The Extrapulmonary Origin of Fibroblasts
Stem/Progenitor Cells and Beyond

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Although intrapulmonary progenitor cells are traditionally believed to be the source for regenerating cells in response to lung injury, recent mounting evidence indicates that a significant proportion of the mesenchymal cells involved in this repair/remodeling process may be derived from extrapulmonary sources, such as the recently described circulating fibrocyte as well as other bone marrow–derived progenitor cells. Studies tracking CD34 and/or CD45 markers of fibrocytes show their presence in injured murine lung tissue. Moreover, bone marrow chimeric mice with green fluorescence protein (GFP)–expressing marrow cells show abundant GFP-expressing fibroblasts in their lungs in response to lung injury. However, although fibrocytes express CD34 and CD45, and appear to have the capacity to differentiate to myofibroblasts, these properties are not evident in the bone marrow–derived fibroblasts. Induction of CCL21 (SLC) and CXCR12 (SDF1α) in injured lung tissue, and their respective cognate receptors, CCR7 and CXCR4, in fibroblasts from injured lungs, suggests recruitment of these extrapulmonary progenitor cells via these chemokines. This is supported by evidence that antibody neutralization of CXCR12 reduces recruitment of fibrocytes and pulmonary fibrosis. In contrast, other studies suggest a protective effect for bone marrow–derived cells. Thus, although suggesting that influx of extrapulmonary fibroblast progenitor cells occurs in response to lung injury, these recent studies do not yet provide clear insight as to the actual phenotype and fate of the recruited cells, the identity of the progenitor cell population in bone marrow, and most important, the function or role of these cells in pathogenesis of the idiopathic interstitial pneumonias.

Keywords: fibroblasts; myofibroblasts; stem cells

Fibroblasts, especially in their activated differentiated state when they are referred to as myofibroblasts, are considered to be key elements in the pathogenesis of fibrosis. These cells are found localized in sites of active fibrosis and in morphologically distinct areas termed "fibroblastic foci." These foci, which consist of fibroblast-like cells and myofibroblasts, are considered to be a key element in the histopathologic diagnosis of usual interstitial pneumonitis (UIP), and their presence is believed to be indicative of progressive fibrosis (1–3). Thus, understanding the origin of these cells and the mechanism of recruitment should shed further insight into the basis for the development of progressive fibrosis instead of successful healing and regeneration. The mechanism underlying the emergence of these cells is not fully understood, although the derivation of myofibroblasts from fibroblasts is well studied using cells in culture. Previously, it has been suggested that myofibroblasts are potentially derived from preexisting intrapulmonary mesenchymal precursors, such as peribroncholiolar and perivascular adventitial fibroblasts. Recent studies, however, have suggested potential derivation from extrapulmonary progenitor cells as well. Part of the impetus for these studies is based on recent evidence of greater plasticity of bone marrow stem cells than has been previously believed. Although it is accepted that hematopoietic elements, such as leukocytes, are derived from bone marrow hematopoietic stem cells (HSCs), the situation is much less clear with respect to the origin of the progenitor cells that give rise to the structural cells involved in lung repair and regeneration. However, given the recent evidence of plasticity of adult bone marrow stem cells and existence of circulating progenitor cells, potential derivation of lung fibroblasts and myofibroblasts from extrapulmonary cells needs to be considered.

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EXTRAPULMONARY CELLS AS LUNG STRUCTURAL CELL PROGENITORS

For the purposes of this discussion, lung structural cells can be simplistically divided into epithelial, endothelial, and mesenchymal elements because repair and regeneration in response to injury would require reconstitution of all these key components of lung tissue. Although the focus of this review is on the fibroblastic component, mention will be made of the other elements, partly in support of the concept that extrapulmonary origin of fibroblasts may not be unique to only this mesenchymal element. A key element in repair/regeneration is vasculogenesis, with the endothelial cell being the main component. In addition to proliferation of surviving endothelial cells at the sites of injury, the presence of bone marrow–derived circulating endothelial progenitor cells also has been shown to play a role in vasculogenesis in tissue repair (4–7). Moreover, these cells appear to be important in angiogenesis associated with the response to elastase-induced lung injury (7) and their presence in the circulation correlates with survival in acute lung injury (8).

With respect to the epithelial element in the context of alveolar epithelial repair/regeneration, surviving type II pneumocytes are considered to be the progenitor cells for type I pneumocytes critical for reepithelialization. In view of the importance of reepithelialization in successful repair and prevention of fibrosis (9–12), any impairment of this process will likely contribute to formation of fibroblastic foci and progressive fibrosis. Recent studies suggest that there may be an additional mechanism for alveolar epithelial regeneration, whose failure may reduce the lung repair response to injury. These studies reveal HSCs may give rise to lung epithelial cells under certain conditions, including when the lung is injured (13–20). However, this appears to occur at a relatively low frequency, and other studies cannot confirm this degree of HSC plasticity (21, 22). Additional studies are needed to clarify the extent and significance of this extrapulmonary contribution to lung epithelial regeneration and its potential importance for prevention and/or cell therapy of fibrosis.

