

Cytokines and pulmonary fibrosis

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There is acceptance of the concepts implicating acute and chronic inflammation in the pathogenesis of pulmonary fibrosis, either of known aetiology or of idiopathic origin (IPF). In the same way, the process is thought to involve the stimulated response of tissue cells such as fibroblasts resulting in increased proliferation and collagen secretion.¹ Inflammatory cells including alveolar macrophages participate through the release of mediators such as eicosinoid metabolites, destructive proteolytic enzymes and inflammatory growth and differentiation factors, including interleukin 1 (IL-1) and tumour necrosis factor (TNF). These factors act directly on resident tissue cells to modify their behaviour and alter matrix gene expression. More recently the resident cells have been shown to be effector cells themselves, secreting various cytokines such as interleukin 6 (IL-6), interleukin 8 (IL-8), and monocyte chemotactic peptide (MCP), both in a spontaneous manner and, more profoundly, after stimulation by monocyte derived cytokines IL-1 and TNF.²⁻⁵ IL-8 and MCP can mediate the accumulation of neutrophils and mononuclear cells into the lung tissue as seen in many acute inflammatory responses.⁶ In the examination of the chronic phase of disorders leading to pulmonary fibrosis, however, it is clear that other cytokines, including transforming growth factor β (TGF- β), platelet derived growth factor (PDGF), and granulocyte macrophage colony stimulating factor (GM-CSF) may play an important part in the overall matrix distortion, fibroblast proliferation and alteration of structural cell phenotype seen in this disorder.⁷⁻¹⁰ This short review will concentrate on recent evidence for the presence of these three powerful growth and differentiating cytokines, derived from both inflammatory and structural cells, and speculate on their role in the pathogenesis of chronic disease. A fuller treatment of cytokines in the lung is the topic of a recent publication.¹¹

Evidence for the presence of TGF- β , PDGF, and GM-CSF in chronically diseased tissue comes from both in vitro and in vivo experiments. Studies of cell lines derived from fibrotic human tissue and the examination of tissue biopsy samples by techniques of immunohistochemistry and mRNA expression by Northern analysis and in situ hybridisation have been used to investigate the source and distribution of these factors. Chronically inflamed or fibrotic tissues can be obtained from the upper respiratory tract by

polypectomy (nasal polyposis) or from the lower respiratory tract by lung biopsy or resection (fibrosis). Histopathological evaluation of samples obtained from patients with these two chronic disorders has shown that they share a number of common features, including fibroblast proliferation, inflammatory cell infiltrate, basement membrane thickening, and disordered collagen metabolism. Two types of polymorphonuclear granulocytes are notable participants in these disorders. In the initial stage of fibrosis polymorphonuclear neutrophils are present and are prominent during acute active inflammation. In polyposis neutrophils are present in varying amounts, but eosinophils are the most prominent granulocyte seen in the tissue. The presence (and participation) of these effector cells in polyposis and lung fibrosis which are of a chronic nature suggests that these features, including the presence of inflammatory cells and mediators, may be present in other chronic inflammatory diseases including chronic inflammation of the airways such as asthma. However, there are also likely to be factors involved in pathogenesis that are different in diseases at different sites, which are temporal or tissue specific giving rise to the different outcomes. Direct examination of respiratory tissues for the presence and cellular source of factors believed to be central to disease evolution will lead to a more specific definition of the inflammatory response typical for each individual disease.

Animal models of fibrosis, induced by such agents as bleomycin or silica dust, have also been used to establish cell lines and examine tissue distribution of inflammatory mediators such as TGF- β , PDGF, and GM-CSF. These models have the added advantage that specifically timed and multiple or repeated tissue samples can be obtained. Taken together, the human and animal data argue for a significant role for these three growth and differentiation factors in the generation of the chronic remodelling of the tissues seen in chronic inflammation including pulmonary fibrosis.

Transforming growth factor β

TGF- β refers to a family of cytokines named for their ability to induce transformation of cells to a state in which they are capable of anchorage-independent growth. There are five known subtypes of TGF- β but only three are known to be present in mammalian tissue and, of these, TGF- β_1 is the most prominent.

