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RAPID COMMUNICATION

Evaluation of Fibronectin Gene Expression by In Situ *Hybridization*

Differential Expression of the Fibronectin Gene Among Populations of Human Alveolar Macrophages

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Populations of alveolar macrophages recovered from the lower respiratory tract secrete fibronectin, a multifunctional glycoprotein capable of influencing cell migration, attachment, differentiation, and proliferation. Using *in situ* hybridization of ^{35}S-labeled antisense and sense RNA fibronectin probes, the present study demonstrates that most, but not all, normal alveolar macrophages contain fibronectin mRNA transcripts, and that among those macrophages expressing this gene, the relative amount of fibronectin mRNA transcripts varies from cell to cell. Interestingly, while 66 ± 3% of normal alveolar macrophages contain fibronectin mRNA transcripts, this is increased to 82 ± 2% ($P < 0.01$) of alveolar macrophages recovered from the lungs of individuals with idiopathic pulmonary fibrosis (IPF), a chronic inflammatory disorder associated with exaggerated amounts of fibronectin in the lower respiratory tract. Furthermore, of the macrophages expressing the fibronectin gene, those from IPF patients contain more fibronectin mRNA transcripts than those from normals. Consistent with this observation, evaluation of tissue samples from IPF patients demonstrated that of all cells present, alveolar macrophages showed the greatest numbers of fibronectin mRNA transcripts per cell. These observations demonstrate that there can be marked cell-to-cell variation in the expression of the gene for a macrophage product such as fibronectin, suggesting that there are processes that modulate similar cells in the same anatomic compartment to vary their expression of the same gene. (Am J Pathol 1988, 133:193–203)

FIBRONECTIN, A 440 KD glycoprotein present in plasma and tissues, plays a central role in cell-matrix interactions through its ability to bind to cells and a variety of connective tissue components.[1-5] In this context, fibronectin is considered important in development, normal tissue turnover, wound repair, and acute and chronic inflammatory processes characterized by tissue remodeling.[1-12] Detailed studies of fibronectin structure have identified a number of binding domains within the molecule, each with different functional properties.[1-5] Fibronectin is coded for by a gene of at least 50 exons of over 50 kb in length.[13,14] The gene is expressed in a variety of cells, including mesenchymal cells, epithelial cells, endothelial cells, and mononuclear phagocytes.[1-6,8,15-23]

Of the various sources of fibronectin, mononuclear phagocytes are of interest because of the fundamental role of these cells in normal and abnormal tissue turnover and repair.[11,24] In this regard, while the fibronec-

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tin gene is not expressed by blood monocytes, it is expressed by mature mononuclear phagocytes, including alveolar macrophages and peritoneal macrophages.[6,8,16–23] Because mature mononuclear phagocytes are capable of migrating, the ability of these cells to express the fibronectin gene provides a mechanism by which this important macromolecule can be deposited at sites of local tissue growth and injury.[6–12,25]

Studies of the control of fibronectin production by mononuclear phagocytes have shown that the rate of fibronectin secretion parallels the steady state fibronectin mRNA levels of the mononuclear phagocyte population.[26] For example, blood monocytes contain no fibronectin mRNA transcripts and do not produce fibronectin, while alveolar macrophages, the pulmonary component of the mononuclear phagocyte system, express fibronectin mRNA transcripts and produce fibronectin. Furthermore, in circumstances in which alveolar macrophages down-regulate the amounts of fibronectin mRNA, such as when activated by surface stimuli, the amount of fibronectin produced by the cells is decreased. In inflammatory diseases of the lungs such as idiopathic pulmonary fibrosis (IPF), however, in which alveolar macrophages contain high steady-state levels of fibronectin mRNA, the amount of fibronectin produced is increased in parallel.[26]

In the context of these observations, the present study was designed to evaluate whether the steady-state fibronectin mRNA levels observed in a population of alveolar macrophages reflect the fibronectin mRNA levels for all macrophages in the population, or whether fibronectin mRNA levels vary from cell to cell. To approach this question we have used *in situ* hybridization techniques with fibronectin RNA probes to evaluate the proportion of alveolar macrophages expressing fibronectin mRNA transcripts, to determine whether there are differences in the morphologic characteristics of the macrophages expressing or not expressing the fibronectin gene, and, for those macrophages expressing the fibronectin gene, whether there are differences in the number of fibronectin mRNA copies per cell. As models for circumstances in which *in vivo* fibronectin expression may differ on a cell to cell basis, we have used alveolar macrophages from normal individuals and from patients with IPF.

Materials and Methods

Study Population

Normal individuals (n = 5; 1 man, 4 women; age, 27 ± 3 years) had no history of respiratory problems. Physical examination, chest x-ray, and lung function tests were normal in all individuals. All data are presented as mean ± standard error of the mean; all statistical comparisons were made using the two-tailed Student's *t*-test.

