Knowns and Unknowns of the Alveolus

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Our current alveolar paradigm includes three highly specialized cell populations. Alveolar type I cells are flat, elongated cells that presumably enable gas exchange. Alveolar type II cells are small, cuboidal cells with metabolic, secretory, progenitor, and immunologic functions. Alveolar fibroblasts secrete extracellular matrix proteins that support alveolar structure. These cells work together to facilitate respiration. Many years of high-quality research have defined our understanding of alveolar biology. However, there is much to be determined about the factors controlling cellular phenotypes and crosstalk. Moreover, specific questions remain regarding origin, repopulation, and previously unrecognized functions of each cell. This article summarizes the current data for each cell type and highlights areas that would benefit from further investigation.

Keywords: alveolus; pneumocyte; fibroblast

The structural, cytologic, and morphometric features of the mammalian alveolus have been extensively described (1). Our current understanding of the alveolus includes the following: type I cells that mediate gas exchange; type II cells with secretory, proliferative, and innate immune functions; endothelial cells with critical gas exchange and metabolic functions; alveolar macrophages that coordinate host defense; and interstitial fibroblasts that support alveolar structure through the secretion of extracellular matrix (ECM) proteins. Many questions remain about the origin, phenotypic regulation, and crosstalk of these cells in the normal and diseased lung. This article reviews alveolar unknowns and suggests areas for future study that could impact our understanding of human disease. The focus of this article will be on type I cells, type II cells, and interstitial fibroblasts.

TYPE I CELLS

The exquisitely thin alveolar type I (ATI) cells cover 95% of the alveolus while comprising only 8% of the total cells in the normal adult human lung (2). This “flatness” presumably facilitates gas exchange. We have no understanding of the factors that regulate ATI cell shape. Many investigators think that this morphology confers a large surface area for exposure to inhaled toxins and limited numbers of mitochondria in these cells cause the ATI cell’s extreme sensitivity to injury (MC Williams, personal communication). Because this flatness hampers the study of ATI cells on tissue sections and in primary culture, development of better methods to identify and isolate ATI cells would aid in their study.

Type I cells develop from an as yet unidentified fetal progenitor and are present at birth (3). Most studies of ATI cell

turnover in the injured, mature rodent lung suggest that adult ATI cells are derived from type II (ATII) cells during alveolar repair (4). It is not known how this cell type is repopulated in the normal lung. We also do not know if the *in vitro* reversibility of ATII to ATI transition (5) occurs in the mammalian lung. The type I-like cells derived from type II cells *in vitro* may be quite different from type I cells *in vivo*. Type I cells express a large number of proteins (reviewed in Reference 3), among them T1- α (6), aquaporin 5 (AQP-5) (7), functional ion channels (8), caveolins, adenosine receptors (9), and multidrug resistance genes.

Fluid and Ion Transport

Despite its importance in air breathing, alveolar fluid maintenance in the normal and diseased lung remains poorly understood. Although water transport may occur across ATI cells via AQP-5, the actual function of this protein is debated. Isolated perfused lungs from AQP-5-null mice show normal lung development but disrupted airspace-capillary osmotic permeability (7). *In vivo* studies of acute lung injury and resolution of alveolar flooding in AQP-5-null mice show no role for it in this function (10). Interestingly, AQP-5-null mice exhibit increased airway hyperresponsiveness, but this characteristic probably relates to AQP-5 absence in airway epithelium and not to ATI cells (11). Water transport is believed to follow osmotic gradients so fluid transport is secondary to solute transport.

The best-characterized marker for type I cells, T1- α , is not functionally characterized. This protein is expressed in other tissue sites known to carry out active ion transport (6). Animal modeling of this protein, which is relatively specific for the ATI cell in the distal rodent lung, has been difficult because newborn T1- α -null mice show severely abnormal sacculation and die of neonatal asphyxiation within minutes of birth. Microarray analysis of this developmental phenotype demonstrates decreased mRNA for negative regulators of cell proliferation and increased mRNA for proliferating cell nuclear antigen (PCNA) in T1- α -null lungs when compared with the normal murine lung at term (12). Microarrays also identify a large number of genes showing relatively small changes that may have achieved significance if the study had analyzed RNA derived only from ATI cells. This problem highlights the need for better isolation techniques of ATI cells.

