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Minireview

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INTRODUCTION

Although the precise definition of pulmonary fibrosis depends to some extent on one's discipline, for the purposes of this review it will be somewhat loosely defined as "abnormal deposition of collagen in the lung." The abnormality may thus be construed to be aberrations in the amount of collagen, the site of collagen deposition, and/or the nature of the collagen itself. This review will focus on early events, at both the cellular and molecular level, that culminate in such abnormalities. The lung is a particularly complex organ to study in this respect as there are many cell types present whose function is often poorly understood, even in normal lungs. Clearly it would be impossible to review the vast literature on cellular defense mechanisms in the lung in a single paper. This review will thus focus selectively on what is currently known about lung cell function and interaction during the early phases of specific types of pulmonary fibrosis. Studies discussed will illustrate the range of experimental approaches available for investigating early cellular events, both in animal models and in human diseases. Although the various cell types present in the lung are probably constantly interacting during fibrogenesis, our knowledge is too fragmentary at this point to present a coherent picture of all their interactions chronologically. Thus, cell types will be considered individually in terms of their behavior and response to various types of fibrogenic stimuli. Of particular interest is the question of how stereotyped is the lung's response to fibrogenic stimuli. Can we detect, for example, important differences in the sequence of cellular events following administration of particulate, gaseous, and systemic fibrogens: If so, is there a "final common pathway" to fibrosis

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at a certain point? Of interest also is the question of how fibrosis induced by known etiologic agents compares with the human fibrotic diseases of unknown etiology.

EPITHELIAL CELLS

The first lung cells to encounter inhaled or intratracheally instilled fibrogenic agents are the epithelial cells lining the respiratory tract. There appears to be a wide range of response of the type I and III pneumonocytes to such agents. Brody et al. [1, 2] have studied the deposition and epithelial transport of inhaled asbestos and silica. They found that most particles were deposited on the alveolar duct bifurcations, where they are rapidly taken up by the alveolar epithelial cells. Some particles appear to be transported to the alveolar interstitium within 3–4 hours after exposure. In these experiments, the epithelial cells did not appear to be damaged by their contact with the particulates. Bowden and Adamson [3, 4], however, found focal necrosis of type I cells in mouse lungs 24 h after instillation of 2 mg of silica.

Oxidant gases such as NO_2, ozone, or pure oxygen have long been known to directly damage epithelial cells; there is a large literature on the mechanisms by which oxygen metabolites may exert their cytotoxic effects [5, 6]. Systemically administered fibrogenic agents such as paraquat, radiation, and possibly bleomycin, may also exert a direct cytotoxic effect on lung parenchymal cells through generation of free radicals in vitro [6].

In general, type I pneumonocytes appear to be more sensitive to damage than type II pneumonocytes, although the relatively much greater surface area of type I cells (about 95% of normal alveolar epithelium) may merely present a correspondingly larger target for injury. Exposure of an animal to a pneumotoxin often results in a characteristic pattern of injury and repair in which type II cells begin proliferating to repair the epithelial lesions resulting from injury and death of type I cells. Despite their greater cytoplasmic differentiation, type II cells apparently serve as progenitor cells of type I pneumonocytes [7].

It has been suggested [8] that type II cell proliferation defines a critical period in terms of repair; disruption during this period may favor the development of pulmonary fibrosis. For example, systemic administration of butylated hydroxytoluene (BHT) to mice results in widespread necrosis of type I cells within 24 hours. On days 2 and 3, most dividing cells are type II pneumonocytes. If mice are exposed to relatively high levels of oxygen (70%) during this period, type II cells are severely affected, whereas dividing interstitial cells are not. Inhibition of the epithelial cell proliferation allows the interstitial cells to proliferate relatively unchecked, resulting in increased collagen production and pulmonary

fibrosis in this hypothesis. Similar results were found when animals were exposed to x-rays instead of O_2 [9]. These experiments also showed that the timing of the second insult was critical. If it did not coincide with the period of reepithelialization, then the enhanced fibrosis was not observed. Other investigators have found a similar enhancement of fibrosis in rats exposed to bleomycin followed by high levels of oxygen [10, 11]. Interestingly, paraquat, which by itself can induce irreversible fibrosis, has been observed to destroy both type I and type II cells [12, 13, 14]. These data suggest that epithelial cell control of fibroblast proliferation may be an important early mechanism in pulmonary fibrosis of various etiologies [15].

MACROPHAGES

The macrophage has been allotted a central role by many investigators in the initiation of fibrosis. The role of the macrophage in inflammatory and immunologic processes is enormously complex; the topic is periodically reviewed [16, 17]. Here we are primarily concerned with the effects of fibrogenic agents on pulmonary alveolar macrophages in terms of their recruitment and functional response.

In many models of fibrosis, macrophages begin accumulating extremely rapidly after exposure to the fibrogenic agent. For example, Brody et al. [2] have studied early cellular events in the lungs of rats exposed to asbestos or silica. After a 1-hour exposure to asbestos or a 3-hour exposure to inhaled silica, local populations of alveolar macrophages migrated to sites of particle deposition. Of particular interest was the observation that there was excellent correlation between the percentages of particle-containing macrophages lavaged from the lung and particle-containing macrophages remaining attached to alveolar surfaces.