The diagnosis of IPF was established in five individuals (three men, two women; mean age 57 ± 5 years) using previously described criteria, including an open lung biopsy.[27] All had chest x-rays showing a diffuse reticulonodular infiltrate. On the average, lung function testing[28] revealed vital capacity 57 ± 11% predicted, total lung capacity 53 ± 7% predicted, forced expiratory volume in 1 sec/forced vital capacity 101 ± 17% predicted, and single breath diffusion capacity (DLCO; corrected for volume and hemoglobin) 43 ± 5% predicted. Bronchoalveolar lavage analysis and gallium-67 scans were typical for individuals with mid-course IPF.[27,29,30]

Preparation of Alveolar Macrophages

Alveolar macrophages were obtained by bronchoalveolar lavage as described previously.[31] The viability of the alveolar macrophages as assessed by trypan blue exclusion was >90% in all cases. Cells used for Northern analysis of fibronectin mRNA were handled as described previously.[32] For *in situ* hybridization, the recovered cells were washed once with cold phosphate-buffered saline (PBS; pH 7.4) and then immediately centrifuged onto slides prepared as described[33] at a density of 2×10^5/sq cm using a Cytospin 2 (Shandon Instruments, Pittsburgh, PA) at 600*g* for 7 minutes. The cells were air dried for 5 minutes, 23 C, fixed with 3.7% formaldehyde in PBS for 2 minutes, and kept in 70% ethanol at 4 C until use. Preliminary studies demonstrated no loss of signal with storage up to 3 months.

Preparation of Lung Biopsies

Small pieces of lung from the open lung biopsies of the IPF patients were rinsed twice in PBS containing 15% sucrose and fixed for 2 hours in a freshly prepared solution of 4% paraformaldehyde (Fisher, Pittsburgh, PA) in PBS. To make up the fixative, paraformaldehyde was dissolved in PBS with stirring and low heat for 3 hours, and the resulting solution filtered. The fixed tissues were washed in several changes of cold PBS containing 15% sucrose and incubated overnight in the same solution. After washing, one of the tissue blocks was frozen rapidly with liquid nitrogen for making cryostat sections and was kept in liquid nitrogen until sectioning. Another block was used for preparation of the usual paraffin-embedded tissue sec-

tions. Cryostat sections (6–8 μ), were cut at −20 C, and collected on polylysine-coated, 0.1% diethyl pyrocarbonate (DEPC) treated slides. Sections were thawed at 25 C, and dried with an air jet for 10 minutes, 23 C. The slides were postfixed with 3.7% formaldehyde in PBS for 10 min at 23 C, rinsed twice with PBS, and kept in 70% ethanol at 4 C until use.

Preparation of RNA Probes

A cloned human fibronectin cDNA (plasmid pFH154; provided by F. E. Baralle, University of Oxford)[34] was digested with PstI, and the resulting 1.5 kb fragment was digested with BamH1. The resulting 0.9 kb PstI-BamHI fragment was subcloned into the transcription vector pGEM 4 (Promega Biotec) to prepare single-strand RNA sense and anti-sense probes.[35] Radiolabeled RNA probes (specific activity, 1.3×10^8–1.9×10^8 dpm/μg DNA template) were prepared by transcription of the linearized double-strand DNA template using ^{35}S-UTP (1000 Ci/mmol, Amersham) as described by the supplier. As a control, ^{35}S-labeled sense and anti-sense γ-actin RNA probes were prepared in the same manner. These RNA probes were based on a 0.34 kb PstI-BglII fragment of plasmid pHFγA-1 (provided by P. Gunning and L. Kedes, Stanford University).[36] As controls of the specificity of the probes used, Northern analyses, using ^{32}P-labeled sense and anti-sense fibronectin RNA probes, prepared as described for ^{35}S-labeled RNA probes, were performed with RNA extracted from cells known to contain fibronectin mRNA transcripts (normal human diploid fetal lung fibroblast strain HFL-1, American Type Culture Collection [ATCC], CCL 153) as well as from cells known not to contain fibronectin mRNA transcripts (human blood monocytes[26] and the human histiocytic lymphoma strain U937 [ATCC, CRL 1593], unpublished observation from this laboratory).

In Situ Hybridization

The prehybridization preparation of the lavage samples and tissue were based on the procedure described by Brigati et al.[37] The slides containing the lavage samples were washed twice in 2× standard saline-citrate buffer (SSC; 150 mM NaCl, 15 mM Na citrate, pH 7.0) for 1 minute each with frequent agitation, immersed in 0.1 M triethanolamine buffer, pH 8.0, for 2 minutes, and incubated in 0.25% acetic anhydride in triethanolamine buffer for 10 minutes.[38] The slides were then washed (1 minute) in 2× SSC, (1 minute) in PBS, immersed for 30 minutes in 0.1 M Tris-HCl, pH 7.0, containing 0.1 M glycine, and rinsed twice in 2× SSC for 5 minutes each. Cell samples were kept in the second change of 2× SSC until hybridized.

The tissue samples were predigested with freshly prepared pronase (Calbiochem, San Diego, CA) at a final concentration of 0.25 mg/ml in 50 mM Tris-HCl, pH 7.6, containing 5 mM ethylenediaminetetraacetate (EDTA) for 10 minutes at 23 C. The slides were washed twice (30 seconds each) in PBS containing 2 mg/ml glycine, postfixed in 4% formaldehyde in PBS for 20 minutes, and then washed twice (5 minutes each) in PBS containing 2 mg/ml glycine. The tissue samples were then dehydrated twice in 70% ethanol for 5 minutes each, in 95% ethanol for 5 minutes, dried with an air jet for 5 minutes, and then rinsed in 2× SSC until hybridized.