The functional relevance of T1- α could be resolved by the creation of conditional knockout mice or use of other methods of gene silencing, such as exogenously or transgenically delivered siRNA to delete gene expression after maturity. T1- α is also expressed on the lymphatic endothelium, and this is especially evident in the human lung. Possible human homologs of T1- α include podoplanin, although the role of this molecule in the human lung is not known (3).

Cultured rat ATI cells express highly selective and non-selective cation channels, the cystic fibrosis transmembrane conductance regulator, and cyclic nucleotide gated channels. mRNA and protein for these transporters are present in various degrees in this cell population (8). Although intriguing, these data remain difficult to interpret because the cultured ATI cells have been removed from their physical boundaries and lack

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normal cellular interactions. In addition, the dearth of mitochondria in ATI cells raises questions about the metabolic ability of these cells to sustain active ion transport. In the absence of *in vivo* data showing a role for these molecules in the fluid balance of the normal and/or flooded lung, the functional relevance of these channels remains unknown. Animal modeling of these transporters would be an extremely helpful approach to this problem. Development of a promoter with expression restricted to type I cells, perhaps T-1 α , would be useful for gene targeting studies.

Other Putative Functions

ATI expression of caveolins also supports an expanded role for this cell type in alveolar homeostasis. Caveolins form caveolae, whose major functions are receptor inactivation and sequestration of proteins into subcompartments of the cell membrane. They can also mediate bulk transport across cells and participate in cell signaling, modulate reactive oxygen species, and may have tumor suppressor activities (13). Caveolin (Cav)-1 is expressed on ATI cells, endothelial cells, and fibroblasts in the murine lung. Targeted disruption of this protein leads to pulmonary fibrosis at about 4 to 5 months of age (14). The specific cellular abnormalities causing this phenotype remain obscure. Creation of cell-specific knockout mice or development of other means of targeted Cav-1 deletion would clarify this important issue. The regulatory patterns of Cav-1 expression described below indicate that this goal is reasonable.

Rat alveolar epithelial (but not endothelial) cells increase Cav-1 mRNA and protein by induction of E26 transformation-specific (ETS) protein/ETS-related molecule (ERM) (15). The physiologic significance of this variable gene induction may play an important role in the pulmonary fibrosis phenotype described for the Cav-1-null mice. In addition to specific information about Cav-1, the general concept illustrated by these data is the specificity of phenotypic regulation of different cell populations in the lung. We do not know how this specificity is achieved, whether cells removed from the lung exhibit the same functional properties as those with intact cell-cell and matrix contacts, and whether cellular crosstalk is required for ATI cells to maintain their phenotype.

Conclusions

ATI cells may have an unexpected role in the maintenance of lung homeostasis. However, relatively little is known about this cell population. The inability to isolate and phenotype this population limits study of this important cell type. There are almost no data on the human type I cell and observations made in rodents will need to be verified in humans before being extrapolated to human disease. The development of new markers, reagents, and assays is essential to advance our understanding of the ATI cell biology and may allow answers to the following questions (summarized in Table 1):

1. What is the natural history of the human type 1 cell? How does it achieve and maintain its flattened morphology? Does baseline ATI cell turnover occur and, if so, from what precursor(s) do new ATI cells arise in the absence of lung injury?
2. What signals induce the development of ATI cells from ATII cells? Is this transition reversible *in vivo*? What regulates the independent fate of the daughter cells arising from type II cell division?
3. What cellular crosstalk and matrix interactions determine gene expression in ATI cells?

4. What is the contribution of ATI cells to ion and fluid clearance in the normal and injured lung?

TYPE II CELLS

The cuboidal ATII pneumocyte accounts for approximately 15% of total cells but only about 5% of the alveolar surface area in the healthy human lung (2). Mainly located in the corner of the mammalian alveolus, ATII cells perform many tasks to keep the alveolus functioning normally.

