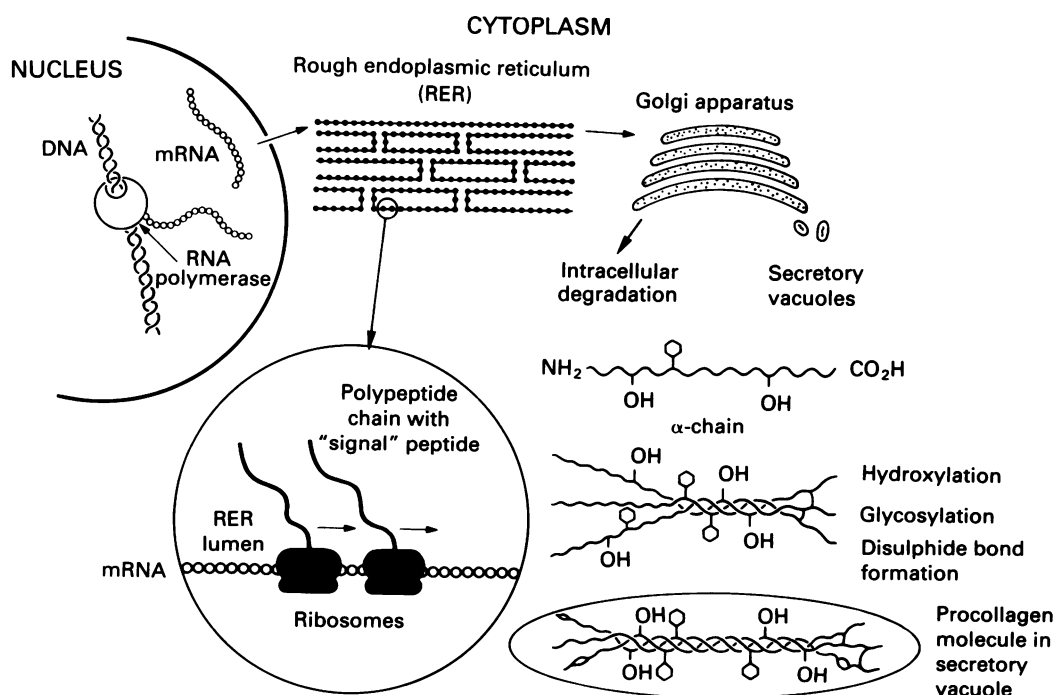
stability of collagen, make it more susceptible to intracellular degradation and delay secretion.⁹¹ The anthracyclines doxorubicin and daunorubicin, both potent agents of irreversible inactivation of prolyl hydroxylase,⁹² have been shown to impair wound healing in experimental animals.⁹³

PROCOLLAGEN SECRETION

As procollagen molecules are secreted from cells the procollagen peptides are cleaved by proteases, which are specific for either the *N* or the *C* terminal ends, but not for each collagen type. Several biological functions have been ascribed to these so called procollagen peptides. Before secretion they are thought to prevent premature fibril formation but after secretion may direct fibril assembly. Cleavage of the *C* terminal propeptide has been shown to be the rate controlling step for assembly of type I fibrils in vitro.⁹⁴ The *N* terminal propeptides of collagen types I and III have been shown to exert a negative feedback on collagen synthesis by fibroblasts cultured in vitro.⁹⁵

Procollagen peptides have recently assumed clinical importance as potential markers of collagen production in vivo. Several features make them useful for this purpose. They are thought to be released in stoichiometric amounts when collagen molecules are secreted from cells; they are immunologically distinct for each collagen type; and their high carbohydrate content renders them antigenic. Consequently, radioimmunoassays that detect procollagen peptides in biological fluids have been developed and several are commercially available. Most widely used are assays which measure the *N* terminal propeptide of type III collagen. Increased concentrations of type III procollagen *N* terminal peptides have been detected in the serum of patients with various fibroproliferative disorders,⁹⁶ and these assays

Figure 4 Collagen gene activation and protein production.



have also been applied to bronchoalveolar lavage fluid from patients with pulmonary fibrosis.⁹⁷

Two areas of research have been important in improving our understanding of the regulation of lung collagen production. These are the development of molecular biological techniques that permit the investigation of elements regulating collagen gene expression, and the discovery and characterisation of polypeptide mediators that have potent effects on the mesenchymal cells producing collagen.