EXTRAPULMONARY ORIGIN OF FIBROBLAST-LIKE CELLS

Mesenchymal cells are considered to be key contributors of the abnormal deposition of extracellular matrix that is the hallmark

of fibrosis. However, the heterogeneity of these cells has complicated efforts at understanding the fibrotic process. Significant phenotypic differences characterize these fibroblast-like cells found in fibrotic lesions, such as fibroblastic foci, although *in situ* analysis of type I collagen expression in rat lung tissue sections indicates localization to a distinct subpopulation, known as the myofibroblast (23). Although other fibroblast-like cells have the capacity to also elaborate interstitial collagens, the level of expression by the myofibroblast is significantly higher and also is accompanied by higher levels of profibrotic cytokines, such as transforming growth factor β (TGF-β) (23–25). These properties of the myofibroblast embody many of the hallmarks of fibrosis, which have resulted in greater focus being placed on analysis of the origin and fate of these cells. A kinetic study to monitor the *de novo* appearance of the myofibroblast in the bleomycin-induced rat model relies on expression of α-smooth muscle actin as a marker for this cell (23). The adventitia of the distal airways and adjacent blood vessels is normally sparsely populated by scattered fibroblasts, which do not show much in the way of collagen gene expression. Increasing cellularity is noted in these areas after bleomycin-induced injury, associated with significantly higher levels of collagen expression by the third day after injury. The first α-smooth muscle actin–expressing cell is found in these areas at about this time point, in an area that is normally devoid of such cells. This evidence indicates that the myofibroblast likely originates from the peribronchiolar and/or perivascular adventitial fibroblast (23). Indeed, there is abundant evidence that isolated fibroblasts can be stimulated to differentiate to myofibroblasts on treatment with cytokines, such as TGF-β, interleukin 4 (IL-4), and IL-13 (26–28). Because these and other cytokines are known to be highly induced in injured lung, it is conceivable that this may represent an important pathway by which myofibroblasts arise *de novo* in pulmonary fibrosis. These studies, however, do not rule out the possibility of an extrapulmonary contribution to the fibroblast population in fibrotic lesions, with subsequent differentiation to myofibroblasts.

Part of the impetus for reexamining the issue of the extrapulmonary origin of lung fibroblasts is the observation that a circulating leukocyte subpopulation may mediate tissue repair (29). This observation, in conjunction with the recent interest in potentially greater plasticity of adult bone marrow stem cells, provides the rationale for recent interest in this issue. Although myofibroblasts can arise from fibroblasts, the origin of these latter cells and the basis for their expansion in pulmonary fibrosis are not fully understood. Thus, a key question is whether the proliferation of precursor cells present in the adult lung can account for the entire population of fibroblast-like cells in fibrotic lesions. The discovery of the circulating leukocyte subpopulation known as fibrocytes argues for the possibility that an extrapulmonary component may be important in contributing to the fibroblast-like cells in the fibrotic lung. These fibrocytes represent a very low percentage (0.1–0.5%) of the circulating leukocyte population, but can be isolated by flow cytometry and a combination of immunoselection techniques based on the markers ascribed to these cells (29, 30). These cells can be defined by their expression of collagen I (COL I), CD11b, CD13, CD34, CD45RO, major histocompatibility complex class II, and CD86. As with cells in the circulation, these cells are likely derived from bone marrow progenitors, probably HSCs based on their expression of CD34 and CD45. After isolation, these cells can be expanded in culture and shown to resemble fibroblasts morphologically and on the basis of their ability to express COL I. Moreover, on stimulation with TGF-β, they appear to have the capacity to express α-smooth muscle actin, indicative of myofibroblast differentiation (30). Reinfusion of these cells after isolation and dye tagging indicates that they can migrate to sites of dermal wound healing. Additional support for fibrocytes as potential sources of tissue fibroblasts in fibrotic lesions is provided by subsequent studies monitoring migration of these cells as identified by expression of CD34. Airway remodeling in asthma and an allergen-induced animal model is associated with significant airway wall fibrosis. Cells expressing CD34, α-smooth muscle actin, and collagen can be identified in these remodeling airways (31). Their origin from circulating fibrocytes can also be shown using the reinfusion of isolated, dye-tagged fibrocytes. Although isolated fibrocytes have the capacity to differentiate to myofibroblasts *in vitro* when stimulated by TGF-β, the ability of dye-tagged reinfused fibrocytes to migrate and differentiate to myofibroblasts has not been directly demonstrated in the wound-healing model (30). Thus, the phenotypic features of the fibrocyte-derived fibroblast-like cells at remodeling sites remain to be fully characterized. Nevertheless, these studies do suggest that circulating precursor cells represent a potential additional source of fibroblast-like cells in remodeling lung tissue.