Warheit et al. [18] hypothesized that complement may play a key role in particle-induced macrophage migration. Rats depleted of complement by cobra venom and mice genetically deficient in complement had far less macrophage accumulation following asbestos exposure. In addition, preliminary characterization by molecular weight of the chemotactic factor from lavage fluid was consistent with its being C5a. They have speculated that the complement component is derived from serum transudate [2].

Other investigators have shown that macrophages themselves may participate in macrophage recruitment. For example, Dauber and Daniele [19] found that guinea pig lung macrophages secrete a chemoattractant for macrophages, as well as for neutrophils and lymphocytes. Similarly, Kagan et al. [20] showed that alveolar macrophages from rats exposed to asbestos appear to secrete a chemoattractant for macrophages; they did not examine the effects of this chemoattractant on other effector

cells. Partial characterization suggested it to be a protein and heterogeneous with regard to molecular weight. A somewhat different mechanism has been proposed for macrophage recruitment in the human lung disease idiopathic pulmonary fibrosis (IPF). Circulating immune complexes, whose etiology is unknown, are believed to stimulate the macrophage accumulation [21].

The time course of macrophage accumulation in relation to the fibrogenic stimulus appears to vary depending on the insult, and to some extent perhaps on the experimental design. For example, Bowden and Adamson [3, 4] recently reported on the sequence of cellular events in mouse lungs following a single intratracheal instillation of silica. In contrast to the findings of Brody et al. [2] they found that neutrophils initially predominated (at 24 hours), while peak macrophage accumulation did not occur until 3 days later. Haschek et al. [22] reported a surge in macrophages in lung tissue of mice administered BHT + O_3 four days after the insult. Chandler et al. [23] examined cellular infiltration into interstitial lung tissue in hamsters instilled with bleomycin. They found macrophages to be the predominant cell initially; however, the first time point examined was 4 days after exposure. Their results certainly do not rule out a transient flux of neutrophils, for example, preceeding the macrophage accumulation.

The direct effects of fibrogenic agents on macrophages seem to vary considerably, depending on the specific agent and the experimental design. The effects of silica on macrophages in vitro, for example, have been extensively studied by many investigators (reviewed in reference 24). Such studies have emphasized the pronounced cytoxicity of silica, in particular its disruptive effect on lysosomal membranes. The role of the macrophage in silicosis has often been seen as somewhat passive; that is, its injury and death results in the release of a variety of soluble mediators that play a role in fibrogenesis. More recent studies suggest that macrophages may respond to silica in various ways. Brody et al. [25] have examined ultrastructural and functional changes in alveolar macrophages in rats exposed to silica for 1 and 5 hours. Immediately after exposure, numerous silica-containing macrophages could be observed both in lavage fluid and in situ; high percentages of silica-containing macrophages could still be observed 6 weeks later. Ultrastructural examination revealed that the silica-containing macrophages appeared normal with highly ruffled surfaces and numerous filopodia. Functional studies (measurement of oxygen consumption and phagocytosis) of lavaged macrophages revealed no abnormalities. Although the total amount of silica exposure was very low in this study, it shows that silica ingestion per se is not necessarily cytotoxic to macrophages.

Other investigators have observed a variety of macrophage responses to silica. Bowden and Adamson [3, 4] observed ultrastructural damage

to macrophages in mice instilled with 2 mg of silica. In contrast, Davis et al. [26] suggested that silica instilled in rats may actually stimulate macrophages (as measured by staphylococcal ingestion and killing and increased oxygen consumption). SEM of lavaged cells showed increased surface complexity of exposed macrophages, many of which contained silica. The dose of silica used in these studies was sufficient to produce fibrosis 9 months after exposure. Somewhat different results were found by Dauber et al. [27]. Macrophages obtained by bronchoalveolar lavage (BAL) from guinea pigs 6 months after silica exposure had impaired adherence and motility. Differences in route of exposure, species, amount of silica, and duration of exposure may account for the apparent variability in macrophage response to silica.

Early macrophage changes in response to asbestos exposure have also been examined [18]. Rats were exposed for 1 and 5 hours to asbestos. Macrophages were examined both in lung tissue and in lavage fluid. The 5-hour exposure resulted in a decrease in ruffled macrophages which correlated with diminished phagocytic capacity and decreased chemotactic responsiveness to zymosan-activated rat serum. The effects on macrophages of oxidant gases such as ozone and NO_2 have been studied both in vivo and in vitro. Both are directly toxic to PAMs and result in decreased phagocytosis and antibacterial activity [28–32].

It should be noted that a decrease in phagocytic capacity is not necessarily synonymous with functional impairment. For example, Tryka et al. [33] evaluated alveolar macrophages lavaged from lungs of hamsters exposed to bleomycin and oxygen at time points ranging up to 120 days. The macrophages were increased in number but had a decreased amount of cell surface antigen, indicating they were relatively immature. Tryka et al. attribute their markedly decreased phagocytic capability as being at least partly due to the decrease in surface antigen. This study also suggests that fibrosis may be associated with increased macrophage turnover.