One such mediator is transforming growth factor β (TGF- β), a 25 kDa dimeric peptide composed of two identical disulphide bonded subunits, which occurs in at least five different isoforms. TGF- β was originally purified from human platelets, human placenta, and bovine kidney but is widely distributed throughout the animal kingdom, being totally conserved in human, bovine, and porcine species.⁹⁸ TGF- β has potent stimulatory effects on the synthesis of procollagen and fibronectin by lung fibroblast cell lines, and this is associated with an increase in steady state levels of mRNA for these proteins.⁹⁹ It also has the potential to inhibit collagenase secretion by fibroblasts.¹⁰⁰ Recent studies have shown that TGF- β is present at sites of extracellular matrix gene expression in human pulmonary fibrosis¹⁰¹ and that fibroblast cell lines derived from lungs of patients with pulmonary fibrosis respond to TGF- β by upregulating procollagen gene expression¹⁰² and by reducing the rates at which newly synthesised procollagen is degraded.¹⁰³

In addition to TGF- β , various mediators released by activated resident or inflammatory cells could influence expression of collagen genes in the lungs after injury. Their interactions are complex and some observations appear contradictory. For example, IL-1 increases type I procollagen mRNA concentrations,¹⁰⁴ yet in combination with TNF concentrations are reduced. Interferon γ reduces steady state type I procollagen mRNA concentrations,¹⁰⁵ but does not appear to affect type I

collagen transcription.¹⁰⁶ The cytokine network, by which inflammatory cells regulate fibroblast function, with fibroblasts in turn feeding back to regulate inflammatory cell function, is complex (fig 5). The effect of the individual cytokine varies with the state of activation of the target cells, the presence of other cytokines, and the microenvironment.⁶⁷ Progress in this area of research is important as it should lead to the development of agents that have the potential to block lung collagen production at the molecular level.

Collagen degradation

Reduced degradation of collagen may also lead to an increase in lung collagen content. Regulation of this process occurs at intracellular and extracellular sites.

INTRACELLULAR MECHANISMS

Over the last 30 years two independent lines of evidence have suggested the existence of pathways by which newly synthesised procollagens are rapidly degraded *in vivo*. Firstly, experiments by Jackson and Bentley¹⁰⁷ and by Nimni and coworkers¹⁰⁶ indicate that procollagen turnover is most rapid for collagens soluble in solutions of low ionic strength (that is, those most recently synthesised). Secondly, experiments by Barnes and coworkers¹⁰⁹ show the rapid appearance of radiolabelled hydroxyproline after the injection of radiolabelled proline into guinea pigs. As hydroxylation of proline occurs as a post-translation event during procollagen synthesis, this observation suggests that the radiolabelled hydroxyproline is derived from the degradation of a larger substrate molecule. Bienkowski and coworkers¹¹⁰ consider that the substrate is most likely to be procollagen and that degradation occurs intracellularly by proteases located in the endoplasmic reticulum or Golgi apparatus.¹¹¹

Rates of intracellular degradation of procollagen are known to increase in response to agents that inhibit the hydroxylation of proline.¹¹² This "enhanced degradation," defined by Bienkowski¹¹¹ as an increase above basal concentrations, can be blocked by inhibitors of lysosomal proteases and by colchicine. It can also be increased by PGE₂ and epidermal growth factor,¹¹³ and this represents a potential site at which procollagen may be regulated.

EXTRACELLULAR MECHANISMS

The extracellular degradation of collagen is thought to result largely from the action of neutral proteases. Fibroblasts actively produce collagenase, gelatinase, and stromelysin, enzymes that cleave collagen or its breakdown products.¹¹⁴ Leucocytes also produce collagenases, which may contribute to remodelling of lung extracellular matrix in disease states.