Hybridization of the labeled RNA probes to lavage and tissue samples was performed using methods described by Harper et al[39] with minor modifications.[40] The cells or tissues were placed in 50% formamide (Fluka), 2× SSC for 10 minutes at 55 C before hybridization. The hybridization mixture contained the labeled RNA probe (2.8×10^5–4.2×10^5 dpm/μl), 50% formamide (International Biotechnologies), 10 mM dithiothreitol (DTT), salmon sperm DNA (100 μg/ml), yeast tRNA (600 μg/ml), bovine serum albumin (2 mg/ml), and 2× SSC. After hybridization at 50 C for 5 hours, the slides were immersed in 3 changes of 50% formamide-2× SSC containing 5 mM DTT at 52 C, washed in 2× SSC at 23 C, and incubated in a RNAse solution (100 μg/ml RNAse A [Sigma, St. Louis, MO], 1 μg/ml RNAse T1 [Boehringer Mannheim, Indianapolis, IN]) at 37 C, 30 minutes. The slides were subsequently washed in 50% formamide-2× SSC containing 5 mM DTT at 52 C for 5 minutes, and 3 changes of 2× SSC at 23 C. They were then dehydrated sequentially in 70%, 80%, and 90% ethanol for 1 minute each with agitation, dried with an air jet for 5 minutes, and stored with anhydrous $CaCl_2$ at 4 C. Autoradiography was performed by dipping in Kodak NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with sterile distilled water at 42 C. After a 3-day exposure at 4 C the slides were developed in Kodak D-19 developer (4.5 minutes, 15 C), and fixed with Kodak rapid fixer for 5 minutes, 15 C. The developed slides containing the lavage cells were stained with modified Giemsa stain and the slides containing the tissue samples were stained with hematoxylin and eosin (H & E).

A number of controls were used to evaluate the specificity of the hybridization of the ^{35}S-labeled anti-sense fibronectin RNA probe to the lavage cells and tissue samples. These included: 1) *in situ* hybridization with the sense probe; 2) *in situ* hybridization of the sense and anti-sense probes to cell culture samples

from a normal human diploid fetal lung fibroblast strain, HFL-1, as well as from human blood monocytes and the human histiocytic lymphoma strain U937; 3) *in situ* hybridization with the γ-actin RNA probe; and 4) *in situ* hybridization with the anti-sense fibronectin probe after RNase treatment of the cell and tissue samples.

To quantitate the relative number of fibronectin mRNA transcripts per cell, the autoradiographs of the samples hybridized with the ^{35}S-labeled anti-sense RNA fibronectin probe were screened systematically at high power (×1000) every 1 mm. An average of >100 cells were evaluated for each sample. Macrophages were identified as "positive" if they contained 6 or more grains/cell; the maximum of 5 grains/negative cell in preparations hybridized with the anti-sense probe was based on the number of grains observed in macrophages hybridized with the ^{35}S-labeled sense RNA fibronectin probe. To quantitate the size distribution of the lavage cells containing fibronectin mRNA transcripts the cell diameter was evaluated using an ocular eyepiece equipped with a standardized micrometer grid (Carl Zeiss Inc., Thomwood, NY). To compare the amount of fibronectin mRNA transcripts according to the cell size in normals *vs.* IPF patients, the mean number of grains per cell were evaluated in seven different categories of cells grouped according to the cell diameter. As for fibronectin expression the relative number of γ-actin mRNA containing cells was evaluated in normals and IPF patients after hybridization with the anti-sense γ-actin probe. To compare the amount of γ-actin mRNA in normals and IPF patients, the mean number of grains per cell was determined by evaluating 100 alveolar macrophages per sample.

To assess the relative intensity of fibronectin gene expression by alveolar macrophages compared with the parenchymal cells comprising the alveolar walls, lung biopsies were hybridized with the labeled anti-sense probe. The degree of binding of the anti-sense probe to alveolar macrophages compared with the parenchymal cells was determined by counting the number of silver grains over alveolar macrophages and parenchymal cells (all epithelial, endothelial, and mesenchymal cells comprising the alveolar walls grouped together). In the areas with densely packed cells, the total number of grains in that area was counted and the mean number of grains per cell was evaluated and quantified based on the total number of cells in that area. A minimum of 130 cells in each category were counted. Evaluation of parallel samples with the labeled sense fibronectin probe demonstrated ≤5 grains/cell; in this context, ≤5 grains/cell was considered "negative."

Results

Evaluation of RNA extracted from normal and IPF alveolar macrophages with either labeled cDNA[32] or anti-sense RNA fibronectin probes demonstrated on Northern analysis the expected 7.8 kb mRNA transcripts (not shown; see reference 32 for a typical example). Under identical conditions in parallel lanes on the Northern analysis, the labeled sense fibronectin RNA probe did not hybridize to any RNA. Likewise, identical results were observed using sense and anti-sense RNA fibronectin probes on RNA extracted from a normal human lung fibroblast line (HFL-1). In contrast, RNA extracted from human blood monocytes and the histiocytic lymphoma cell line U937, cells that do not produce fibronectin, showed no positive signals on Northern analysis with either the anti-sense or sense probes.

Fibronectin mRNA Transcripts in Alveolar Macrophages Recovered by Lavage

Consistent with the knowledge that normal human alveolar macrophages produce fibronectin,[6,16] many normal alveolar macrophages demonstrated hybridization with the labeled anti-sense RNA fibronectin probe. In those cells that were positive, the silver grains were diffusely distributed throughout the cytoplasm. Qualitative evaluation of the samples demonstrated that more than half of the cells had mild to moderate deposition of silver grains, and that most of the positive cells appeared to be mature macrophages (Figure 1a, b). In contrast, most of the small, basophilic younger macrophages showed fewer grains in the cytoplasm. In contrast to the anti-sense RNA probe, the labeled sense RNA fibronectin probe showed little hybridization to normal macrophages; most cells showed no grains, or at most 1 grain per cell (Figure 1c, d). The anti-sense fibronectin probe gave positive results on *in situ* hybridization with HFL-1 cells and negative results with human blood monocytes and the histiocytic lymphoma cell line U937. These three cell types all gave negative results with the sense fibronectin probe. As an additional control, the macrophages were pretreated with RNAse before adding the labeled anti-sense RNA probe; such pretreatment completely abolished the hybridization.