An alternative approach to identifying potential extrapulmonary precursor cells for lung fibroblasts relies on availability of transgenic mice expressing easily identifiable proteins, which can be used to track the migration and fate of these uniquely tagged cells on transplant to wild-type mice. As discussed above, using such an approach has identified adult HSCs as a potential extrapulmonary source of regenerating alveolar epithelial cells (13–20). This method has been applied to study of the origin of fibroblast-like cells in animal models of lung injury and fibrosis using enhanced green fluorescence protein (GFP) bone marrow chimera mice (15, 32). These mice are created by transplanting bone marrow from GFP transgenic mice into lethally irradiated syngeneic recipient mice. Although bone marrow, peripheral blood, and spleen cells show virtually complete engraftment as manifested by more than 95% GFP-positive cells 8 wk after transplant, no clear morphologic evidence of GFP-positive cell engraftment is found for structural cells of the lung in normal control GFP bone marrow chimera mice (32). This is in contrast to injured lung, wherein significant numbers of GFP-expressing cells with morphologic characteristics of fibroblasts can be identified in areas of active fibrosis, in addition to GFP-positive mononuclear and other leukocytic cells (15, 32). This also has been shown using a model of elastase-induced lung injury in parabiotic animals, one of which is a GFP-expressing transgenic (33). Further analysis of these GFP-expressing cells by flow cytometric analysis of single suspensions prepared from injured lung tissue confirms the significant presence of bone marrow–derived GFP-positive cells that express COL I, but not CD34 or CD45 (32). As many as 80% of the COL I–positive cells express GFP, indicating a substantive contribution of bone marrow–derived cells to the fibroblast-like lung cell population. It is likely, however, that the percentage of actual fibroblasts derived from bone marrow may be less than this because some 5% of cells that are immunostained by anti–COL I antibodies also express macrophage markers (32). Nevertheless, bone marrow–derived progenitor cells contributed significantly to the total population of fibroblasts in the injured lung. Unambiguous confirmation is obtained by analysis of cells isolated and cultured from these injured lungs using standard methods and media for fibroblast culture. Essentially pure fibroblasts obtained by culturing these cells also reveal that a substantial portion express GFP. Although all these cells, both GFP-positive and GFP-negative, immunostain positively for COL I, virtually none of the GFP-positive cells express α-smooth muscle actin. Direct dual immunofluorescence assessment also fails to show colocalization of α-actin and GFP, indicative of failure of the bone marrow–derived fibroblasts to differentiate to myofibroblasts. Indeed, treatment of these cultured cells with TGF-β fails to induce differentiation

of the GFP-expressing fibroblasts. Thus, myofibroblasts in fibrotic lung tissue may not arise from bone marrow–derived progenitors. Interestingly, a similar observation is reported in bone marrow–derived cells in dermal wound healing (34). In contrast to α-smooth muscle actin expression, most of the bone marrow–derived cells appear to express telomerase reverse transcriptase, which has been found to be induced in the bleomycin model (35, 36). The basis for this difference between bone marrow– versus intrapulmonary-derived fibroblasts remains to be determined, but does suggest heterogeneity of the fibroblast population in fibrotic lung tissue on the basis of their origin.

RECRUITMENT OF BONE MARROW PROGENITOR CELLS

The preceding paragraphs describe the body of evidence that suggests mobilization and recruitment of bone marrow progenitor cells to the injured lung, giving rise to fibroblasts that appear to differ in a number of important characteristics from intrapulmonary-derived cells. Previously, circulating fibrocytes have been shown to express several chemokine receptors, and to respond chemotactically to the cognate ligands (30), thus suggesting their possible roles in recruitment of bone marrow progenitor cells. Of these, two chemokine ligands, CCL21 and CXCL12, are found to be induced in bleomycin-injured murine lungs, whereas their respective cognate receptors are up-regulated in fibroblasts isolated from these lungs (32). The chemotactic activity of these two chemokines is confirmed using these isolated lung fibroblasts. Thus, bone marrow progenitor cells can be mobilized and recruited to the lung in response to distal lung injury in response to chemokine signals released from the injured tissue. This appears to be the case when recruitment of fibrocytes expressing COL I, CD45, and CXCR4 are analyzed in the bleomycin model (37). Treatment with neutralizing antibodies to the CXCR4 ligand CXCL12 results in diminished recruitment of these cells to the injured lung. In view of the expression of CXCR4 in other bone marrow cells and their responsiveness to CXCL12, it is unclear if the observed reduced fibrocyte recruitment is a direct or an indirect effect mediated by other cells expressing this receptor. Additional studies are needed to clarify the mechanisms underlying the mobilization of these progenitor cells from the bone marrow and their recruitment to the lung. An additional point is the mechanism governing the differentiation of the progenitor cell to assume the phenotype of the fibroblast.

