Expression of the Integrin $\alpha 8 \beta 1$ during Pulmonary and Hepatic Fibrosis

David Levine,* Don C. Rockey,[†]
Teresa A. Milner,[‡] Johannes M. Breuss,[§]
John T. Fallon,[¶] and Lynn M. Schnapp*

From the Division of Pulmonary and Critical Care Medicine,*
Department of Medicine, Mount Sinai School of Medicine, New
York, New York; the Division of Gastroenterology,[†] Duke
University Medical Center, Durham, North Carolina; the
Department of Neurology and Neuroscience,[‡] Weill Medical
College of Cornell, New York, New York; the Institute for Vascular
Biology and Thrombosis Research,[§] University of Vienna, Vienna,
Austria; and the Cardiovascular Institute,[¶] Departments of
Medicine and Pathology, Mount Sinai School of Medicine, New
York, New York

The fibrotic response after diverse forms of injury is characterized by the accumulation of extracellular matrix proteins, proliferation of myofibroblast-like cells, and organ contraction. Myofibroblasts are key effector cells in the development of the fibrotic response. They contribute to fibrosis through both increased cell number (proliferation) and enhanced matrix synthesis. Integrins, a class of cell adhesion molecules, are mediators of cell-extracellular matrix protein interactions that are important in the proliferative and migratory response of cells to matrix proteins. We have previously cloned the human integrin subunit $\alpha 8$, documented its high expression in lung tissue, and established it as a receptor for the matrix proteins fibronectin, vitronectin, and tenascin. We now demonstrate that alveolar interstitial cells are the primary cell type expressing $\alpha 8 \beta 1$ in the lung parenchyma. Expression of $\alpha 8 \beta 1$ is concentrated primarily along the thinned extensions of cells and at the tips of filopodia. Because of its unique distribution in alveolar interstitial cells, we hypothesized that it may play a role in the fibrotic response after injury. In bleomycin-induced pulmonary fibrosis, there is increased expression of $\alpha 8 \beta 1$ by interstitial fibroblasts, the majority of which coexpress α smooth muscle actin, a marker of tissue myofibroblasts. To establish a more general role for $\alpha 8 \beta 1$ during organ fibrosis, we further examined its expression in two rat models of liver fibrosis. During hepatic injury due to either carbon tetrachloride injury or bile duct ligation, we demonstrate *de novo* expression of $\alpha 8 \beta 1$ in activated hepatic stellate cells, the myofibroblast equivalent in liver. Taken together, the data localize $\alpha 8 \beta 1$ to myofibroblast-like cells during wound healing and suggest

that signal transduction through the $\alpha 8 \beta 1$ integrin may contribute to the fibrotic response of organs to injury. (*Am J Pathol* 2000, 156:1927–1935)

Myofibroblasts are essential effectors of the fibroproliferative response to injury in lung, liver and other parenchyma. Key features of this response to tissue injury include the proliferation of myofibroblasts, increased extracellular matrix (ECM) production, and remodeling of the extracellular matrix. Increased matrix production in this setting results from both increased myofibroblast cell number (proliferation) and enhanced cellular extracellular matrix synthesis.

In normal lung, the interstitium contains a population of mesenchymal cells called alveolar myofibroblasts or contractile interstitial cells (CICs).¹ CIC-mediated functions are particularly important during the establishment of pulmonary fibrosis. These cells express myogenic intermediate filaments, including desmin and vimentin. CICs are postulated to control the ventilation-perfusion ratio by alveolar wall contraction during breathing.^{1–3} In contrast to pericytes (myofibroblasts associated with capillaries) and alveolar ring cells (smooth muscle-like cells found at the corners of alveolar ducts),⁴ CICs/myofibroblasts do not express α -smooth muscle actin under normal conditions.⁵ However, during pulmonary fibrosis, CICs undergo a phenotypic modulation to a smooth muscle-like phenotype characterized by expression of α -smooth muscle actin⁵ and proliferation and increased synthesis of ECM proteins, including collagen and fibronectin. As pulmonary fibrosis progresses, the percentage of CICs in the lung increases from 30% to 80%, accompanied by a 10-fold increase in cell number.⁶

In the liver, the cell type analogous to the alveolar contractile interstitial cell is the hepatic stellate cell (formerly known as the lipocyte or Ito cell).⁷ In the normal liver, stellate cells manifest a quiescent phenotype and do not express smooth muscle-specific proteins. After liver injury, stellate cells “activate,” whereby they express α -smooth muscle actin,⁸ proliferate, and produce abundant extracellular matrix proteins.⁹ Activated stellate cells

Supported by AHA-Heritage Affiliate Grant-in-Aid (L. M. S.) and NIH HL18974 (T. A. M.).

Accepted for publication February 16, 2000.