Once macrophages have been recruited, they are capable of releasing a great many mediators that modulate the behavior of other effector cells in the lung. Kazmierowski et al. [34] and Merrill et al. [35] have reported that alveolar macrophages secrete at least two factors chemoattractant for neutrophils. Dauber and Daniele [19], as noted earlier, reported that guinea pig lung macrophages secrete chemoattractants for macrophages, neutrophils and lymphocytes.

Macrophages may also modulate effector cells in another way. Stimulated macrophages are capable of releasing arachidonic acid from cell phospholipid pools. Further metabolism through the cyclooxygenase pathway results in the production of PGE_2 and PGI_2 among other products. In vitro data show that the E prostaglandins and PGI_2 suppress a variety of neutrophil, macrophage and lymphocyte functional responses;

they may also participate in the induction of suppressor T cells. In marked contrast, metabolic products of arachidonic acid resulting from the lipooxygenase pathway appear to enhance the inflammatory response [36].

Macrophage secretion of effector cell chemoattractants appears to be increased in fibrosis. Schoenberger et al. [37] showed that asbestos stimulates alveolar macrophages to increase neutrophil migration to the lung. Wesselius et al. [38] found that macrophages from bleomycin-exposed animals secreted increased amounts of neutrophil chemoattractant as compared with macrophages from controls. Since bleomycin did not directly stimulate macrophages, the mechanism for the increased macrophage secretion is unclear. The authors postulate that the stimulus may result from cell injury caused by bleomycin. Kaelin et al. [39] also studied macrophage-derived chemoattractants from hamsters instilled with bleomycin. Interestingly, they found that neutrophil chemoattractant secretion was increased (relative to controls) at day 4, while secretion of lymphocyte chemoattractant was increased at day 8. Ultrastructural analysis of lung tissue revealed the presence of a predominantly neutrophilic inflammatory infiltrate at day 4, while at 8 days there were both lymphocytes and neutrophils. These data suggest that macrophages may be regulating the sequence of effector cell migration following injury. Schoenberger et al. [40] found that BAL cells from monkeys exposed to paraquat released large amounts of neutrophil chemoattractant 1–2 weeks after exposure; by the third week release was not detectable. Macrophages lavaged from patients with IPF have also been shown to release a neutrophil chemotactic factor [41].

The interaction between macrophages and fibroblasts is particularly complex since macrophages appear to have the capacity both to stimulate fibroblasts and to suppress them. Stiles et al. [42] have proposed that fibroblast growth factors may be divided into competence factors (which provide a signal early in the G_1 phase of the cell cycle) and progression factors (which provide a signal later in G_1, stimulating the cell to replicate). Alveolar macrophages have been shown to secrete a growth factor (AMDGF, MW = 18,000) for fibroblasts that is distinct from other described growth factors, including epidermal growth factor, platelet-derived growth factor (PDGF), growth hormone, fibroblast growth factor, and interleukin-1 [43]. AMDGF is believed to function as a progression factor and to stimulate fibroblasts to produce their own progression factor. Macrophages also secrete fibronectin, a large glycoprotein known to mediate cell-matrix interactions through a variety of functions, including its chemotactic properties [36]. Fibronectin is believed to act as a competence factor for fibroblast growth [44]. In addition, fibronectin is chemotactic for fibroblasts. Indeed, macrophage-derived fibronectin is 1000-fold more potent as a chemoattractant than is plasma

fibronectin [45]. Macrophages also appear to be capable of suppressing fibroblast growth. Elias et al. [46] demonstrated that supernatants from normal human alveolar macrophages inhibit growth of log-phase fibroblasts. The inhibitory capacity of the supernatant was directly related to its capacity to stimulate fibroblast prostaglandin production. Macrophage subpopulations were separated by density gradients; the factor(s) appeared to be preferentially elaborated by smaller and denser macrophages. In some respects this factor resembles those described by Korn et al. [47] and Clark et al. [48].

Investigation of the effects of specific fibrogenic agents on macrophage regulation of fibroblast growth illustrates the complexities involved. For example, Lugano et al. [49] examined the effects of lavaged macrophages on fibroblasts at various times after silica exposure. At 2 and 14 days after exposure, macrophages depressed fibroblast proliferation, while at 42 days macrophages stimulated fibroblast proliferation. Clark et al. [48] found that macrophages from hamsters instilled with bleomycin had a greater suppressive effect on fibroblast proliferation and collagen synthesis compared with control macrophages. Suppression was associated with increased PGE_2 and intracellular cAMP. Interestingly, fibroblast suppressive activity decreased in the first days after bleomycin instillation, and then increased after 8 days. The authors suggest that this suppressive activity may be a mechanism for modulating fibroblast proliferation and fibrosis following fibrogenic exposure. Schmidt et al. [50] recently published data suggesting that macrophage-derived interleukin-1 (IL-1) may play a role in fibroblast proliferation. Although other groups have not found that IL-I has fibroblast-stimulating properties, the authors point out differences in experimental design that might account for this discrepancy. In this particular study, peripheral blood monocytes were used; it should be noted that alveolar macrophages are also capable of secreting IL-I.