Neutrophil collagenase, a metalloproteinase found preformed in neutrophil specific granules, is one such protease. It is produced in latent form and activated extracellularly. It cleaves collagens type I, II, and III but has the greatest activity against type I.¹¹⁵ Neutrophils

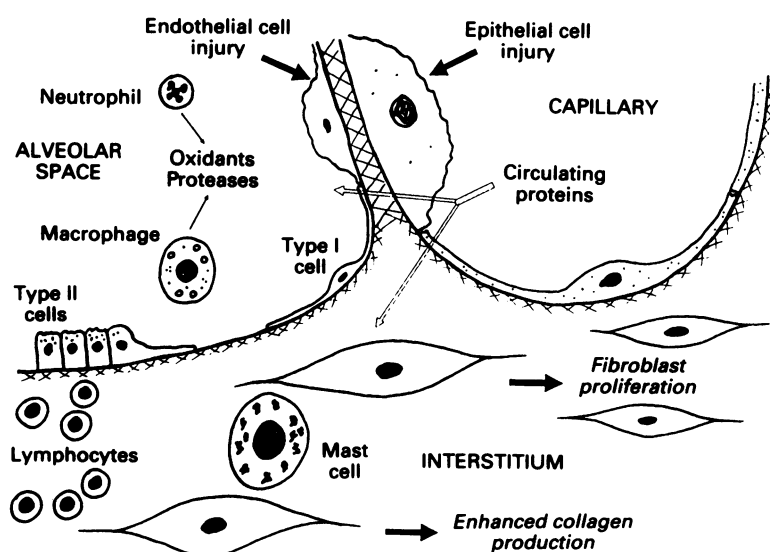


Figure 5 Inflammatory cells and the cytokine network influencing fibroblast function.

also possess a gelatinase capable of degrading collagen.¹¹⁶ Macrophages secrete both collagenase and stromelysin and these have properties identical to those of the enzymes produced by fibroblasts. They also produce a gelatinase that is immunologically identical to neutrophil gelatinase. In the circulation collagenases are inhibited by α_2 macroglobulin and β_1 anticollagenase and in tissues by the tissue inhibitor of metalloproteinases. The latter is produced by fibroblasts¹¹⁷ and represents another site at which collagen deposition could be regulated.

The relevance of changes in rates of collagen degradation to the pathogenesis of pulmonary fibrosis has received relatively little attention. It has been reported that a reduction in the degradation of newly synthesised collagen contributes to the development of bleomycin induced pulmonary fibrosis in rabbits.¹¹⁸ There is also evidence that the increase in procollagen production induced by TGF- β in rat fetal fibroblasts is associated with a reduction in the rate at which newly synthesised procollagen is degraded.¹¹⁹

Role of epithelial and endothelial barriers

As described earlier in this review, injury to pulmonary endothelial and epithelial cells is known to occur in pulmonary fibrosis. It has been proposed that epithelial cells normally release inhibitory mediators such as PGE₂ (which inhibit fibroblast proliferation and collagen production) and that epithelial cell loss results in an imbalance of mediators and a shift towards fibroblast activation.⁶

Regenerating epithelial cells might also produce platelet derived growth factor like molecules with the capacity to stimulate fibroblast proliferation (see above), and this might be a further mechanism by which epithelial injury contributes to interstitial fibrosis. This hypothesis may also be relevant to sub-epithelial fibrosis in asthmatic patients, in whom bronchial epithelial cell loss is a characteristic feature.¹²⁰

It is increasingly recognised that the endothelial cell produces mediators that may interact with mesenchymal cells and connective tissue beneath its basement membrane. These include heparin binding growth factors, platelet derived growth factor, TGF- β ,¹²¹ and endothelin, which, in addition to regulating vasomotor tone, may have important effects on mesenchymal cell proliferation and fibrosis.