Evaluation of alveolar macrophages with the anti-sense and sense RNA fibronectin probes under hybridization conditions identical to those used for normal macrophages demonstrated that many macrophages from IPF patients contained fibronectin mRNA transcripts (Figure 1e, f). Fully mature, older appearing cells containing dust particles in the cyto-

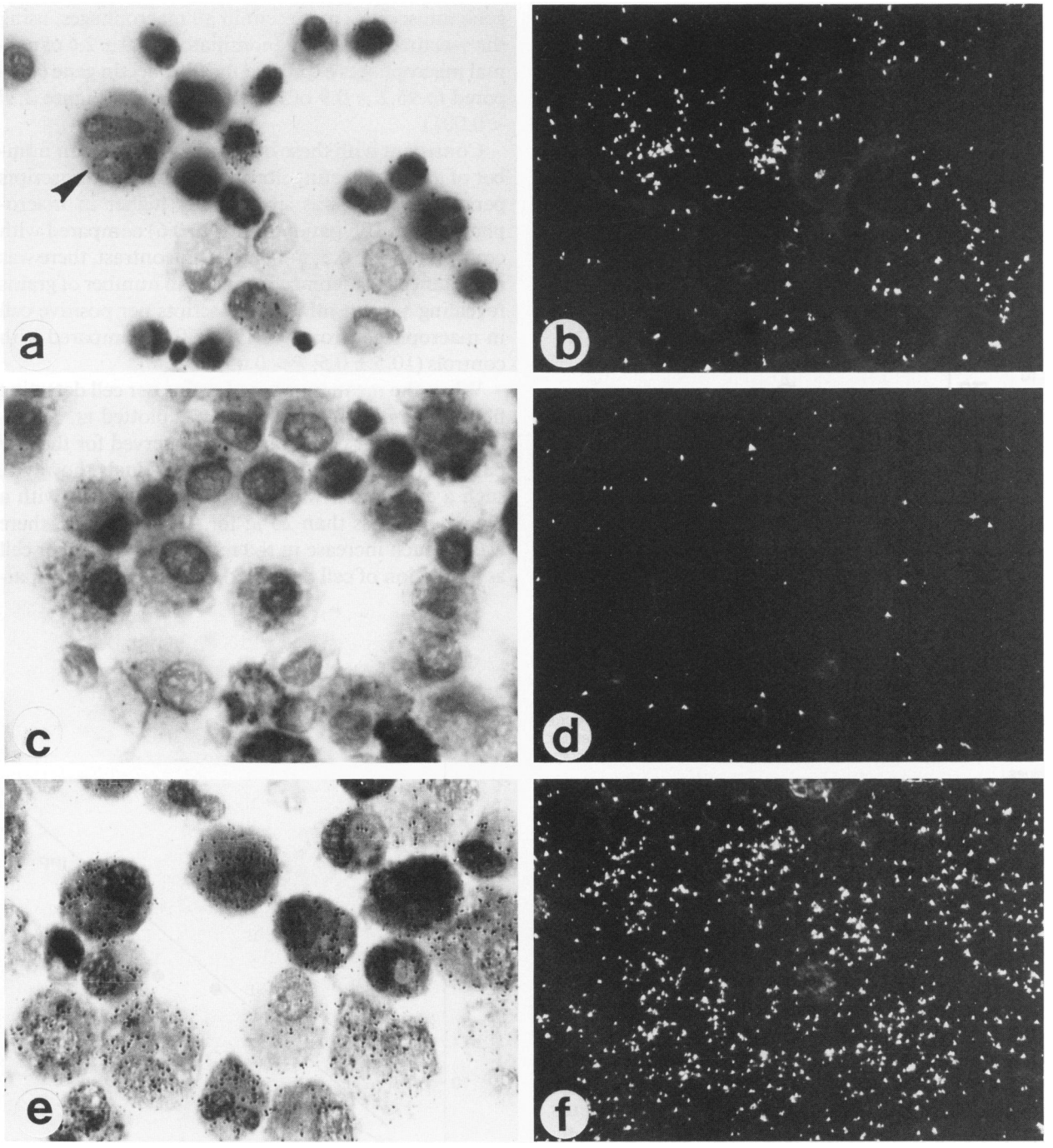

Figure 1—Examples of autoradiographs demonstrating *in situ* hybridization evaluation of fibronectin mRNA transcripts within alveolar macrophages. **a and b**—Autoradiographs demonstrating *in situ* hybridization of ^{35}S-labeled fibronectin RNA anti-sense probe to fibronectin mRNA transcripts within alveolar macrophages of normal individuals. **a**—Alveolar macrophages hybridized with the ^{35}S-labeled fibronectin RNA anti-sense probe. Many, but not all macrophages exhibit fibronectin transcripts. A large macrophage (arrow) with multiple nuclei demonstrates very positive hybridization. Bright field illumination, modified Giemsa stain, ×500 **b**—Identical to a, but with dark field illumination to highlight the location of the silver grains in the autoradiographs. **c and d**—Similar to panels a and b, respectively, but using ^{35}S-labeled fibronectin sense probe. Note the relative lack of grains compared to panels a and b. **e and f**—Autoradiographs demonstrating *in situ* hybridization of ^{35}S-labeled fibronectin RNA anti-sense probe to fibronectin mRNA transcripts within alveolar macrophages from a patient with IPF. **e**—Example of alveolar macrophages from a patient with IPF hybridized with the ^{35}S-labeled fibronectin anti-sense probe. Most of the macrophages exhibit numerous fibronectin mRNA transcripts. Bright field illumination, modified Giemsa stain, ×500 **f**—Identical to panel e, but with dark field illumination.

plasm and vacuolated cells usually contained very few grains. As with the normal macrophages, when IPF macrophages were evaluated with the sense probe silver grains rarely were observed. Likewise, pretreatment with RNAse completely abolished labeling with the anti-sense RNA probe.