Changes in macrophage-derived fibronectin have been described in several specific types of fibrosis. Lavage fluid from monkeys exposed to paraquat [40] contained large amounts of fibronectin up to 3 weeks after exposure. Macrophages cultured from lavage fluid had increased secretion of fibronectin at 1–2 weeks compared with controls. The exposed macrophages also secreted AMDGF at one week; no AMDGF was detected in controls. Recently, Rennard et al. [51] studied fibronectin production by alveolar macrophages obtained by BAL from patients with various fibrotic lung diseases. Macrophages from patients with IPF produced almost twenty times as much fibronectin (on a per cell basis) than did macrophages from control patients. Patients with sarcoidosis and various other fibrotic diseases also had eleveated secretion of fibronectin. The macrophage-produced fibronectin was chemotactic for fibroblasts. These data suggest that increased production of fibro-

nectin may be a common feature of several interstitial lung diseases. Fibronectin may also participate in the clearance from lung of partially degraded tissue [51, 52] that may occur in fibrosis.

One other potential role of macrophages deserves mention. It has long been proposed that a key event in silicosis is the release of a "fibrogenic factor" from silica-damaged macrophages that specifically increases collagen synthesis by fibroblasts. One of the earliest suggestions of the existence of such a factor was by Heppleston and Styles [53]. Since then, several researchers have attempted to further characterize and isolate such a fibrogenic factor, with somewhat mixed results (reviewed in reference 24). Recently, Brown and Gormley [54] attempted to duplicate precisely the experimental conditions of Heppleston and Styles; they were unable to demonstrate the existence of a fibrogenic factor. They suggested that some of the conflicting results reported in the literature might result from the possibility that macrophages may produce factors that both inhibit and stimulate collagen synthesis by fibroblasts, perhaps analogous to proliferative and suppressive factors. Work by Kulonen's group [55] supports such a hypothesis.

In many of the studies discussed above, bronchoalveolar lavage has been used to obtain macrophages for study. Given the heterogeneity of macrophages, it is unclear if the populations present in lavage fluid accurately reflect those populations actually present in lung tissue, particularly in disease states. The problem is compounded by the fact that there is no consensus as to the appropriate functional and/or structural definitions of such macrophage subpopulations. Brain et al. [56] and Mason [57] have reviewed some of the potential problems in studying lavaged macrophages. Lum et al. [58] recently presented data illustrating the problems that can arise in studying lavaged cells. They performed detailed morphometric analyses on centriacinar macrophages in situ and lavaged pulmonary macrophages from control and ozone-exposed rats. They found significant differences in most parameters studied between the lavaged and in situ macrophages in both the control and ozone-exposed rats. These data suggest that, at least in this model of fibrosis, lavaged macrophages are not representative of those present at the sites of greatest lung damage. Clearly, similar problems may exist in studying any of the effector cells obtained by lavage.

NEUTROPHILS

Neutrophils, with their large array of potentially damaging secretory products, clearly have the capacity to affect lung structure and function. However, their precise role, if any, in the etiology of pulmonary fibrosis remains unclear.

Neutrophils have been specifically implicated as having a critical role

in early cellular events occurring in several types of chronic interstitial lung disease in humans, including IPF, familial pulmonary fibrosis, interstitial fibrosis associated with the collagen-vascular diseases, asbestosis, and histiocytosis-X. Crystal's group at NIH has investigated a wide number of interstitial lung diseases for some years; they have classified such diseases as either neutrophilic or lymphocytic. Crystal et al. [41] have recently reviewed the data underlying this classification scheme. They believe that in most interstitial lung diseases an alveolitis precedes the derangements in structure. The classification scheme is based on the relative proportions of the different effector cells participating in the alveolitis. In the neutrophilic disorders, neutrophils may represent anywhere from 5 to 50% of all cells recovered in lavage fluid. Of particular interest is their capacity to secrete collagenase. Gadek et al. [52] showed that active collagenase is present in BAL fluid from IPF patients but not from controls or patients with sarcoid. No elastase activity was found. Since the collagenase from the IPF patients was found to be specific for type I collagen, they suggested that the neutrophil is the most likely source, since macrophage collagenase attacks both type I and type III collagen. The destruction of the normal collagen framework of the alveoli by collagenase may thus set the stage for remodelling with abnormal collagen deposition. However, it should be emphasized that to our knowledge there is no direct evidence for breakdown of lung collagen as an obligatory step in the development of lung fibrosis, a group of diseases associated with increased collagen content of the lung.

The role of neutrophils in acute respiratory distress syndrome (ARDS) has also been examined. Jacob et al. [59] have presented evidence that neutrophils as well as platelets can aggregate intravascularly, and that this phenomenon of leukoembolization may be the underlying cause of tissue damage in the pulmonary fibrosis-calcinosis syndrome found in long-term hemodialysis patients as well as in ARDS. In the case of hemodialysis, it was found that when plasma contacted the cellophane membrane of the dialyzer, complement was activated. This led to increased neutrophil stickiness, aggregation, and sequestration in the lung. The authors postulate that repeated plugging of lung microvasculature by the aggregated leukocytes may result in permanent damage. They suggest that similar events may occur in ARDS, since the complement system may be activated during some of the events that can precede ARDS, such as trauma, shock, and sepsis. Prolonged circulation of C5a has been observed in the plasma of ARDS patients. As in the case of IPF, collagenase activity was directed against both type I and type III collagen; the contribution of neutrophils is unclear.