FUNCTIONAL IMPORTANCE OF EXTRAPULMONARY-DERIVED FIBROBLASTS

The above discussion indicates that ample evidence exists to suggest significant contribution of extrapulmonary cells to the fibroblast population in lung fibrosis. Delineation of the functional or pathophysiologic importance of these extrapulmonary cells to the development of fibrosis is at an early stage. Currently available data suggest conflicting roles for these cells. On the one hand, neutralization of CXCL12 results in reduced fibrocyte recruitment to the bleomycin-injured lung, which is accompanied by reduced lung fibrosis (37). This seems to argue for a profibrotic role for the fibrocyte, perhaps by increasing the number of fibroblasts in the lung. In contrast, another recent study indicates that myelosuppression causes increased susceptibility to bleomycin, whereas transfer of bone marrow–derived mesenchymal stem cells (MSCs) is protective and accompanied by presence of MSC-derived fibroblasts in the lung (38). This protective effect of MSCs has been previously noted but the focus is on regeneration or engraftment as alveolar epithelial cells (17). This protective effect of bone marrow–derived cells appears to extend to acute lung injury in humans, as well as endotoxin- and elastase-induced lung injury in animal models (7, 8, 20). However, in these studies, the focus is on the endothelial progenitor cell or alveolar epithelial regeneration, whereas the connection to fibroblast recruitment is undocumented (7, 20). Additional studies are needed to resolve these apparently conflicting roles for the extrapulmonary-derived cells in lung injury and fibrosis.

HUMAN STUDIES

Although considerable effort has been expended in determining the origin of fibroblasts in wound healing and fibrotic disorders using animal models, surprisingly few studies in humans have addressed the potential sources of mesenchymal cell/fibroblast lineages in the lung. On the basis of the expression of CD34 by fibrocytes (29, 30), the presence of $CD34^+$ cells that also express COL I and α-smooth muscle actin in the bronchial mucosa of patients with allergic asthma (31) suggests that circulating fibrocytes are potential precursors for the fibroblast/myofibroblast in the remodeling airway wall. An ideal circumstance to try to distinguish the bone marrow (extrapulmonary) versus intrapulmonary origins of fibroblasts in patients is afforded by studies of solid organ transplantation, especially in cases of sex-mismatched transplants. However, aside from the asthma study, all studies, to date, in human lung transplant recipients have focused on nonfibroblastic cells (primarily epithelial and endothelial cells), often arriving at different conclusions with regard to pulmonary versus extrapulmonary origins (39–42). In a study of patients with HSC transplantation, significant rates of lung endothelial and epithelial chimerism have been reported (18). Although the origin of fibroblasts is not the primary endpoint in that study, it is noted that the "fibroblastic tissue" appears not to be of donor origin. A concern with phenotyping of cells in complex tissues en bloc by immunostaining and immunofluorescence microscopy is the potential for staining artifacts, autofluorescence, and misinterpretation of hematopoietic cells in close proximity to cell(s) of interest as being "engrafted." Indeed, a recent study has challenged the notion of bone marrow–derived progenitors of lung epithelium on the basis of more rigorous genetic and flow cytometric analyses (22). Further studies at a single-cell level are needed to further delineate the origin of fibroblasts in fibrotic processes of the lung in humans.

CONCLUSIONS

Although there is increasing evidence for extrapulmonary progenitors for the expanded lung fibroblast population in injured and fibrotic lungs, several questions remain as to their significance to the pathogenesis of pulmonary fibrosis. Another key issue is that virtually all of the evidence is derived from animal model studies, whereas the human data are quite meager. Another unresolved issue is the potential of additional sources of fibroblasts, such as from endothelial– and/or epithelial–mesenchymal transition, which is discussed in a separate review (at least with respect to epithelial–mesenchymal transition). Because, as noted above, the endothelial and epithelial elements may also be derived from extrapulmonary progenitor cells, they can contribute as well to the fibroblast population via these mechanisms. Finally, the identity of the specific subpopulation of bone marrow progenitor cells that give rise to the lung fibroblast remains uncertain, with evidence both for HSCs (e.g., $CD45^+$ fibrocytes) and MSCs. Future studies will be directed at these unresolved issues as well as the potential implications on potential novel cell-based therapies for the idiopathic interstitial pneumonias.

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