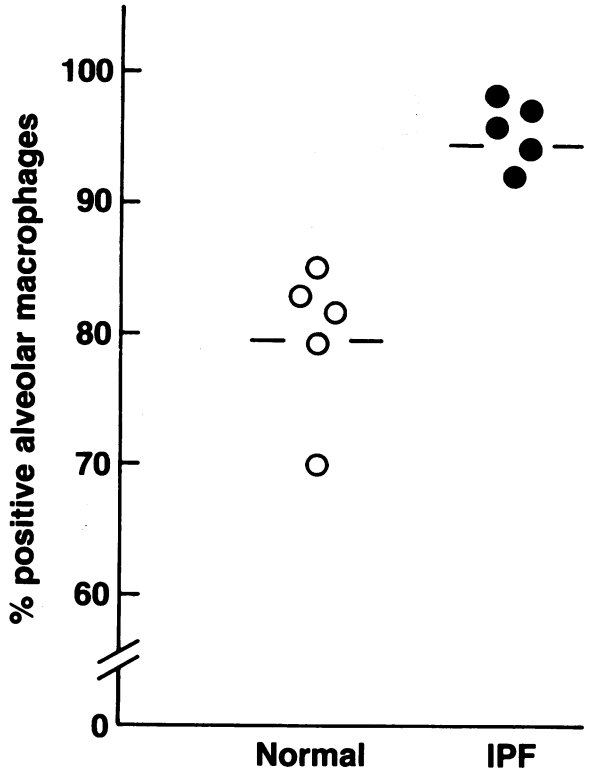

Figure 2—Proportion of alveolar macrophages from normals and patients with IPF expressing the fibronectin gene evaluated using ^{35}S-labeled anti-sense and sense fibronectin RNA probes. The percentage of positive alveolar macrophages is expressed relative to the percentage of alveolar macrophages expressing the γ-actin gene (see Materials and Methods for details). Each data point represents one individual. Horizontal lines indicate average values.

By counting the number of grains per cell, the percentage of macrophages showing fibronectin gene expression was compared between controls and IPF patients. With the sense RNA probe, for both controls and IPF patients, almost all macrophages had ≤1 grain/cell, at most a few cells showed up to 5 grains/cell. Accordingly, cells having ≥6 grains/cell with the anti-sense RNA probe were considered to indicate positive fibronectin gene expression. Using these criteria, in controls, 66 ± 3% of the alveolar macrophages expressed the fibronectin gene. In the IPF patients, however, 82 ± 2% of the cells were positive, a value significantly higher than that for normal individuals ($P < 0.01$). In contrast to the anti-sense RNA fibronectin probe, a general distribution of grains was observed among all macrophages after hybridization with the anti-sense γ-actin RNA probe (not shown). Quantification of parallel samples of normal and IPF alveolar macrophages with the ^{35}S-labeled anti-sense RNA γ-actin probe showed that a similar proportion of cells were positive in the two study populations (controls, 82 ± 2%; IPF, 86 ± 2%; $P > 0.3$). In this context, if the assumption is made that the γ-actin gene transcripts are present in all macrophages, using the γ-actin data as the denominator, 79.9 ± 2.6 of normal macrophages expressed the fibronectin gene compared to 95.2 ± 0.9 of IPF macrophages (Figure 2; $P < 0.001$).

Consistent with these observations, the mean number of grains detecting fibronectin mRNA transcripts per positive cell was significantly higher in macrophages from IPF patients (18.3 ± 0.6) compared with controls (13.5 ± 0.5; $P < 0.001$). In contrast, there was no significant difference in the mean number of grains revealing γ-actin mRNA transcripts per positive cell in macrophages from IPF (9.8 ± 0.6) compared with controls (10.5 ± 0.5; $P > 0.05$).

When the mean number of grains per cell detecting fibronectin mRNA transcripts was plotted *vs.* cell diameter, a linear correlation was observed for the IPF patients (Figure 3). In normal individuals, however, such a correlation was only observed in cells with a diameter of less than 25 μ; for the larger cells, there was no such increase in the number of grains per cell as a function of cell diameter. Overall, when compar-