Address reprint requests to Dr. Lynn M. Schnapp, Pulmonary and Critical Care Medicine, Box 1232, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029. E-mail: lynn.schnapp@mssm.edu.

also acquire the ability to contract, a key feature of the cirrhotic liver.¹⁰

Altered extracellular matrix composition in fibrosis leads to cellular responses that are mediated through integrins. Integrins are a family of cell adhesion receptors composed of α and β subunits that influence many cellular activities, including adhesion, migration, proliferation, apoptosis, and cell survival, all of which can modulate the development of pulmonary fibrosis. For example, treatment of mice with antibodies to the leukocyte integrin $\beta 2$ subunit dramatically decreases collagen deposition after lung injury.¹¹ Mice lacking the $\beta 6$ integrin subunit, which is expressed in airway epithelium after injury,¹² are protected from the development of bleomycin-induced pulmonary fibrosis.¹³ The prevention of pulmonary fibrosis in $\beta 6$ -deficient mice is due to a defect in the activation of TGF- $\beta 1$ in the lung.¹³

Previous work from our laboratory has identified a potential role for the integrin $\alpha 8 \beta 1$ in lung fibrosis. We have cloned the human integrin subunit $\alpha 8$, which is found to be highly expressed in the normal lung,¹⁴ and showed that $\alpha 8 \beta 1$ is a receptor for the matrix proteins fibronectin, tenascin, and vitronectin.^{15–17} We now demonstrate that alveolar contractile interstitial cells are the predominant $\alpha 8$ -expressing cells in the lung parenchyma. Because interaction of CICs with the ECM may be important for the development of pulmonary fibrosis, we examined the expression pattern of $\alpha 8$ during bleomycin-induced pulmonary fibrosis. We also examined two models of hepatic fibrosis to determine whether this integrin has a generalized role in the response to organ injury. We find the integrin $\alpha 8 \beta 1$ is a marker of activated CICs/myofibroblasts and that proliferation of $\alpha 8$ -expressing cells appears to be a common feature of fibrosis.

Materials and Methods

Reagents and Antibodies

Rabbit polyclonal antibodies to the human $\alpha 8$ cytoplasmic domain were previously characterized.^{14,15} The immortalized rat hepatic stellate cell line HSC-T6 was a generous gift from Dr. Scott Friedman (Mount Sinai School of Medicine, New York). The HSC-T6 cell line was generated by transfection of primary hepatic stellate cells with an expression plasmid encoding the SV40 large T antigen as previously described.¹⁸ HSC-T6 cells retain all features of activated stellate cells, including expression of desmin, smooth muscle actin, and glial acidic fibrillary protein, and it can esterify retinol into retinyl esters.¹⁸

Immunoelectron Microscopy

Lungs from adult Sprague-Dawley rats were sequentially perfused through the thoracic aorta with 1) normal saline (0.9%) containing 1000 U/ml of heparin, 2) 50 ml of 3.75% acrolein and 2% paraformaldehyde in phosphate buffer (pH 7.4) (PB), and 3) 200 ml of 2% paraformaldehyde in PB. The lungs were removed and stored in the last fixative for an additional 30 minutes. Sections (50 μ m thick)

were cut on a vibratome, collected in PB, and treated with 1% sodium borohydride in PB for 30 minutes before immunocytochemical labeling. Rabbit polyclonal antibody to human $\alpha 8$ subunit¹⁴ (1:1000, with 0.035% Triton in the diluent) was localized immunocytochemically, using the avidin-biotin complex (ABC) peroxidase method, and processed for electron microscopy as previously described.¹⁹

Isolation of Rat Lung Fibroblasts

Lungs were initially perfused with phosphate-buffered saline (PBS)/10 mmol/L EDTA via the right ventricle, during simultaneous inflation and deflation through cannulated trachea. The lungs were removed *en bloc* and lavaged with PBS/10 mmol/L EDTA, followed by instillation of prewarmed collagenase (250 mg/ml)/trypsin (2.5 μ g/ml) in PBS with penicillin/streptomycin (P/S)/gentamycin for 30 minutes at 37°C and then instillation with cold trypsin inhibitor (type II-S (Sigma T9128)/DNase (Sigma D-5025) (250 mg TI, 1 mg DNase, 10 ml fetal bovine serum, 90 ml PBS). Trachea, bronchi, major structures, and other extraneous tissues were excised, and the remainder of the tissue was minced into 2–4-mm pieces. Tissue was suspended in PBS/0.5% trypsin, stirred at 37°C for 30 minutes, and then filtered through nylon mesh. Cells were washed with PBS and resuspended in Dulbecco's modified Eagle's medium/10% fetal bovine serum/P/S/fungizone. Macrophages were depleted by repeated adherence to IgG-coated plates. The remaining cells were plated and incubated overnight.

Immunofluorescence

Freshly isolated fibroblasts were grown on fibronectin-coated chamber slides for 1 day and fixed and permeabilized with 2% paraformaldehyde/0.1% Triton X-100 for 10 minutes. Slides were blocked with 3% bovine serum albumin and then incubated with anti- $\alpha 8$ antibody (10 μ g/ml) for 1 hour at room temperature, washed with PBS, and incubated with biotin-conjugated donkey anti-rabbit IgG (1:50) (Amersham, Arlington Heights, IL) for 1 hour at room temperature, followed by PBS wash. Coverslips were then incubated with fluorescein-conjugated streptavidin (1:100) (Amersham) for 15 minutes at room temperature, washed with PBS, and mounted with Vectashield (Vector Laboratories, Burlingame, CA).

Western Blot

Freshly isolated fibroblasts were grown for 3–5 days, and cells were lysed with 200 mmol/L octyl- α -D-glucopyranoside/100 mmol/L Tris-HCl/1 mmol/L phenylmethylsulfonyl fluoride for 1 hour at 4°C and centrifuged twice at 2000 rpm for 10 minutes each. The protein concentrations were determined by the Bradford Assay. Samples (30 μ g/well) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions on 7.5% acrylamide gel, and electrophoretically transferred to a nylon membrane

(Hybond-N; Amersham). Filters were blocked with 1% dry milk/1% bovine serum albumin/0.02% Tween-20/PBS for 2 hours, probed with the anti- α 8 antibody for 1 hour, rinsed in PBS, and then probed with an alkaline phosphatase-conjugated goat anti-rabbit antibody at a dilution of 1:3000 for 30 minutes. After rinsing in PBS, color was developed with the Protoblot alkaline phosphatase detection system (Promega).