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The molecule is a protein of 25 kDa made up of two identical chains linked by disulphide bonds. TGF- β is normally secreted as an inactive high molecular weight precursor that requires either acid treatment or enzymic action for activation.¹² In some cases, such as with monocytes and neutrophils, the cytokine may be released in active form, presumably undergoing activation processing either within the cell or as the cytokine is passed to the extracellular space.¹³

There are three actions of TGF- β that potentially have a role in fibrosis: it can directly affect the gene expression of extracellular matrix molecules in stromal cells to induce collagen synthesis and inhibit collagenase production; it can induce proliferation of fibroblasts, most probably in an indirect fashion through the induction of other growth factors such as PDGF; and it can establish an apparent state of autocrine stimulation in structural cells, including fibroblasts, resulting in chronic activation and possible differentiation to a more "aggressive" phenotype, consistent with the expression of disease.^{14,15}

TGF- β has been detected in a number of animal models of fibrosis and in human IPF tissue and fluids during the active phase of the disease. Raghov *et al*¹⁶ using hamsters and Hoyt and Lazo¹⁷ using mice showed a significant increase in the steady state expression of TGF- β transcripts about one week after intratracheal instillation of bleomycin. The increased expression preceded the onset of increased matrix deposition suggesting involvement of this cytokine in the process. Further evidence was reported by Khalil *et al*¹⁸ using immunohistochemistry to show the presence of significant levels of TGF- β in the lung one week after instillation of bleomycin to rats. The cytokine was predominantly localised to macrophages at this time and suggests that these cells have a prominent role in the remodelling or fibrosis that occurs in the chronic inflamed lung. In vitro bleomycin has been shown to induce TGF- β mRNA expression and protein synthesis in endothelial cells and fibroblasts.^{19,20} Whether these are also cell sources in vivo is not known. In studies using immunohistochemical localisation, however, TGF- β was observed in bronchiolar epithelial cells of patients with advanced IPF²¹ and was not found in biopsy specimens from patients who had an ongoing acute inflammatory reaction in the lung with little or no associated fibrosis.

The relationship of the appearance of TGF- β to the process of fibrosis was highlighted by the studies of Phan and Kunkel.²² They carried out a kinetic study on the appearance of two cytokines (TNF and TGF- β) in a rat bleomycin model and showed that in early phases there is an induction of TNF, presumably monocyte derived, and subsequently TGF- β is increased at both the mRNA and protein level. The kinetics of response imply that TNF is released first and then TGF- β enhanced expression occurs subsequently when enhanced collagen gene regu-

lation is occurring. The integrated nature of this sequential activation and cascade of cytokines is seen in the studies of Piguet *et al*²³ who found that pretreatment of rats with antibody to TNF abrogated the fibrotic response in a silica model of pulmonary fibrosis. Similar requirements for early activation of TNF were seen by these authors in a study with intratracheal instillation of bleomycin.²⁴

The relationship of TGF- β expression and collagen gene activation in the tissues was elegantly shown in a study involving combined immunohistochemistry for TGF- β expression and in situ hybridisation for collagen gene expression in human IPF tissue.²⁵ There was colocalisation of these two activities in the areas of fibrotic tissue involvement suggesting that expression of TGF- β results in enhanced collagen synthesis and deposition in the disease process.

A broad range of cells is capable of expressing TGF- β . Originally obtained from bone and platelets, it is known to be produced by various lung cells. Alveolar macrophages appear to contain large amounts of TGF- β in the rat bleomycin model.¹⁸ Studies of the monocyte suggest that the protein is only released when the cell is activated²⁶ but it is not yet clear if it can release active rather than precursor cytokine. Whether the alveolar macrophage behaves in a similar fashion or has different regulation and expression is not known but, given the differences between these cells, it is possible that it is capable of the release of active cytokine even if monocyte is not. Such is certainly the case for the neutrophil which releases the active form of TGF- β .¹³ Since the lung fibroblast is a major target and effector cell population in fibrosis, it will come as no surprise that these cells respond to TGF- β and also actively express and secrete the same cytokine in an apparent paracrine/autocrine cascade in vitro.¹¹