Type II cells develop from a surfactant protein (SP)-C-expressing embryologic precursor (16) and, in most animals, form primitive airspaces at term gestation. In most small-animal species (with the notable exception of the morphologically advanced guinea pig [17]), alveolarization occurs after birth. The factors controlling the transition from primitive saccules to well-formed alveoli are poorly understood. In the adult rodent lung, ATII cells cycle every 28 to 35 days, and this slow mitotic rate is also believed to occur in humans (18). These kinetics are accelerated in response to lung injury (4) and growth factors such as keratinocyte growth factor (KGF) (18). The bulk of the evidence indicates that new ATII cells arise from a pool of transit amplifying ATII cells, which may or may not represent a distinct subpopulation of the ATII cell pool.

Reports of adult ATII cells arising from cells other than ATII cells themselves have appeared in the last few years. These include SP-C, Clara cell secretory protein (CCSP⁺) cells at the bronchoalveolar junction (bronchoalveolar stem cells [BASCs] [19]) and a wide variety of cells derived from the circulation (20). Although BASCs derived from mice give rise to cells expressing markers of either ATI (AQP-5) and ATII (pro-SP-C) cells *in vitro*, *in vivo* lineage tracing experiments are lacking and their contribution to the normal alveolar epithelium is not known. The cell migration required to repopulate the alveolar wall from these restricted sites would be significant. In addition, an analogous population has yet to be identified in humans. Dedicated studies including cre-lox lineage tracing in mice show only a minimal contribution of bone marrow to the ATII cell pool (21). This type of nuclear reprogramming results in morphologically and karyotypically abnormal cells that appear to have arisen through cell-cell fusion (22). Thus, the biological relevance of this finding in the healthy lung is viewed as minimal, given current data. Whether bone marrow-derived cells with an ATII phenotype contribute to the pathogenesis of certain types of lung disease remains to be seen.

Surfactant Production

The ATII cell produces, secretes, and recycles the lipid and protein components of pulmonary surfactant. One area of interest is the synthesis of dipalmitoylphosphatidylcholine and the role of phosphatidylcholine remodeling through a highly specific deacylation/reacylation reaction (23, 24). Because rodent and human surfactant formation and processing are likely different, studies of surfactant protein and phospholipid synthesis and assembly in human type II cells need to be performed.

Other questions concern the lamellar body. We do not know if the transporter ABCA3, which is required for lamellar body biogenesis (25), also regulates phospholipid import and specificity. Furthermore, the actual role of surfactant proteins in these processes is unclear. The profound respiratory failure caused by deficiency of SP-B (26) indicates this protein's importance in surfactant processing and function. The chronic, fibrotic phenotype of SP-C deficiency in certain mouse strains and humans implies a different, but possibly equally important role for this protein (27). These issues highlight the need for

TABLE 1. ALVEOLAR UNKNOWNNS

Cell Type	Function	Areas Needing More Study Which Are Likely to Impact Human Disease
Type I cell	1. Gas exchange 2. Likely role in fluid and ion transport	1. What factors regulate their shape? 2. Do type I cells have the ability to proliferate <i>in vivo</i> ? What is the origin of type I cells in the normal lung? 3. What is the contribution of ATI cells to ion and fluid clearance in the normal and injured lung? 4. What signals induce development of ATI cells from ATII cells, and is this transition reversible <i>in vivo</i> ? 5. What cellular crosstalk and matrix interactions determine gene expression in ATI cells?
Type II cell	1. Surfactant production 2. Fluid and ion transport 3. Progenitor cell 4. Innate immunity	1. How are surfactant phospholipids and proteins produced and organized, especially in the human lung? 2. What are the triggers and genes involved in "transitional states" in the alveolar compartment? 3. Does EMT contribute to lung fibrosis in a meaningful way? 4. Are all ATII cells equipotent in terms of surfactant production, fluid transport, reepithelialization, and immune responses, or does functional heterogeneity exist within this cellular compartment? 5. What cellular crosstalk determines gene expression in ATII cells?
Fibroblast	1. Matrix production 2. Modulation of epithelial phenotype	1. Does EMT occur in the lung? 2. To what extent does cellular crosstalk influence the phenotype of interstitial fibroblasts? 3. To what extent do fibroblasts/activated myofibroblasts influence the phenotype and survival of the alveolar epithelium? 4. What are the mediators of cell growth and matrix production and what are the roles of the alveolar epithelial cells and the interstitial cells in the epithelial-mesenchymal interaction in pulmonary fibrosis? 5. What is the pathogenesis of fibroblastic foci?