Bleomycin-Induced Pulmonary Fibrosis

C57Bl/6 mice were maintained in a pathogen-free environment. Bleomycin (Mead-Johnson) was dissolved in sterile saline, and 0.075 U was administered intratracheally by direct visualization after cutdown. As a control, saline alone was administered intratracheally. Mice were sacrificed at 2 days, 4 days, 6 days, 2 weeks, 4 weeks, and 9 weeks after initial bleomycin installation. Lungs were perfused with PBS/heparin and then immersed in 10% sucrose solution overnight at 4°C. Tissues were embedded in OCT and then frozen in nitrogen-chilled butane. Five-micron sections were processed as previously described.¹⁴ Sections were incubated in 5% bovine serum albumin for 1 hour at room temperature and then incubated with the primary antibody overnight at 4°C. After washing in PBS, sections were incubated with peroxidase-conjugated goat anti-rabbit secondary antibody (Vector Laboratories) for 1 hour at room temperature. The reaction product was visualized after incubation of the sections with diaminobenzidine (DAB Plus kit; Zymed Laboratories). Sections were counterstained with hematoxylin, sequentially dehydrated in ethanol solutions, transferred to xylene, and mounted with Permount (Fisher).

For colocalization immunofluorescence studies, lung sections were incubated with α 8 antibody (1:1000) and Cy3-conjugated α -smooth muscle actin antibody (Sigma), followed by biotin-conjugated donkey anti-rabbit IgG (1:100) (Amersham), then fluorescein-conjugated streptavidin (1:100) (Amersham) for 15 minutes at room temperature, washed with PBS, and mounted with Vectashield (Vector Laboratories). Slides were examined on a Zeiss Axioskop (Thornwood, NY) equipped with filter sets for fluorescein isothiocyanate (FITC) and rhodamine/Cy3 (Chroma Technology, Brattleboro, VT). Images were collected using a Spot 2 cCCD camera (Diagnostic Instruments, Sterling Heights, MI). Black-and-white images (8-bit) were collected for each channel (ie, FITC and Cy3) and then merged into the respective RGB channels in Adobe Photoshop. Colocalization of α 8 expression (green) and α -smooth muscle actin expression (red) was determined by overlap of the two fluorochromes (yellow).

To determine the proportion of α 8-expressing cells that did not express α -smooth muscle actin in fibrotic lesions, we counted the number of α 8-positive/ α -smooth muscle-negative and α 8-positive/ α -smooth muscle-positive cells in four independent, $\times 40$ fields containing areas of fibrosis from three different mice, 4–6 days after bleomycin administration.

Models of Hepatic Fibrosis

Hepatic fibrosis was induced in adult Sprague-Dawley rats by carbon tetrachloride (CCl₄) administration or bile duct ligation as previously described.⁸ To induce biliary obstruction, the common bile duct was ligated and sectioned. Periductal fibrosis occurred by 5 days, and biliary cirrhosis occurred by 14 days. CCl₄ was administered at a dose of 1.0 ml CCl₄/kg body weight at weekly intervals via gavage. Early bridging fibrosis was apparent by 3–4 weeks. Tissues were processed for immunohistochemistry as above.

Results

α 8 β 1 Is Expressed in Alveolar Interstitial Cells

In the normal lung parenchyma, immunoreactivity for α 8 β 1 was seen within the alveoli septa by light microscopy.¹⁴ To determine the cell type expressing α 8 β 1 in the lung parenchyma, we performed immunoelectron microscopy of normal rat lung. Immunoreactivity for α 8 was localized to the interstitial cells (Figure 1, A–D). In contrast, epithelial cells and endothelial cells lacked immunoreactivity for α 8. Many of the interstitial cells expressing α 8 labeling contained contractile filaments (Figure 1A, asterisk), identifying them as CICs.² In these cells, α 8 immunoreactivity was found at discrete patches along the cell membrane and was often concentrated at the tips of the filopodia cells as they protruded into the fused basement membrane of the endothelial and epithelial cells (Figure 1C, arrow). This pattern is reminiscent of the localization of α 8 β 1 at synaptic densities in the hippocampus.¹⁹ α 8-labeling was frequently concentrated at areas along the thin extensions of interstitial cells. It is of interest that a lipid droplet was identified within an α 8-immunoreactive cell (Figure 1D, arrowhead), consistent with the notion that these cells are similar to the hepatic stellate cells that contain cytoplasmic droplets of retinyl esters.

To confirm expression of α 8 β 1 in lung interstitial fibroblasts, we analyzed isolated primary rat lung fibroblasts by Western blot and immunofluorescence. Fibroblasts isolated from normal rat lung express α 8 β 1 by Western blot (Figure 2A). Immunofluorescence of isolated fibroblasts identified a subpopulation of α 8 β 1-expressing cells. When pulmonary fibroblasts were grown on fibronectin-coated plates, α 8 β 1 was concentrated at focal contacts, consistent with its role as a receptor for fibronectin (Figure 2B).