Neutrophils have been studied in animal models of fibrosis with somewhat conflicting results. For example, Warheit et al. [18] found no evidence for neutrophil accumulation in lungs of rats immediately after

exposure to asbestos. In contrast, Schoenberger et al. [37] found increased numbers of neutrophils in lavage fluid of guinea pigs intratracheally instilled with asbestos. However, it is difficult to interpret the significance of neutrophils appearing soon after this method of exposure, since a nonspecific response may well be elicited by instillation of saline or inert particulates. Adamson and Bowden [3, 4] recently presented data in mice suggesting that neutrophils may play an important role in silicosis. Increased neutrophils seen 2 days after instillation rapidly declined and probably represented a nonspecific response to instillation. Neutrophils were observed to increase again at 8 weeks, paralleling the response of macrophages. The authors suggest that neutrophils may release enzymes after interacting with silica that contribute to continued fibroblast stimulation.

Sykes et al. [61] have done similar studies investigating cells and cell products in rats intratracheally instilled with silica. They found a dose-dependent increase in macrophages, neutrophils, and lymphocytes 50 and 100 days after instillation. Enzymes in lavage fluid were also measured. LDH activity was correlated with neutrophil and lymphocyte increases, suggesting it might be derived from these two cell types. Collagenase and peptidase, also derived from neutrophils, were found to be increased as well. These data suggest that neutrophils may have an ongoing role in fibrosis induced by silica. It remains unclear whether or not neutrophils participate in very early events.

Another experimental approach to investigating the role of neutrophils in fibrosis is to examine the effects of neutrophil depletion. Thrall et al. [62] examined the effects of bleomycin in rats depleted of neutrophils by anti-neutrophil serum. The authors suggest that the increased amount of total lung collagen at one week in depleted rats as compared with control rats exposed to bleomycin may indicate that neutrophils play a role in restricting collagen deposition, possibly through secretion of collagenase. It should be noted, however, that unexpectedly large increases in collagen content were observed in this study in the bleomycin-exposed animals at one week.

One of the problems inherent in using anti-neutrophil serum is that neutrophil levels return to normal within 7 days. Phan et al. [63] therefore examined the effects of bleomycin in beige mice, a mutant strain of mouse that has a functional deficit in neutrophil response. They found that beige mice responded more intensely to bleomycin than did heterozygote controls both 2 weeks and 1 month after drug administration as assessed by measurement of lung collagen synthesis rates and of lung collagen content, again suggesting that neutrophils might play a role in attenuating fibrosis.

LYMPHOCYTES

The secretory products of lymphocytes, like those of macrophages, are involved in a great many immunological and inflammatory processes, and are capable of regulating the behavior of macrophages, neutrophils, and fibroblasts. Lymphokines that may be of particular importance include macrophage activating factor [64], macrophage migration inhibition factor (MIF) [65], leukocyte inhibitory factor [65], monocyte chemotactic factor [66], fibroblast chemotactic factor [67], and a fibroblast activating factor that stimulates both proliferation and collagen synthesis [68].

Lymphocytes are believed to play an important role in the pathogenesis of several human interstitial lung diseases such as sarcoidosis, hypersensitivity pneumonitis, and berylliosis [41]. In these disorders, BAL analysis reveals that lymphocytes usually comprise more than 15% of the cells participating in the alveolitis. Sarcoidosis is perhaps the best characterized of these disorders. Forty percent of the lavaged cells may be lymphocytes, and of these 90% may be T cells. In addition to the expansion of the T-cell population as a whole, there is also a relative increase in the proportions of T helper cells to T suppressor cells [41]. These activated T cells release such lymphokines as monocyte chemotactic factor and MIF that are important in granuloma formation. The hypergammaglobulinemia and increased angiotensin-converting enzyme usually seen in sarcoidosis may be indirect consequences of T-cell activation [69]. As noted earlier, macrophages may play a major role in expanding the T cell population by their release of interleukin-1. Why macrophages may be activated in a different fashion in sarcoidosis than in IPF remains unclear.

Kravis et al. [70] examined lymphocytes from patients with various fibrotic disorders including IPF, fibrosis secondary to known etiologic agents, and hypersensitivity pneumonitis. They found that T-lymphocytes from patients with fibrosis were sensitized to type I collagen. Exposure of lymphocytes from these patients to type I collagen in vitro resulted in the production of MIF and specific cytolysis of collagen-coated cells. It is unclear whether this cell-mediated reactivity to collagen plays a primary role in the pathogenesis of pulmonary fibrosis, or whether it is secondary to chronic lung injury induced by other effector cells.