Definition of abbreviations: ATI = alveolar type I; ATII = alveolar type II; EMT = epithelial-mesenchymal transition.

more information in this area. There are no known clinical means of increasing the endogenous pool of surfactant in acute lung injury.

Transepithelial Transport

Both human and rodent ATII cells help keep the alveolar space relatively free of fluid and transport sodium through well-described apical sodium channels and the basolateral Na^+/K^+ ATPase (28). Recent *in vivo* studies using siRNA to delete Na^+/K^+ ATPase expression found that this transporter clears most of the alveolar fluid induced by terbutaline but only a small part (30%) of baseline alveolar fluid (29). These results may not apply to acute lung injury in which the fluid has a high protein concentration and the epithelial barrier is not intact. The factors controlling baseline regulation of alveolar fluid volume and pH remain unclear. Last, we also do not know the significance of the sodium-phosphate transporter located on the apical membrane of ATII cells (30) or how the other components of alveolar fluid are processed.

Proliferative and Stem Cell Properties

In response to a variety of ATI cell injuries, hyperplastic ATII cells (5) cover the basement membrane and then differentiate into ATI cells, maintain their ATII cell phenotype, or undergo programmed cell death (31). The factors controlling induction, differentiation, and clearance of hyperplastic ATII cells are not well understood and many questions remain. Renewal of the ATII cell population in the normal lung is also not well characterized. For example, does the ATII cell dedifferentiate to a less committed state before asymmetric cell division? What is the role of the ECM in this process? Is there a stem cell "niche" in the alveolus? Do epigenetic or post-transcriptional mechanisms control this process? Do ATII cells exhibit heterogeneity in terms of plasticity? Last, do BASCs contribute to lung repair? Development of models to further characterize this progenitor response will aid in answering these important questions. Finally, there is some evidence that ATII cells may undergo epithelial to mesenchymal transition (EMT) (32). Does this process contribute to pulmonary fibrosis in a meaningful way?

Innate Immunity

An emerging area of interest in ATII cell biology is the important role this cell plays in host defense. In addition to producing SP-A and SP-D, which are opsonins and regulate inflammatory cell function, ATII cells produce cytokines and

growth factors that also affect immune cells (33, 34). The importance of this paracrine signaling and cellular crosstalk between injured or infected cells remains unknown. Type II cells express major histocompatibility class II antigens (35) but little is known about their ability to present antigen and initiate inflammatory responses or their responses to respiratory viruses and other inhaled organisms.

Conclusions

ATII cells are powerful integrators of alveolar biology and participate in important secretory, transport, stem cell, and immune functions. The accumulating knowledge regarding type II cells would be enhanced by further studies of the following areas (*see also* Table 1):

1. How is phospholipid production organized for surfactant production and association with the hydrophobic surfactant proteins?
2. What regulates the fate of dividing ATII cells and what regulates their proliferation in the normal lung?
3. Does EMT occur in the lung and does it contribute to pulmonary fibrosis?
4. Are all ATII cells equipotent in terms of surfactant production, fluid transport, reepithelialization, and immune responses, or does functional heterogeneity exist among these cells?
5. What cellular crosstalk determines gene expression in ATII cells and their innate immune response?

INTERSTITIAL FIBROBLASTS

The spindle-shaped interstitial fibroblast comprises 30–40% of the cells in the normal adult human lung and secretes the ECM scaffold for the alveolus (2). Because the ECM has profound effects on epithelial biology (36), this role cannot be understated. Although fibroblasts in the normal lung synthesize very little matrix, activated myofibroblasts are major contributors to fibrotic lung disease through matrix production and their α -smooth muscle actin (α -SMA)-mediated contractile phenotype. The factors regulating activation and differentiation of myofibroblasts are poorly understood, although the importance of transforming growth factor (TGF)- β in this process has been widely appreciated (37).