Expression of α 8 β 1 during Fibrosis in Vivo

Pulmonary Fibrosis

Because α 8 β 1 is expressed in CICs in the lung, which are important in the development of pulmonary fibrosis, we examined the expression pattern of α 8 β 1 in an experimental model of pulmonary fibrosis. Pulmonary fibrosis in C57/black wild-type mice was produced by intratracheal

Figure 1. Electron microscopy of normal rat lung demonstrating $\alpha 8$ -immunoreactivity in contractile interstitial cells. **A–D:** $\alpha 8$ immunoreactivity is visualized at the tips and along the membrane (**arrows**) of contractile interstitial cells (CIC). **A:** Actin filaments (*) are noted in an $\alpha 8$ -expressing CIC. **C:** Higher magnification of CIC illustrating $\alpha 8$ -reactivity (**arrow**) along the filopodia as it protrudes into the basement membrane. **D:** Lipid droplet (**arrowhead**) within an $\alpha 8$ -expressing CIC. Immunoperoxidase staining. Scale bars, 0.5 μ m in **A**, **B**, and **D**; 0.2 μ m in **C**.

Figure 2. A: Western blot of $\alpha 8\beta 1$ in primary lung fibroblasts. Fibroblasts isolated from rat lung were lysed, and aliquots of lysates were analyzed under nonreducing conditions. The samples were separated on SDS-PAGE gel and immunoblotted with anti- $\alpha 8$ antiserum. **Lanes 1–3** represent three different rat fibroblast preparations. The **arrow** indicates the position of $\alpha 8$. Positions of molecular size markers in kd are shown to the left. **B:** Immunofluorescence of $\alpha 8\beta 1$ in primary lung fibroblasts. Primary rat lung fibroblasts were grown on fibronectin-coated plates and then fixed, permeabilized, and labeled with anti- $\alpha 8$ antibody. $\alpha 8$ -containing focal contacts are indicated by **arrows**.

instillation of bleomycin, a standard technique resulting in reproducible, nonfatal pulmonary inflammation followed by fibrosis in mice (see Materials and Methods). In the saline-treated control mouse lung, there was preservation of normal alveolar architecture with baseline expression of $\alpha 8\beta 1$ in the alveolar interstitial cells (Figure 3A). The expression pattern of $\alpha 8\beta 1$ in saline-treated mouse lung was identical to normal mouse lung. Within 2–4 days after bleomycin instillation, alveolar wall thickening was present, with the collapse of some alveoli. The vast majority of cells within areas of alveolar wall thickening were immunoreactive for $\alpha 8$ (Figure 3, B–F). Patches of inflammatory cells that were negative for $\alpha 8\beta 1$ were observed within alveolar spaces (Figure 3D). Vascular smooth muscle and airway smooth muscle were positive for $\alpha 8\beta 1$ (Figure 3C), as observed in normal lung.

Within 4–6 weeks, more advanced fibrosis was established. Areas of dense $\alpha 8$ -positive cellular infiltration were observed, with an expansion in the number of $\alpha 8$ -reactive cells and complete obliteration of normal alveolar archi-

ture (Figure 3, G–I). Although the majority of cells within the fibrotic lesions were $\alpha 8\beta 1$ -positive, occasional $\alpha 8\beta 1$ -negative cells were also identified. In areas of more established fibrosis, there was decreased cellularity.

During pulmonary fibrosis, CICs activate and newly express α -smooth muscle actin. To investigate whether the activated CICs coexpressed $\alpha 8\beta 1$ and α -smooth muscle actin, we double-labeled sections for $\alpha 8\beta 1$ and α -smooth muscle actin (Figure 4). Colocalization of $\alpha 8\beta 1$ and α -smooth muscle actin occurred in vascular and airway smooth muscle. In the parenchyma, α -smooth muscle actin-positive cells were identified in areas of alveolar wall thickening. The vast majority of these cells were $\alpha 8\beta 1$ immunoreactive. However, a subpopulation of $\alpha 8\beta 1$ -positive cells were identified that were α -smooth muscle actin-negative (Figure 4, white arrows). In areas of fibrosis, the percentage of $\alpha 8\beta 1$ -positive/ α -smooth muscle actin-negative cells (compared to $\alpha 8$ -positive/ α -smooth muscle-actin-positive cells) ranged from 8% to 55%, with an average of 16%. In contrast, normal-appearing areas of lung parenchyma contained >90% cells that were $\alpha 8$ -positive/ α -smooth muscle-actin-negative.

Hepatic Fibrosis

The expression of $\alpha 8\beta 1$ in two mechanistically different models of hepatic fibrosis was examined using carbon tetrachloride-induced hepatic fibrosis and bile duct ligation-induced hepatic fibrosis. In normal liver, $\alpha 8\beta 1$ immunoreactivity was confined to the smooth muscle cells surrounding vessels and bile ducts (Figure 5, A and D), with none detected in the parenchyma or hepatocytes. After bile duct ligation (2 weeks), extensive bile duct proliferation occurred, with portal inflammation and fibrosis. The cells surrounding the proliferating bile ductules were immunoreactive for $\alpha 8\beta 1$, and the bile duct epithelium remained negative for $\alpha 8\beta 1$ (Figure 5, B and E). The increased $\alpha 8\beta 1$ immunoreactivity was limited to areas of ductal proliferation and perisinusoidal cells; $\alpha 8\beta 1$ was not seen within the parenchyma. The pattern of expression of $\alpha 8\beta 1$ is identical to that observed with stellate cells after bile duct ligation.⁸ After CCl_4 administration (4 weeks), extensive bridging fibrosis was present between portal areas of the lobule. Cells within the fibrotic bands of tissue were immunoreactive for $\alpha 8\beta 1$ (Figure 5, C and F), an expression pattern identical to that of stellate cells after CCl_4 administration.⁸