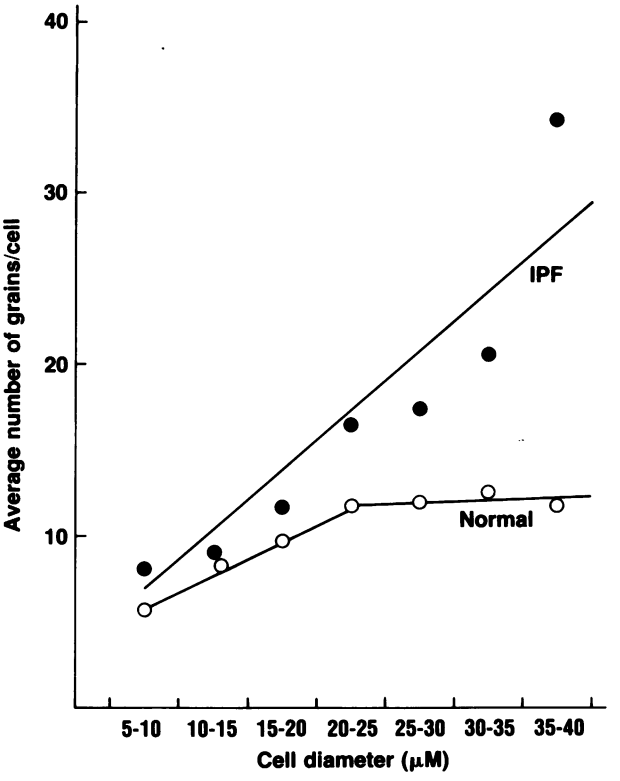

Figure 3—Relationship of the hybridization of the ^{35}S-labeled fibronectin anti-sense probe to cell diameter of alveolar macrophages of controls and individuals with IPF. Shown are the average number of grains per cell for cell diameters for individuals with IPF (●) and controls (○) grouped in 5 μ increments from 5 to 40 μ. For the IPF individuals, there is a linear correlation between the number of grains and cell diameter (r = 0.92). For the normals, there is a direct correlation for cell diameters 5 to 25 μ (r = 0.97), but for the larger cells, the average number of grains per cell was relatively constant.

ing IPF patients with the controls, there were significantly larger numbers of grains associated with cells of ≥20 μ for the IPF patients (for diameters 20–25, 25–30, 30–35, and 35–40 μ, the differences were significant at the $P < 0.01$, $P < 0.01$, $P < 0.01$, and $P < 0.001$ levels respectively). For patients with IPF, most (71%) of the fibronectin mRNA transcripts were expressed in cells having a cell diameter above 20 μ (Figure 4A). In contrast, in controls only 49% of the grains were observed in cells with a diameter of more than 20 μ. Moreover, in IPF patients, 60% of the positive cells had a cell diameter above 20 μ compared to 40% in controls (Figure 4B). Thus, not only are more IPF patients' cells expressing the fibronectin gene than controls, but the cells that express most of the fibronectin mRNA transcripts are in alveolar macrophages greater than 20 μ in diameter.

Fibronectin mRNA Transcripts in Biopsy Specimens

Evaluation of open lung biopsy specimens from IPF patients with the anti-sense fibronectin probe demonstrated that almost all macrophages contained silver grains located over the cytoplasm (Figure 5). This was in striking contrast to the parenchymal cells, some of which appeared to contain fibronectin mRNA transcripts, but to a lesser extent than the macrophages. Analysis of the biopsies demonstrated that hybridization with the fibronectin RNA probe was strikingly positive in alveolar macrophages but present to much less extent in other parenchymal cells (Figure 6). In this regard, parenchymal cells contained significantly fewer silver grains than macrophages. In contrast to the autoradiograms obtained with the anti-sense probe, tissue hybridized with the sense probe showed only the background level of silver grains throughout.

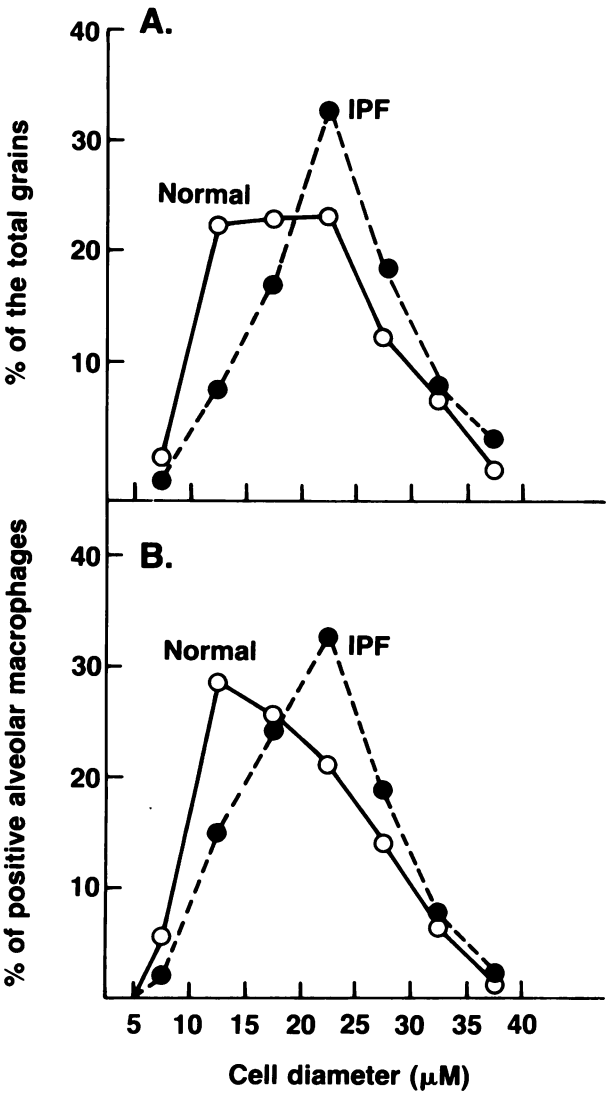

Figure 4—Relationship of the hybridization of the ^{35}S-labeled fibronectin anti-sense probe to cell diameter of alveolar macrophages from controls and individuals with IPF expressed as % of the total grains observed over 100 cells (**A**) and % of positive alveolar macrophages (**B**).