One of the most basic, yet complex questions regarding the fibroblast is its origin and recruitment. Data obtained over recent years redefine the concept that these cells are solely derived from intrapulmonary mesenchymal progenitors (38). Convincing animal studies from a number of investigators now suggest that the intrapulmonary origin of collagen-producing cells is augmented by bone marrow-derived fibrocytes (39–41) and/or mesenchymal stem cells (42). However, because these cells do not appear to express α -SMA, their effector significance is not known. Some investigators propose a paracrine role for fibrocytes and mesenchymal stem cells in the development and treatment of lung injury (43), but evidence supporting this hypothesis is lacking. Thus, the role of bone marrow-derived fibroblast progenitors in lung disease remains unclear.

Another possible site of extramesenchymal fibroblast development is the diseased lung epithelium. In this paradigm, epithelial cells respond to TGF- β 1 by undergoing EMT (32, 43). This process is well described in the kidney (which is derived from mesenchyme). Because the lung is endoderm derived, the renal data may be less applicable to alveolar biology. Nevertheless, the physiologic importance of EMT remains a critical area for future study.

Functions

This potential heterogeneity in origin of fibroblasts may cause the functional differences noted between subpopulations of this cell type. For example, cells in various states of development and activation express high or low levels of the cell surface marker thymus cell antigen (Thy)-1. Rat fibroblasts with high levels of Thy-1 show a less contractile phenotype and α -SMA expression than Thy-1-low fibroblasts (44). Similar phenotypic differences are found in humans, with the fibroblastic foci characteristic of idiopathic pulmonary fibrosis (IPF) containing a fibroblast population whose Thy-1 expression is far below that of the rest of the lung (45). Whether Thy-1 expression causes or results from fibroblastic foci, as well as the relevance of this finding to the pathogenesis of IPF, are questions that have yet to be answered.

Regulation of Phenotype

Two other unexplored aspects of fibroblast biology concern the regulatory patterns of their activation and proliferation. As demonstrated by the β 6-integrin knockout mouse, initiation and maintenance of these functions result from signals different from those that induce inflammation. Microarray data obtained from bleomycin-resistant (β 6-integrin null) and bleomycin-susceptible mice (β 6-integrin present) show a stepwise expression of genes for each response, with proinflammatory genes characterizing inflammation, and DNA damage and ECM genes characterizing the fibrotic response (46).

Real-time polymerase chain reaction data from cells dissected from fibroblastic foci in human IPF show increased matrix metalloproteinase (MMP)-2, MMP-9, and tissue inhibitor of metalloproteinase (TIMP) expression compared with cells outside these matrix-rich islands (47). Similar to the Thy-1 data described above, this finding shows that cells within the fibroblastic foci are unique within the IPF lung. What is not known is whether this phenotypic heterogeneity results from differences in origin or activation. Furthermore, although we know that fibroblasts influence epithelial phenotype, we do not know how epithelial cells affect fibroblasts and whether fibroblastic foci result from abnormalities in cellular crosstalk.

One special aspect of these gene expression data is the finding that these differences were only noted when the lung was partitioned into different cell populations using laser capture microscopy. Analysis of whole lung lysate, as is usually

done in gene expression studies, failed to find any difference between cell populations. Such an extreme sampling effect supports the critical need for better isolation techniques for microscopy, flow cytometry, and gene expression studies.

Conclusions

The heterogeneity of interstitial fibroblasts has long been recognized. Within the transition from fibroblast to activated myofibroblast lies a continuum of cells that vary in their gene expression patterns and functional phenotypes. Our understanding of this complicated paradigm will be enhanced by exploration of the following topics, as outlined in Table 1:

1. Does EMT occur in the human lung?
2. To what extent does cellular crosstalk influence the phenotype of interstitial fibroblasts?
3. Conversely, to what extent do fibroblasts/activated myofibroblasts influence the phenotype and survival of the alveolar epithelium?
4. What are the mediators of cell growth and matrix production and what are the roles of the alveolar epithelial cells and the interstitial cells in the epithelial-mesenchymal interaction in pulmonary fibrosis?
5. What is the pathogenesis of fibroblastic foci seen in human usual interstitial pneumonia?

We know a great deal about the resident cells of the alveolus. However, questions remain on specific aspects of the cellular phenotypes and general questions regarding cellular plasticity, heterogeneity, and communication. Addressing these and other issues requires new, improved models and/or better characterization of the models currently in use. The compelling nature of the questions above argues that developing these would be a useful effort.

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