To confirm expression of $\alpha 8\beta 1$ by activated stellate cells, we immunoprecipitated lysates from immortalized activated rat stellate cells (HSC T6 cells) with the anti- $\alpha 8$ antibody. HSC T6 maintains the phenotype of activated stellate cells, including gel contraction, and α -smooth muscle actin expression. Although transfection with SV40 T antigen may alter some patterns of gene expression compared to primary cells, its phenotype is faithful to primary activated stellate cells in all major parameters studied to date. Immunoprecipitation of biotinylated cell lysates revealed two bands corresponding to the α and β subunits (Figure 6). In contrast, $\alpha 8\beta 1$ was not detected in endothelial cells, Kupffer cells, or hepatocytes derived from normal or fibrotic livers (data not shown). Thus im-

Figure 3. Immunohistochemistry of $\alpha 8 \beta 1$ in normal lung and pulmonary fibrosis. Immunohistochemical staining with anti- $\alpha 8$ antibody of mouse lung after saline (A) or bleomycin (B–J) treatment. Frozen sections of lung were stained with hematoxylin and affinity-purified anti- $\alpha 8$ antibodies. In the control (A) 4 weeks after saline treatment, normal lung architecture is preserved, and $\alpha 8$ -immunoreactive cells are seen in the interstitium. In the bleomycin-treated animals, alveolar wall thickening, increased cellularity, and obliteration of air spaces are seen at early time points (B–F). The majority of cells within alveolar wall thickening are $\alpha 8 \beta 1$ -positive. At higher magnification, some areas of $\alpha 8 \beta 1$ -negative inflammatory cells are observed (asterisk) (D). Vascular smooth muscle and airway smooth muscle are $\alpha 8 \beta 1$ -positive (arrowheads) (C). By 4 weeks (G and H), areas of dense cellular infiltration with $\alpha 8 \beta 1$ -positive cells are seen with complete loss of normal alveolar architecture. I: Nine weeks. Higher magnification of a less involved area shows $\alpha 8 \beta 1$ -positive cells within thickened alveolar septa. Control specimens incubated with preimmune rabbit serum displayed no significant staining (data not shown). Immunoperoxidase staining. Original magnifications: A–C and E–G, $\times 10$; D and H, $\times 20$; I, $\times 40$.

munohistochemistry and immunoprecipitation suggest that activated hepatic stellate cells are the primary source of $\alpha 8 \beta 1$. Given the increasing recognition that stellate cells represent a heterogeneous population in liver,^{20,21} these data do not exclude the possibility that only a subset of this cell type expresses $\alpha 8 \beta 1$ *in vivo*.

Discussion

We have demonstrated that in normal lung, the alveolar interstitial cell is the primary parenchymal cell in the lung expressing $\alpha 8 \beta 1$ by electron microscopy. The cell surface localization is consistent with its role as a cell adhesion molecule. The ultrastructural localization concentration at tips of cell protrusion is suggestive of a role of $\alpha 8 \beta 1$ in the migration or adhesion of CICs and/or matrix remodeling. During pulmonary fibrosis, there is increased immunoreactivity of $\alpha 8 \beta 1$. Although we cannot determine whether increased $\alpha 8 \beta 1$ expression represents an increased number of $\alpha 8$ -positive cells or increased cellular

expression per cell, the pattern is consistent with expression by the proliferating population of interstitial cells. It is likely that increased $\alpha 8 \beta 1$ expression facilitates interaction of cells with the provisional matrix laid down after injury and mediates cell matrix signal transduction important in the development of fibrosis.

By providing an essential link between the cell and the extracellular matrix, integrins play a pivotal role in the fibrotic response to injury. Interaction of integrins with ECM ligands results in intracellular signal transduction and modification of cell behavior. Many of these behaviors, such as migration, contractility, ECM production and degradation,²² cell survival, and proliferation,²³ contribute to the development of fibrosis. Thus information on the expression of integrins that mediate these behaviors may help us understand the mechanisms involved during the development of fibrosis.

$\alpha 8 \beta 1$ is in a unique position to relay specific signals to myofibroblast-like or smooth muscle cells. Much of the focus in the lung has been on the expression of the

Figure 4. $\alpha 8\beta 1$ and α -smooth muscle actin expression during pulmonary fibrosis. Mouse lung sections obtained 4 days after bleomycin treatment were stained for $\alpha 8\beta 1$ (FITC) and α -smooth muscle actin (Cy3). Superimposition of the images reveals that most cells that express α -smooth muscle actin also express $\alpha 8\beta 1$ (yellow). However, some cells express $\alpha 8\beta 1$ but not α -smooth muscle actin (white arrows). Control specimens incubated with preimmune serum displayed no significant staining (data not shown). Original magnification, $\times 40$.

classic fibronectin receptor $\alpha 5\beta 1$. $\alpha 5\beta 1$ is expressed in a variety of cell types found in the lung after injury, such as macrophages, lymphocytes, neutrophils, endothelial cells, and epithelial cells, in addition to fibroblasts,^{24,25} whereas $\alpha 8\beta 1$ expression is limited to interstitial fibroblasts and smooth muscle cells.¹⁴ Therefore, the binding of $\alpha 8\beta 1$ to its ligands, such as fibronectin, vitronectin, or tenascin, may transduce specific signals to activated

myofibroblasts that are important in the generation of fibrosis.