Several investigators have examined the role of lymphocytes in animal models of pulmonary fibrosis. Thrall et al. have shown that anti-lymphocyte globulin suppresses fibrosis in rats administered bleomycin [71]; T-cell depletion by adult thymectomy had a similar effect [72]. Schrier et al. [73] found that spleen cells obtained from bleomycin-instilled rats exhibited a proliferative response to type I collagen, but not to type III

collagen. This response reached its maximum 14 days after exposure, but decreased to control values between 31 and 56 days. Collagen synthesis rate in the lung also reached maximum values at day 14. The authors suggest that the similarities between the time course of cellular sensitization and of collagen synthesis suggest that cellular immunity may play an important role in bleomycin-induced fibrosis. Schrier et al. [74] pursued their investigation of cellular immunity by studying the effects of intratracheally instilled bleomycin on nude mice. Collagen synthesis rates were significantly higher than control values in both nude and euthymic mice 14 days after bleomycin instillation. However, the values for the nude mice were significantly lower than the values for the euthymic mice. These findings conflict with those of Szapiel et al. [75] who found no differences between nude and euthymic mice. However, Schrier et al. point out that dose, route and schedule of administration, and method of assaying fibrosis were very different. They suggest that their data indicate that the prevention of the full expression of fibrosis by the athymic mutation suggests that T lymphocytes may play an important role in bleomycin-induced fibrosis.

Thrall and Barton [76] examined lymphocyte populations in lung lavage fluid and in lung tissue in rats instilled with bleomycin. After bleomycin administration there was a significant increase in the percentage of B-cells in lung tissue within 3 days; peak levels occurred at 7 days. Changes in the T-cell populations were also observed in lung tissue. Fourteen days after bleomycin the helper: suppressor ratio was 2:1, at 30 and 120 days the ratio was 1:2. Control values were 1:1. These changes in lymphocyte populations were not reflected in lavage fluid analysis. The authors suggest that these data provide further evidence that a specific immune response to collagen is elicited in the fibrosis of bleomycin, perhaps analogous to that seen in IPF. Furthermore, they hypothesize that the helper T cells may contribute to collagen synthesis, while the suppressor cells, which come to prominence later, may suppress collagen synthesis in some manner, thus contributing to the healing and repair process. They also issue an important caveat that the lymphocyte populations observed in BAL may not necessarily reflect those actually present in lung tissue.

EOSINOPHILS

Although eosinophils have long been recongnized as playing a role in such diseases as chronic eosinophilic pneumonia, a disorder associated with eosinophilic infiltrates and interstitial fibrosis [77], their role remains poorly understood either in normal or diseased lungs. Eosinophils certainly have the capacity to injure lung parenchyma and matrix. Davis et al. [78] demonstrated that unstimulated guinea pig eosinophils were

cytotoxic to fibroblasts, epithelial cells and mesothelial cells. Partial inhibition of this cytotoxicity by catalase and by DMSO suggest that the damage may be mediated by hydrogen peroxide or by oxygen-derived free radicals. Davis et al. [78] examined eosinophils from the BAL fluid of patients with interstitial lung disease; these cells were also spontaneously cytotoxic for lung epithelial cells. Another potentially toxic secretory product is major basic protein (MBP), a highly toxic polypeptide that may account for some of the tissue damage seen in chronic hypersensitivity reactions and the pulmonary eosinophilic syndromes. For reviews of current concepts of eosinophil structure and function, see Schatz et al. [79], Butterworth and David [80], and Mahmoud and Austen [81].

Eosinophils have been studied in experimental pulmonary fibrosis. Chandler et al. [23] have provided data on the temporal relationship between various effector cells in the lung interstitium of rats instilled with bleomycin. They found that eosinophils did not begin to increase until 20 days after bleomycin instillation. Peak levels were seen at 30 days, coinciding with secondary increases in macrophages and neutrophils. Similarly, Thrall et al. [82] found eosinophils scattered throughout the interstitial infiltrates of rats instilled with bleomycin. They also examined peripheral blood and found that eosinophil levels in the bleomycin-instilled animals reached 10 times normal levels by day 17. These levels gradually decreased to normal values by 60 days. Pulmonary eosinophilic infiltrates have also been observed in humans receiving bleomycin therapy [83]. As previously noted, T cells are believed to play a role in bleomycin-induced fibrosis. Since T cells are associated with eosinophilia, they may participate in recruitment of eosinophils.

Sun et al. [84] have developed a specific animal model of eosinophilic interstitial disease in guinea pigs exposed to an aerosol of polymyxin B sulfate. Eosinophils recovered from the treated animals were found to have a high level of spontaneous cytotoxicity for human fetal lung cells as compared with eosinophils from control animals. Although there was no evidence of fibrosis after 4 weeks of treatment, the authors speculate that the alveolitis is probably a precursor of permanent parenchymal injury and fibrosis.

MAST CELLS

The role of mast cells in fibrogenesis is perhaps even more elusive than that of eosinophils. It has long been suggested that mast cells have an important role in regulating connective tissue components. Histologic and autoradiographic studies have suggested that mast cell degranulation may affect fibroblast proliferation [85, 86], while EM studies suggest they may alter the structure of the extracellular matrix [87]. Recent

in vitro experiments showed that mast cell granules are taken up by fibroblasts in culture; 24–48 hours later culture supernatants were found to have increased levels of collagenase and β-hexosaminidase and decreased fibronectin [88]. Thus, mast cells in connective tissue appear to be capable of influencing fibroblast behavior, as well as exerting a direct effect on the extracellular matrix.