The association between increased pulmonary vascular permeability and pulmonary fibrosis raises the possibility that proteins derived from the circulation may activate lung fibroblasts. Fibrin deposits are observed to cover bare epithelial basement membrane in patients with fibrosing alveolitis and systemic sclerosis.^{6,14} This provides circumstantial evidence that thrombin has initiated the polymerisation of fibrinogen molecules in the alveolar space, with the consequent release of fibrinopeptides A and B and other soluble products of clot formation. Thrombin has been reported to initiate fibroblast cell division,¹²² to

stimulate human lung fibroblast proliferation *in vitro*,¹²³ and to enhance lung fibroblast proliferation in bleomycin induced pulmonary fibrosis in animals.¹²⁴ Fibrin can act as a matrix on which fibroblasts may grow¹²⁵ and the soluble products of clot formation are known to act as chemoattractants for fibroblasts¹²⁶ and to stimulate human lung fibroblast proliferation.¹²³ Characterised growth factors for fibroblasts, such as fibronectin, platelet derived growth factor, and IGF-1, are all present in blood and are known to be partly responsible for the stimulatory effects of serum on fibroblast proliferation and collagen production.¹²⁷

Taken together, these data provide compelling evidence that pulmonary vascular leakage of proteins may activate lung fibroblasts and initiate deposition of collagen in the lung interstitium.

The fibroblast as an effector cell

Lung fibroblasts are not simply target cells that respond to inflammatory mediators in their environment. They are increasingly recognised as effector cells in their own right.¹²⁸ Their ability to produce fibronectin and an IGF-1 like molecule was discussed earlier in this article. They also secrete PGE₂, which has potent paracrine and autocrine effects inhibiting fibroblast proliferation and collagen synthesis.¹²⁹ There is also evidence that fibroblasts can amplify the production of mediators produced by other cells. Lung fibroblasts are capable of secreting IL-1 in response to the synergistic actions of IL-1 and TNF α .¹³⁰ Thus the production of IL-1 by fibroblasts may represent an amplification mechanism for this inflammatory mediator that can stimulate fibroblast collagen production.¹⁰⁴

Fibroblasts also produce IL-6, also called interleukin β_2 , which stimulates lymphocyte proliferation and induces release of acute phase proteins from the liver.¹³¹ Possibly therefore IL-6 secreted by lung fibroblasts stimulates lymphocyte proliferation in the lung and is responsible, at least in part, for the systemic symptoms of which patients with interstitial lung disease frequently complain, particularly in the early stages.

Human lung fibroblasts have also been shown to spontaneously release granulocyte-macrophage colony stimulating factor, which enhances *in vitro* survival of eosinophils.¹³² This suggests that they have the potential to modulate the inflammatory cell population that infiltrates the lung. Preliminary studies indicate that rat lung fibroblasts are capable of producing both platelet derived growth factor like molecules¹³³ and TGF- β analogues,¹³⁴ though these observations have not yet been confirmed in human lung fibroblast cell lines. Nevertheless, taken with the recent observation made with immunohistochemical techniques that human skin fibroblasts may stain positively for TGF- β ,¹³⁵ these data indicate that in some circumstances fibroblasts produce the very mediators to which they have traditionally been considered responders.

Summary

It is over 25 years since Scadding¹ first defined the term fibrosing alveolitis. It has since been established that complex mechanisms underlie its pathogenesis, including epithelial and endothelial injury, vascular leakage, production of inflammatory cells and their mediators, and fibroblast activation. Only through a detailed knowledge of how these cellular and molecular events are interlinked will we learn how to combat this disease, which is notoriously resistant to present treatments.

So far the only therapeutic advances have been refinements in immunosuppression, and even these treatments are frequently disappointing. We believe that future advances in treatment will come from the development of agents that protect endothelial and epithelial cells from further injury and agents that can inhibit release of inflammatory mediators. A better knowledge of the mechanisms of collagen gene activation and the biochemical pathways of collagen production may also allow the identification of vulnerable sites at which new treatments may be directed. A combined approach to modifying appropriate parts of both the inflammatory component and the fibroblast/collagen component should provide a new stimulus to research. Further epidemiological studies are also needed to identify the environmental causes of lung injury that initiate the cascade of events leading to interstitial fibrosis.

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