Fibronectin, a ligand for $\alpha 8\beta 1$, has been extensively characterized in the context of pulmonary and hepatic fibrosis. Increased fibronectin synthesis and deposition is an important early event following lung injury.²⁶ In addition, increased capillary permeability is seen with lung injury, which results in the exudation of plasma compo-

Figure 5. Immunohistochemistry of $\alpha 8\beta 1$ in normal and fibrotic liver. In normal rat liver, $\alpha 8$ -positive cells are visualized in smooth muscle surrounding vessels in portal veins and hepatic arteries within portal triads. Parenchyma is negative for $\alpha 8\beta 1$. Vascular smooth muscle cells are $\alpha 8\beta 1$ -positive (arrowheads) (A and D). Two weeks after bile duct ligation, $\alpha 8\beta 1$ is detected in cells surrounding proliferating ductules. B and E: After 4 weeks of CCL₄ administration, bridging fibrosis is present within lobules (arrow). Cells within fibrotic bands show strong immunoreactivity for $\alpha 8\beta 1$ (arrow) (C and F). Immunoperoxidase staining. Original magnifications: A–C, $\times 2.5$; D–F, $\times 10$.

Figure 6. Immunoprecipitation of $\alpha 8\beta 1$ from immortalized rat hepatic stellate cells. HSC T6 cells were surface-labeled with biotin, lysed, and then immunoprecipitated with anti- $\alpha 8$ antibody. Proteins were analyzed by SDS-PAGE under nonreducing conditions. The positions of $\alpha 8$ and $\beta 1$ subunits are indicated by arrows on the right; positions of molecular mass markers (kd) are indicated on the left.

nents such as fibronectin into air spaces. Fibronectin is a proliferative and haptotactic signal for fibroblasts^{27,28} and leads to migration of fibroblasts to abnormal locations, such as air spaces, where they deposit ECM proteins and contribute to obliteration of normal lung architecture. Fibronectin staining is concentrated along the slender processes of fibroblasts during pulmonary fibrosis,²⁹ in a distribution similar to that of $\alpha 8\beta 1$ staining. In the liver, EDA fibronectin is synthesized within 24 hours after injury by endothelial cells. Interaction of EDA fibronectin with stellate cells leads to their synthesis of smooth muscle proteins, extracellular matrix proteins (including fibronectin), and stellate cell activation.³⁰ Migration of fibroblasts over a provisional matrix in early wound healing is mediated in part by integrins.³¹ We have shown that $\alpha 8\beta 1$ mediates the migration of cells on fibronectin and therefore may have a role in the migration of fibroblasts over a fibronectin gradient after injury (unpublished data).

Our data also suggest that $\alpha 8\beta 1$ may contribute to the organ contraction by fibroblasts, through interaction with increased fibronectin deposited in the provisional wound matrix. Contractility of activated myofibroblasts leads to tissue distortion and dysfunction in both lung and liver. Because CICs in lung and stellate cells in liver are both important mediators of contraction during fibrosis, information about the receptors mediating this activity is essential. For example, the collagen-binding integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ mediate contraction of collagen gels and play a role in the development of disease in liver.³² Fibronectin contributes to fibrin clot retraction through the integrin $\alpha 5\beta 1$.³³ Other fibronectin receptors, such as $\alpha 8\beta 1$, may also contribute to the contractile response by interacting with components of the provisional wound matrix during injury and facilitate retraction.

Examination of the fibrotic response to pulmonary injury has broad relevance to other tissues, particularly liver. Much like CICs in lung, we show that $\alpha 8\beta 1$ expression by stellate cells is increased during hepatic fibrosis. Nonetheless, there are several differences between the two cell types. Although $\alpha 8\beta 1$ expression increases in both pulmonary and hepatic fibrosis, $\alpha 8\beta 1$ is expressed in resting CICs in the lung but is not expressed in stellate cells in the normal liver. In the normal adult liver, stellate cells contain intracellular droplets of retinoids (once thought to be lipids; hence the former nomenclature "lipocytes"). After injury, retinoid droplets are lost. In the developing lung, a similar population of lipid-containing

interstitial cells (LICs) exists.³⁴ However, with lung maturation, LICs lose the lipid droplets and become CICs. Interestingly, another difference between CICs and stellate cells is that CICs express contractile microfilaments at rest, whereas stellate cells only express contractile microfilaments after injury.³⁵ The data collectively support the notion that there is significant heterogeneity among connective tissue cells in normal and fibrotic tissues. Heterogeneity of cells of fibroblast lineage in the liver is an important, emerging concept.²⁰ In addition to desmin-positive and desmin-negative stellate cells, a recent report suggests that a subpopulation of liver myofibroblasts, distinct from retinoid-containing stellate cells, exists in the liver and may have a role in the development of fibrosis.²¹ The expression of $\alpha 8\beta 1$ may help define a subpopulation of myofibroblast-like cells in the liver that may be important in the development of fibrosis.