Parenchymal mast cell hyperplasia has been reported in patients with IPF and sarcoidosis [89], although Fox et al. [90] could not confirm these findings. Increased histamine levels have been reported in BAL from patients with IPF [91]. Goto et al. [92] studied the distribution, density, and histochemical subtype of mast cells throughout the respiratory tract of rats instilled with bleomycin. Fourteen days after instillation, the mast cell density in the parenchyma was 10 times control values. Although the mechanisms that stimulate this mast cell hyperplasia are unknown, Goto et al. speculate that cellular immunity may be involved. Recent work by Schulman et al. [93] suggest a possible role for macrophages in mast cell function. They showed that alveolar macrophages are capable of stimulating histamine release from mast cells.

FIBROBLASTS

Presumably the cells directly responsible for synthesizing the "abnormal" collagen of pulmonary fibrosis (whether it be abnormal in amount, location, or type) are the fibroblasts. Although they often seem to be perceived as simply responding to signals from the effector cells and their mediators, fibroblasts may on occasion play a more active role in directing the course of fibrosis.

In the previous sections, we have seen a variety of ways in which fibroblasts may be recruited and stimulated by effector cells that have interacted with the fibrogenic stimulus. However, in some cases fibroblasts may directly interact with the fibrogenic agent. The effect of silica on fibroblasts has been controversial. Early studies, which did not investigate the phenomenon in a systematic way, provided no evidence for stimulation of collagen synthesis [24]. Richards and Wusteman [94] and Richards et al. [95] have done the most extensive series of studies on the effects of different concentrations of silica added to fibroblasts throughout their growth cycle. They found no evidence of stimulation with log-phase cells, but ther was an increase in collagen in stationary-phase cells exposed to silica. Some of the potential pitfalls in interpreting such studies, however, have been reviewed [24].

Several workers have examined the effects of bleomycin on fibroblasts. Sterling et al. [96] exposed human fetal lung fibroblasts to bleomycin for 48 hours. Collagen synthesis was increased in the exposed fibroblasts; however, degradation was also increased. To determine the relative

increase in collagen synthesis without the effects of intracellular degradation, polysomes were isolated. Polysomes from bleomycin-treated fibroblasts synthesized twice as much collagen as control polysomes. Noncollagen protein synthesis was not affected. Similar results have been reported by Clark et al. [97] and Phan et al. [98]. Robin and Juhos [99] examined the effects of bleomycin, hyperoxia, and the presence of lung macrophages on collagen synthesis by human WI-38 fibroblasts. They found that bleomycin alone directly stimulated collagen synthesis. The addition of hyperoxia and/or the presence of lung macrophages did not further increase collagen synthesis. Hyperoxia alone significantly decreased collagen synthesis. However, hyperoxia in the presence of lung macrophages increased collagen synthesis about the same amount as bleomycin did. There is also evidence that bleomycin affects fibroblast proliferation. Absher et al. [100] examined the effects of in vivo and in vitro exposure to bleomycin on growth characteristics of fibroblasts. For the in vivo exposure, they isolated fibroblasts from rats 7 days after intratracheal instillation of bleomycin. Both types of bleomycin exposure appeared to decrease growth of fibroblasts in comparison with controls. Phan et al. [98] used a similar system of in vivo exposure and found no effect on growth. However, since they isolated fibroblasts 14 days after bleomycin instillation, they suggest that the difference in timing may account for the apparently discrepant findings. That is, during the first week after instillation, bleomycin toxicity may impair fibroblast growth, while during the second week recovery may be occurring. Furthermore, this recovery may involve recruitment or selection of a population of fibroblasts with different growth characteristics.

Fibroblasts are believed to play a role in epithelial cell growth and function. Fibroblast pneumonocyte factor (FPF) has been isolated from glucocorticoid-treated fibroblasts. It has been shown to stimulate disaturated phosphatidyl choline synthesis in whole lung in vivo [101] and in isolated type II penumonocytes [102]. A small somatomedin-like growth factor specific for penumonocytes is believed to be secreted by fibroblasts after pneumonectomy [103]. Recently, Tanswell [104] has shown that pulmonary fibroblasts exposed to hyperoxia in vitro secrete both an epithelial cell growth factor and a lipid-synthesis stimulating factor. These factors appear to differ from the previously described stimulatory factors.

Fibroblasts may also affect effector cells. Although their predominant function is apparently collagen and mucopolysaccharide production, they have also been shown to secrete a number of biologically active products, such as Clq and interferon [105]. Sobel and Gallin [106] demonstrated that cultured fibroblasts produce a factor chemotactic for both monocytes and neutrophils. In addition, fibroblast culture fluid is capable of generating chemotactic activity from human serum,

probably by cleaving C5a from C5. Tubergen et al. [107] showed that fibroblasts are capable of producing macrophage migration inhibition factor. Whether such "fibrokines" play a role in pulmonary fibrosis has yet to be demonstrated. Collagen itself is another fibroblast product with chemotactic properties. Stecher [108] and Postlethwaite and Kang [109] showed that type I collagen and its isolated chains are chemotactic for monocytes but not neutrophils. In contrast, Chang and Houck [110] showed that rat collagen was chemotactic for rat neutrophils in vivo. Type II collagen was not chemotactic for either monocytes or neutrophils.