Discussion

Fibronectin, an adhesive glycoprotein, is a major constituent of plasma, extracellular matrices, and most basement membranes.[1-5,30,41,42] There are many sources of fibronectin, including fibroblasts, epithelial cells, chondrocytes, myoblasts, amniotic cells, endothelial cells, hepatocytes, and mononuclear phagocytes.[1-6,8,16-23] Among these cells, mononuclear phagocytes are an example of a class of fibronectin-producing cells that are migratory.[43] In the context of the many functions of fibronectin as a mediator of cell attachment, chemotaxis, differentiation and proliferation, mononuclear phagocytes probably provide a mechanism by which fibronectin is deposited at sites of normal tissue turnover and during tissue remodeling after injury.

The population of alveolar macrophages recovered from the normal lung is known to contain fibronectin mRNA transcripts and to spontaneously secrete 400–500 ng fibronectin/10^6 cells in 24 hours.[6,26] Using *in situ* hybridization with a labeled anti-sense RNA fibronectin probe, the present study demonstrates that the expression of the fibronectin gene varies significantly among such a population of alveolar macrophages. The variation of fibronectin gene expression among the normal macrophages was observed in cells of all size classes. This is of interest in the context of the knowledge that blood monocytes do not express the fibronectin gene until they are allowed to ma-

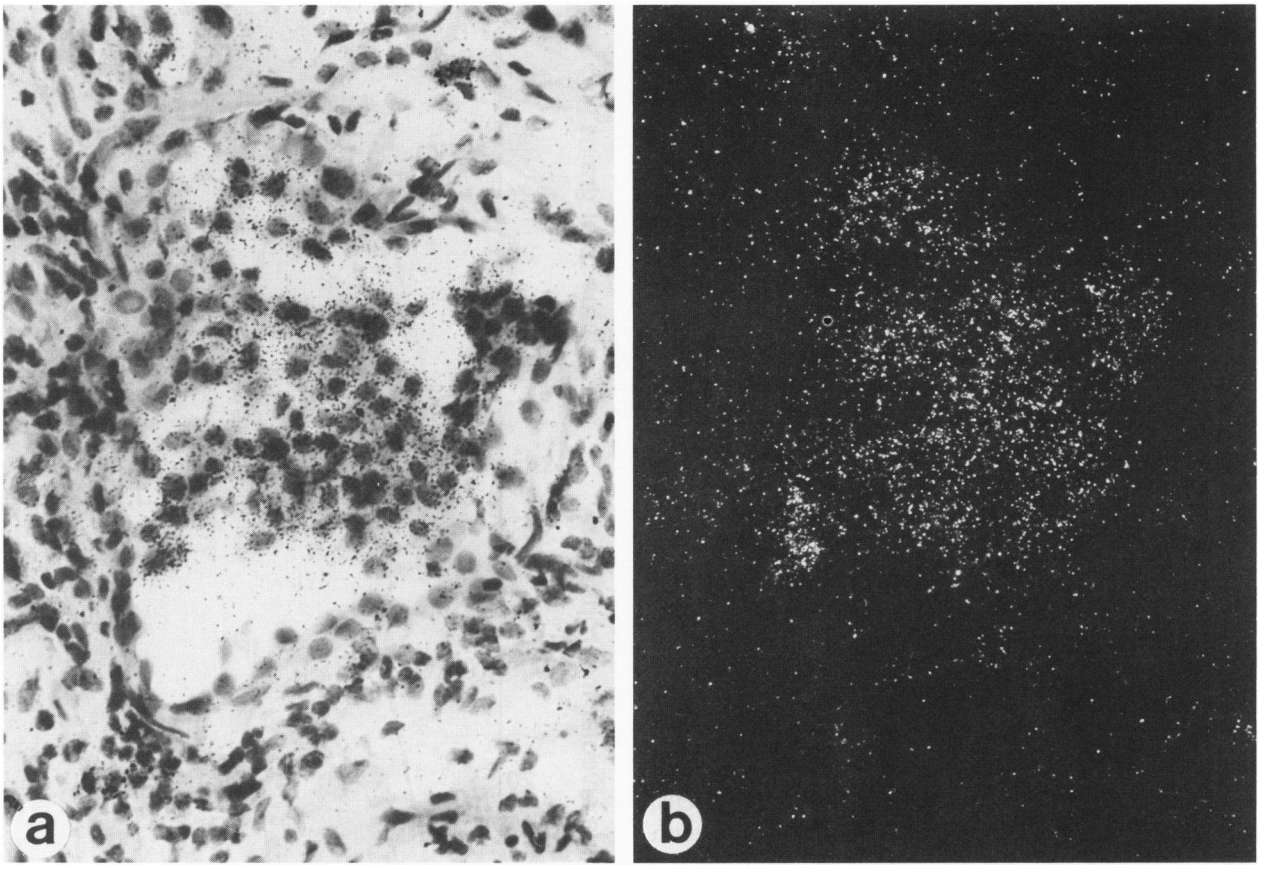

Figure 5—Cells in the lower respiratory tract expressing fibronectin mRNA transcripts. Shown is a section of an open lung biopsy of an individual with mild IPF evaluated with a ^{35}S-labeled fibronectin anti-sense RNA probe and exposed for 3 days. **a**—Bright field illumination of a portion of the biopsy with many alveolar macrophages in the alveolar lumen. Note that while cells comprising the alveolar walls also show silver grains, the alveolar macrophages appear to be the most intensely positive. H & E, ×250 **b**—Identical to a, but with dark field illumination to highlight the silver grains.

ture.[19,26] If the assumption is made that "small" alveolar macrophages include a large proportion of blood monocytes newly recruited into the alveolar tissues,[44] the observation that even small alveolar macrophages contain fibronectin mRNA transcripts suggests that such cells have matured relatively rapidly, at least to the extent of turning on the fibronectin gene.