The transition of myofibroblast-like cells to an activated, smooth muscle-like phenotype is a characteristic response to injury of many other organs. After lung injury, activated CICs (ie, α -smooth muscle actin-expressing cells) express $\alpha 8\beta 1$. Likewise, during experimental glomerulonephritis, mesangial cells in the kidney proliferate and express α -smooth muscle actin.³⁶ It is of interest that mesangial cells express $\alpha 8\beta 1$ in normal kidney.¹⁴ Whether $\alpha 8\beta 1$ is involved in the transition of fibroblasts to an activated phenotype remains to be tested.

In summary, we have shown that the integrin $\alpha 8\beta 1$ is expressed in alveolar CICs by electron microscopy and is predominately expressed along the filopodia and thinned extensions of the interstitial cells. We have shown that fibroblasts isolated from lung express $\alpha 8\beta 1$ by Western blot and that $\alpha 8\beta 1$ localizes to focal contacts by immunofluorescence. Furthermore, expression of $\alpha 8\beta 1$ is increased after the development of pulmonary and hepatic fibrosis and colocalized with α -smooth actin expression. Our data support a potential role for the integrin $\alpha 8\beta 1$ in pulmonary fibroblast response after injury. Moreover, parallel expression patterns of $\alpha 8\beta 1$ after liver injury point to a broader role for this integrin in organizing ECM during wound healing. Expression of $\alpha 8\beta 1$ by interstitial cells may be important for signals generated through interaction with extracellular matrix proteins secreted after injury, such as fibronectin. Further studies examining the mechanisms by which $\alpha 8\beta 1$ contributes to tissue injury and repair are under way.

Acknowledgments

We thank Veronica Guille and Min Lu for technical assistance. We also thank Dr. Scott L. Friedman for helpful discussion and critique of the manuscript.

References

1. Kapanci Y, Assimacopoulos A, Irle C, Zwahlen A, Gabbiani G: "Contractile interstitial cells" in pulmonary alveolar septa: a possible regulator of ventilation-perfusion ratio? Ultrastructural, immunofluorescence, and in vitro studies. *J Cell Biol* 1974, 60:375-392
2. Kapanci Y, Costabella PM, Cerutti P, Assimacopoulos A: Distribution