To explore further the relationship between cells and cytokines in chronic inflammation our group has examined human nasal polyp biopsy tissue by immunohistochemistry and in situ hybridisation for TGF- β expression. As noted previously, this tissue shares many of the same histological features with those seen in interstitial pulmonary fibrosis: a preponderance of fibroblasts and excess collagen deposition; numerous inflammatory cells, especially eosinophils; thickened basement membrane; and a proliferative nature to the tissue.²⁷⁻²⁹ TGF- β protein was found to be associated with the matrix throughout the tissue, but we were surprised to find that the predominant cell type actively expressing mRNA for TGF- β was the eosinophil. Moreover, only a portion of the eosinophils were positive for TGF- β mRNA, implying that these inflammatory blood cells, which had now migrated to the polyp tissue, had become activated and were a potent local source for this cytokine. Whether similar activities can be ascribed to eosinophils or other granulocytes in other chronic respiratory tract tissue remains to be established, but

given the observation that an increased presence of eosinophils in IPF is associated with a worse prognosis,³⁰ such tissue needs to be examined. In addition, it will be clearly important to establish the cell source and temporal expression of TGF- β in each of the animal models of fibrosis—examination of human tissue supplies us with only a single snap shot in time to confirm the presence of the mediator which may then become a target for therapeutic modulation.

Platelet derived growth factor (PDGF)

PDGF is an important growth factor and chemotactic agent first discovered as a granule associated glycoprotein in platelets.³¹ It exists as a homo- or heterodimer of two chains (PDGF A and PDGF B) with a molecular weight of 28 to 35 kDa. The PDGF B chain is related to the product of the *c-sis* oncogene. The growth factor interacts with two types of receptors also having homo- and heterodimeric structure.³² The main role played by PDGF is that of a proliferation factor, particularly for fibroblasts and smooth muscle cells.^{33,34} Stimulation of fibroblasts may occur through both autocrine and paracrine mechanisms^{35,36} as seen in studies showing that IL-1 induced proliferation in fibroblasts was mediated by the induction of PDGF in the responding cells.³⁷ A similar result is seen using TNF as the inducing agent.³⁸

This potent mitogenic cytokine is produced by a number of cells in the lung, including activated fibroblasts, smooth muscle, endothelial and epithelial cells.³⁹⁻⁴¹ The potential for the alveolar macrophage to produce PDGF⁴²⁻⁴⁴ appears to be very relevant in IPF.^{45,46} The use of immunohistochemistry and in situ hybridisation showed that, in established IPF tissue, the alveolar macrophage and epithelial cells were the main sources of this factor.⁴³ These findings were restricted to the fibrotic lung and were not seen in normal tissue.

In a previous study on the effects of pulmonary fibroblasts on peripheral blood derived eosinophil survival in vitro^{47,48} we noticed that, in cocultures of these two cell types, the fibroblasts were induced to proliferate. Similar findings were reported by Pincus *et al*⁴⁸ implying that the eosinophil can directly mediate fibroblast proliferation, possibly via release of molecules such as PDGF while augmenting the deposition of collagen matrix via release of TGF- β , as both factors have been shown to mediate matrix formation in vivo.

Granulocyte macrophage colony stimulating factor (GM-CSF)

GM-CSF is a factor that acts on myeloid stem cells to induce differentiation to granulocytes and macrophage/monocytes. It is a heavily glycosylated protein with molecular weights of 15–30 kDa. The basic non-glycosylated peptide backbone of 15 kDa is fully

active.⁴⁹⁻⁵¹ GM-CSF acts in a species specific manner and interacts with a cell membrane receptor of 45 kDa,⁵² which then transmits signal through formation of a heterodimeric structure with a further surface molecule, KH97, which is a common signal transducing pathway for the cytokines IL-3 and IL-5.⁵³