As noted earlier, some types of pulmonary fibrosis involve abnormalities in the type of collagen being made. Although recruitment and proliferation factors might account for the accumulation of fibroblasts and the increased deposition of collagen, they do not in themselves account for some of the qualitative abnormalities found in the collagen of fibrotic lungs. For example, Seyer et al. showed that collagen type ratios in IPF are abnormal, in that there is an increase in type I collagen relative to type III collagen [111]. Similar shifts have been demonstrated in lungs from patients dying of ARDS [112] and IRDS [113]. Increased type I:III ratios have also been demonstrated in animal models of acute pulmonary fibrosis [114, 22]. A shift in type I:type III ratios has also been demonstrated in several in vitro systems. Clark et al. [97] observed that fibroblasts exposed to bleomycin in vitro not only had increased collagen synthesis rates, but also synthesized relatively more type I collagen when compared with controls. Similarly, Phan et al. [98], found that collagen synthesized by fibroblasts exposed either in vivo or in vitro to bleomycin had an increased ratio of type I to type III collagen. Although the mechanism for this shift is unknown, there are many possible explanations. Clones of fibroblasts responsive to recruitment/proliferation factors may preferentially synthesize type I collagen as compared with resident fibroblasts normally present. In fact, Kelley et al. [115] recently showed that lung macrophages caused cultured fibroblasts to alter the ratios of types I and III collagen being synthesized. They suggested that macrophages may exert an influence on collagen type ratios by selectively stimulating a subpopulation of fibroblasts with a predetermined collagen phenotype. Alterations in the extracellular matrix, resulting from inflammatory mediators secreted by various effector cells, might also cause the fibroblasts to switch the collagen phenotype being synthesized. There is ample evidence from in vitro experiments that altering culture conditions can alter collagen phenotypes [116–121]. Fibrosis collagen may also be abnormal with respect to crosslinking. Alterations in crosslinks in experimental silicosis [122, 123] and in bleomycin-induced fibrosis [124] have recently been described. As in the case of alterations in collagen type ratios, it is unclear if the mechanisms can be ascribed to changes in the

clones of actively synthesizing fibroblasts or to changes in the milieu that secondarily affect the nature of the collagen being made.

SUMMARY AND CONCLUSIONS

In this review we have surveyed recent investigations of early cellular events in pulmonary fibrosis both in animal models and in human diseases. Analysis of the interactions of the numerous cell types in the lung following injury is an almost overwhelmingly complex enterprise. In the animal models experimental design has a profound effect on results, making it difficult to compare studies when species, fibrogenic agent, dose, route of exposure, schedule of administration, time course, and analytical methods may not be equivalent. In human diseases we are rarely able to obtain data at precisely the same time point in the course of the disease even among patients in the same study, and possible confounding variables present are legion. Transcending these difficulties for the moment, can we draw any conclusions from our current knowledge of early cellular interactions in pulmonary fibrosis?

What is striking is not that there are so many agents that can potentially induce pulmonary fibrosis, but that the lung has such capabilities for recovery. Although the major effector cells may all initially participate in damaging the lung and initiating fibrosis, there is evidence that they may also have the capacity to participate in subsequent repair. Macrophages may initially recruit fibroblasts and stimulate them to proliferate, only to suppress them subsequently. Macrophage production of prostaglandins can lead to suppression of macrophage, neutrophil and lymphocyte responses, thus attenuating tissue injury and the development of fibrosis. Neutrophils may initially release toxic metabolites and enzymes that damage parenchyma. However, there is evidence that they may later play a role in attenuating fibrosis, perhaps through collagenase secretion, or through as yet unknown mechanisms. Lymphocytes may initially participate in a number of damaging ways by secreting chemoattractants for other cells and participating in destructive autoimmune processes. However, there is evidence that subpopulations of T cells may dramatically shift during the course of fibrosis, leading to attenuation of the process. It may thus be useful to consider irreversible pulmonary fibrosis as the end result of a process in which the balance of normal injury/repair mechanisms is disrupted. There is clearly no single "fibrogenic event." Rather, there seem to be a number of places where disruption of balance/repair processes may begin. In diseases of unknown etiology such as sarcoidosis or IPF, loss of control may occur at the genetic level, leading to the destructive alveolitis that is the apparent precursor of fibrosis. The "motheaten mouse" appears to be an animal model of this type of genetic disruption [125]. Any systemically overwhelming event, such as

those that can precede ARDS, may be yet another route to loss of control. In the case of some exogenous agents, perhaps there is a critical degree of damage to the alveolar epithelium that leads to irreversible fibrosis; such damage can occur when exposure to bleomycin, for example, is followed by exposure to oxygen. Similarly, exposure to paraquat alone can rapidly lead to destruction of type I and type II pneumonocytes with resulting denudement of large areas of the alveoli. Another way in which control mechanisms may be unbalanced is by total body burden of a fibrogenic agent. Experiments with asbestos and silica have indicated that low-dose exposures (regardless of route) may lead to transient lung injury, while larger doses (even single exposures) may lead to progressive fibrotic changes.

Another important question concerns similarities and differences in the lung's response to various fibrogenic stimuli. Even if the initiating events differ, is there any sort of final common pathway to fibrosis? Although some fibrogenic agents may provoke similar sequences of early cellular events, if we survey pulmonary fibrosis as a whole it seems unlikely that there is any point except at the very late stages of fibrosis where the pathways may actually converge.

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