- and function of cytoskeletal proteins in lung cells with particular reference to "contractile interstitial cells." *Methods Achiev Exp Pathol* 1979, 9:147-168
3. Fukui M, Yasui H, Watanabe K, Fujimoto T, Kakuma T, Yoshida R, Ohi M, Kuno K: Hypoxic contraction of contractile interstitial cells isolated from bovine lung. *Am J Physiol* 1996, 270:L962-L972
4. Weibel ER, Crystal RG: Structural organization of the pulmonary interstitium. *The Lung: Scientific Foundations*, vol 1. Edited by RG Crystal, JB West. New York, Raven Press, 1991, pp 369-380
5. Vyalov SL, Gabbiani G, Kapanci Y: Rat alveolar myofibroblasts acquire alpha-smooth muscle actin expression during bleomycin-induced pulmonary fibrosis. *Am J Pathol* 1993, 143:1754-1765
6. Adler KB, Callahan LM, Evans JN: Cellular alterations in the alveolar wall in bleomycin-induced pulmonary fibrosis in rats. An ultrastructural morphometric study. *Am Rev Respir Dis* 1986, 133:1043-1048
7. Wake K: Perisinusoidal stellate cells (fat-storing cells, interstitial cells, lipocytes), their related structure in and around the liver sinusoids, and vitamin A-storing cells in extrahepatic organs. *Int Rev Cytol* 1980, 66:303-353
8. Rockey DC, Boyles JK, Gabbiani G, Friedman SL: Rat hepatic lipocytes express smooth muscle actin upon activation in vivo and in culture. *J Submicrosc Cytol Pathol* 1992, 24:193-203
9. Friedman SL, Roll FJ, Boyles J, Bissell DM: Hepatic lipocytes: the principal collagen-producing cells of normal rat liver. *Proc Natl Acad Sci USA* 1985, 82:8681-8685
10. Rockey DC, Housset CN, Friedman SL: Activation-dependent contractility of rat hepatic lipocytes in culture and in vivo. *J Clin Invest* 1993, 92:1795-1804
11. Piguet PF, Rosen H, Vesin C, Grau GE: Effective treatment of the pulmonary fibrosis elicited in mice by bleomycin or silica with anti-CD-11 antibodies. *Am Rev Respir Dis* 1993, 147:435-441
12. Breuss JM, Gallo J, DeLisser HM, Klimanskaya IV, Folkesson HG, Pittet JF, Nishimura SL, Aldape K, Landers DV, Carpenter W, Gillett N, Sheppard D, Matthay MA, Albelda SM, Kramer RH, Pytela R: Expression of the beta 6 integrin subunit in development, neoplasia and tissue repair suggests a role in epithelial remodeling. *J Cell Sci* 1995, 224:1-2251
13. Griffiths M, Huang X-Z, Wu JF, Sheppard D: Inactivation of the beta 6 subunit gene protects against bleomycin-induced pulmonary fibrosis. *Mol Biol Cell* 1996, 7:166a
14. Schnapp L, Breuss J, Ramos D, Sheppard D, Pytela R: Sequence and tissue distribution of the human integrin alpha 8 subunit: a beta 1-associated a subunit expressed in smooth muscle cells. *J Cell Science* 1995, 108:537-544
15. Schnapp LM, Hatch N, Ramos DM, Klimanskaya IV, Sheppard D, Pytela R: The human integrin alpha 8 beta 1 functions as a receptor for tenascin, fibronectin, and vitronectin. *J Biol Chem* 1995, 270:23196-23202
16. Varnum-Finney B, Venstrom K, Muller U, Kypta R, Backus C, Chiquet M, Reichardt LF: The integrin receptor alpha 8 beta 1 mediates interactions of embryonic chick motor and sensory neurons with tenascin-C. *Neuron* 1995, 14:1213-1222
17. Muller U, Bossy B, Venstrom K, Reichardt LF: Integrin alpha 8 beta 1 promotes attachment, cell spreading, and neurite outgrowth on fibronectin. *Mol Biol Cell* 1995, 6:433-448
18. Kim Y, Ratzliff V, Choi SG, Lalazar A, Theiss G, Dang Q, Kim SJ, Friedman SL: Transcriptional activation of transforming growth factor beta1 and its receptors by the Kruppel-like factor Zf9/core promoter-binding protein and Sp1. Potential mechanisms for autocrine fibrogenesis in response to injury. *J Biol Chem* 1998, 273:33750-33758
19. Einheber S, Schnapp LM, Salzer JL, Cappelletti ZB, Milner TA: Regional and ultrastructural distribution of the alpha 8 integrin subunit in developing and adult rat brain suggests a role in synaptic function. *J Comp Neurol* 1996, 370:105-134
20. Friedman SL: The virtuosity of hepatic stellate cells. *Gastroenterology* 1999, 117:1244-1246
21. Knittel T, Kobold D, Saile B, Grundmann A, Neubauer K, Piscaglia F, Ramadori G: Rat liver myofibroblasts and hepatic stellate cells: different cell populations of the fibroblast lineage with fibrogenic potential. *Gastroenterology* 1999, 117:1205-1221
22. Huhtala P, Humphries MJ, McCarthy JB, Tremble PM, Werb Z, Damsky CH: Cooperative signaling by alpha 5 beta 1 and alpha 4 beta 1 integrins regulates metalloproteinase gene expression in fibroblasts adhering to fibronectin. *J Cell Biol* 1995, 129:867-879
23. Pozzi A, Wary KK, Giancotti FG, Gardner HA: Integrin alpha1beta1 mediates a unique collagen-dependent proliferation pathway in vivo. *J Cell Biol* 1998, 142:587-594
24. Roman J, Jeon YJ, Gal A, Perez RL: Distribution of extracellular matrices, matrix receptors, and transforming growth factor-beta 1 in human and experimental lung granulomatous inflammation. *Am J Med Sci* 1995, 309:124-133
25. Pilewski JM, Albelda SM: Adhesion molecules in the lung. An overview. *Am Rev Respir Dis* 1993, 148:S31-S37
26. Raghoebar R, Lurie S, Seyer JM, Kang AH: Profiles of steady state levels of messenger RNAs coding for type I procollagen, elastin, and fibronectin in hamster lungs undergoing bleomycin-induced interstitial pulmonary fibrosis. *J Clin Invest* 1985, 76:1733-1739
27. Bitterman PB, Rennard SI, Adelberg S, Crystal RG: Role of fibronectin as a growth factor for fibroblasts. *J Cell Biol* 1983, 97:1925-1932
28. Rennard SI, Hunninghake GW, Bitterman PB, Crystal RG: Production of fibronectin by the human alveolar macrophage: mechanism for the recruitment of fibroblasts to sites of tissue injury in interstitial lung diseases. *Proc Natl Acad Sci USA* 1981, 78:7147-7151
29. Lazenby AJ, Crouch EC, McDonald JA, Kuhn Cd: Remodeling of the lung in bleomycin-induced pulmonary fibrosis in the rat. An immunohistochemical study of laminin, type IV collagen, and fibronectin. *Am Rev Respir Dis* 1990, 142:206-214
30. Jarnagin WR, Rockey DC, Kotliansky VE, Wang SS, Bissell DM: Expression of variant fibronectins in wound healing: cellular source and biological activity of the EIIIA segment in rat hepatic fibrogenesis. *J Cell Biol* 1994, 127:2037-2048
31. Greiling D, Clark RA: Fibronectin provides a conduit for fibroblast transmigration from collagenous stroma into fibrin clot provisional matrix. *J Cell Sci* 1997, 86:1-870
32. Bissell DM: Hepatic fibrosis as wound repair: a progress report. *J Gastroenterol* 1998, 33:295-302
33. Corbett SA, Schwarzbauer JE: Requirements for alpha(5)beta(1) integrin-mediated retraction of fibronectin-fibrin matrices. *J Biol Chem* 1999, 274:20943-20948
34. Vaccaro C, Brody JS: Ultrastructure of developing alveoli. I. The role of the interstitial fibroblast. *Anat Rec* 1978, 192:467-479
35. McGowan SE, Torday JS: The pulmonary lipofibroblast (lipid interstitial cell) and its contributions to alveolar development. *Annu Rev Physiol* 1997, 59:43-62
36. Johnson RJ, Iida H, Alpers CE, Majesky MW, Schwartz SM, Pritzl P, Gordon K, Gown AM: Expression of smooth muscle cell phenotype by rat mesangial cells in immune complex nephritis. Alpha-smooth muscle actin is a marker of mesangial cell proliferation. *J Clin Invest* 1991, 87:847-858