Several aspects of the biology of GM-CSF may apply directly to chronic pulmonary inflammation and fibrosis. Firstly, the cytokine is readily produced by a number of lung cells. T cells stimulated by antigen, monocyte/macrophages stimulated by endotoxin and fibroblasts, endothelial or epithelial cells stimulated by IL-1 or TNF, release significant amounts of GM-CSF.^{47,54-59} Moreover, the cytokine has been shown recently to be present in lavage cells from sarcoid lung.⁶⁰ In addition to its known action on differentiation of progenitor cells, GM-CSF acts on mature granulocytes and monocytes to cause chemotaxis, proliferation, activation and enhanced survival as well as increased phagocytic activity.⁶¹⁻⁶⁷

In our studies on the effector function of lung fibroblasts we found that GM-CSF produced by the pulmonary fibroblast could provide adequate signals to protect peripheral blood eosinophils in vitro resulting in enhanced survival and apparent cell activation.⁴⁷ Furthermore, immunohistochemical studies of eosinophils in nasal polyps showed that they were positive for the EG2 cytoplasmic marker, an indication of activation in the tissue. We have shown that both respiratory fibroblasts and epithelial cell lines derived from chronic inflamed tissue also release significantly greater amounts of GM-CSF than those derived from normal tissue, implying that these cells may have a role in propagation of the inflammation. We have also shown that these same tissue cells release other colony stimulating factors which cooperate with GM-CSF to regulate the enhanced survival of neutrophils and monocytes.^{68,69} The enhanced release of GM-CSF from structural cells in chronically inflamed respiratory tissue may partially explain the accumulation of inflammatory cells in these tissues.^{47,59,70,71}

In an elegant study of cytokine function in vivo Rubbia-Brandt *et al*⁷² used mini-osmotic pumps to deliver low levels of cytokines in a subcutaneous site in the rat. IL-1, TGF- β and GM-CSF all induced a fibroblast proliferative response, but only GM-CSF induced the accumulation of alpha smooth muscle actin containing fibroblasts. This accumulation of myofibroblast-like cells is seen in models of pulmonary fibrosis.⁷³ In our studies the majority of fibroblasts in nasal polyp tissue were seen to be positive for alpha smooth muscle actin, presumably induced by GM-CSF in the tissue (unpublished observations).

When the polyp tissue was stained for GM-CSF by immunohistochemistry a number of cells were positive, including monocytes, epithelial cells and fibroblasts, as well as the eosinophil. When in situ hybridisation was used the eosinophil was the most prominent cell positive for GM-CSF mRNA transcripts.

It is important to recognise that these two methods of evaluating gene expression have different levels of sensitivity of detection and apparent discordance between observation of protein and mRNA can be due to technical factors. A further role suggested for GM-CSF comes from studies on bone marrow stromal cells which can be induced by GM-CSF to form colonies of fibroblasts in anchorage-independent growth conditions.⁷⁴ Indeed, cell lines established from fibrotic lung tissue will form fibroblast colonies under soft agar culture or anchorage-independent conditions.⁷⁵ Whether these cell characteristics (not found in lines from normal lung) are induced by GM-CSF exposure in vivo is not yet established.

Summary

Chronically inflamed and fibrotic tissue of the respiratory tract can be shown to actively express the genes and products of a number of powerful growth and differentiating factors. The initial activation of lung inflammatory cells, including alveolar macrophages, is presumed to result in the release of early acting cytokines such as IL-1 and TNF. Subsequent activation and possible phenotype alteration of the structural cells results in release of other growth factors and accumulation of blood derived inflammatory cells. These cells, once they have entered the tissue and become further activated, may begin to release their own autocrine factors and "feed back" some of the similar signals to the tissue cells in a paracrine manner, further inducing differentiation and phenotype change. These internal tissue cell and cytokine cascades could account for the chronic nature of the inflammation. Therapeutic intervention must therefore take into account the inflammatory component as well as the nature of the cytokines and structural cells involved in the propagation of the disease.

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