Interestingly, the population of alveolar macrophages recovered from the lungs of individuals with IPF releases, on the average, several times more fibronectin per 10^6 cells than an equivalent number of normal alveolar macrophages.[6] Although the mechanism for this "up-regulation" of fibronectin production is not known, the population of alveolar macrophages recovered from the IPF lung contains several times more fibronectin steady-state mRNA levels than do populations of normal alveolar macrophages.[26,32] Consistent with, and expanding on this observation, the present study demonstrates that at least two mechanisms are responsible for the "up-regulation" of the average fibronectin mRNA levels in a population of IPF alveolar macrophages. First, proportionally more IPF alveolar macrophages express the fibronectin gene than do normal alveolar macrophages. Second, for those IPF macrophages that do express the fibronectin gene, especially for large alveolar macrophages, the intensity of expression is greater than among normal alveolar macrophages.

The mechanisms by which fibronectin mRNA levels are controlled are just being elucidated. Portions of the controlling sequences of the fibronectin gene have been isolated[13–15] and there is evidence for complex levels of control of fibronectin mRNA levels.[15] In many instances, the amounts of fibronectin secreted appear to be controlled at the level of fibronectin mRNA levels.[15,26,32,36,45,46] Adding further to the complexity of the control of fibronectin gene expression, it is recognized that alternative splicing mechanisms can result in a family of mRNA transcripts from a single gene.[13,47–49] It is not known if alveolar macrophages use such alternative splicing, and whether such mechanisms are used differentially by macrophages in health and disease. If alternative splicing is used by macrophages, it could theoretically complicate the in-

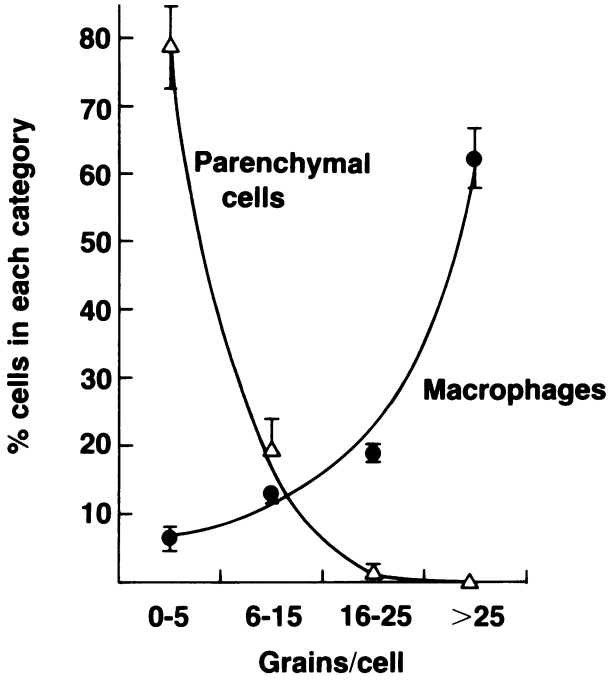

Figure 6—Assessment of the relative intensity of fibronectin gene expression in alveolar macrophages compared to cells comprising the alveolar walls. After hybridization with the ^{35}S-labeled fibronectin anti-sense probe, the numbers of silver grains/cell were determined for alveolar macrophages and parenchymal cells (epithelial cells, endothelial cells, mesenchymal cells grouped together). Hybridization with the sense fibronectin probe showed <5 grains/cell in all cells.

terpretation of these studies, because the probe used encompasses the region known to be used to alternative splicing in other cell types.[34]

The observations in the present study add another level of complexity to the concept of control of this gene by pointing out how cells of a similar lineage, present in the same anatomic compartment, can vary significantly in this expression of the same gene. In this regard, it may be that not only are there tissue-specific controls for the expression of different genes, but additional controls at the levels of cells of the same type and the same tissue.[50–53] Interestingly, in the context that albumin gene expression appears similar among hepatocytes in the intact rat liver,[33] such controls may be present only for some genes and only in some cell types.

Immunohistochemical and electron microscopy studies have demonstrated that fibronectin is distributed in alveolar capillary and epithelial basal lamina, including the basal lamina of interstitial smooth muscle cells, and is associated with interstitial connective tissue.[12,54,55] Furthermore, in fibrotic lung disease, the intensity of fibronectin deposition in the alveolar walls is increased[12] and higher concentrations of fibronectin are present in the fluid lining the alveolar epithelial surface.[7,56] Because a variety of cell types secrete fibronectin *in vitro,* it has been assumed that multiple cell types are responsible for such enhanced fibronectin deposition. It is not known, however, whether this fibronectin is plasma or cell derived, or both. The available supply of fresh lung tissue for the present study only included that from IPF and thus it is not known whether these results can be extended to normal lung, but the observations in the present study do demonstrate intense fibronectin gene expression in macrophages in comparison with that observed in all parenchymal cells. This suggests that alveolar macrophages play a major role in depositing fibronectin in the tissues of the lower respiratory tract, particularly in the enhanced deposition associated with fibrosis.

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