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Transforming growth factor β_1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis

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ABSTRACT **Idiopathic pulmonary fibrosis is an inexorably fatal disorder characterized by connective tissue deposition within the terminal air spaces resulting in loss of lung function and eventual respiratory failure. Previously, we demonstrated that foci of activated fibroblasts expressing high levels of fibronectin, procollagen, and smooth muscle actin and thus resembling those found in healing wounds are responsible for the connective tissue deposition and scarring in idiopathic pulmonary fibrosis. Using *in situ* hybridization and immunohistochemistry, we now demonstrate the presence of transforming growth factor β_1 (TGF-β_1), a potent profibrotic cytokine, in the foci containing these activated fibroblasts. These results suggest that matrix-associated TGF-β_1 may serve as a stimulus for the persistent expression of connective tissue genes. One potential source of the TGF-β_1 is the alveolar macrophage, and we demonstrate the expression of abundant TGF-β_1 mRNA in alveolar macrophages in lung tissue from patients with idiopathic pulmonary fibrosis.**

Idiopathic pulmonary fibrosis (IPF) is a fatal lung disorder afflicting as many as 10,000 individuals in the United States (1). This syndrome is characterized by foci containing activated fibroblasts that deposit connective tissue in the distal air spaces of the lung, progressively involving more and more of the lung parenchyma and culminating in widespread scarring and loss of lung function (2–4). The earliest lesion detected histopathologically is a macrophage-rich fibrinous exudate within an alveolus associated with ultrastructural evidence of epithelial injury. The epithelial ulceration results in the extension of organizing fibroblastic tissue into the alveolar space at the site of epithelial loss. With organization, collagen is deposited, eventually replacing functional gas-exchanging units with a dense connective tissue scar (2–4).

Little is known about the etiology or pathogenesis of this process in humans. Alveolar macrophages from patients with IPF express increased levels of growth-promoting molecules, including platelet-derived growth factor and fibronectin (for review, see refs. 5 and 6). These observations, coupled with the widespread fibroproliferative changes in the lung, support a role for the local expression of cytokines or growth factors in IPF. However, surprisingly little direct evidence for this hypothesis is available. In particular, the cause of the marked alterations in matrix gene expression remains unknown (5, 6). Because of the seemingly pivotal role of transforming growth factor β_1 (TGF-β_1) in wound healing, mediated in part by its strong influence on extracellular matrix gene expression (7, 8), we hypothesized (2) that matrix gene expression in IPF might be driven by the local accumulation of this potent cytokine at sites of lung injury. To test this hypothesis, we utilized the techniques of *in situ* hybridization and immunohistochemistry to detect TGF-β_1 mRNA, TGF-β_1 protein, and cognate mRNAs for procollagen type I and fibronectin in lung biopsies obtained from eight patients with IPF. Our results show marked expression of TGF-β_1 and fibronectin mRNAs by alveolar macrophages associated with sites of active fibrosis. Strikingly, there was intense staining for TGF-β_1 at sites where activated fibroblasts expressed collagen type I and fibronectin. These results suggest a pathogenic role for TGF-β_1 in human pulmonary fibrosis. TGF-β_1 synthesized by alveolar macrophages and associated with extracellular matrices may serve as a chronic stimulus driving matrix gene expression and fibrosis.

METHODS

***In Situ* Hybridization.** Generation of antisense RNA probes and the specific *in situ* hybridization techniques and conditions are described by Prosser *et al.* (9). In brief, plasmids were linearized overnight with the appropriate restriction endonucleases, and the DNA was purified with Geneclean (Bio 101, La Jolla, CA). Uridine 5′-[α-[^{35}S]thio]triphosphate (1000–1500 Ci/mmol; 1 Ci = 37 GBq; NEN, NEG-039H) was transcribed into RNA using the Promega Riboprobe system II (Promega) with T3 (Stratagene 600111) or SP6 (Promega) RNA polymerase promoters. The resultant probe was treated with RQ1 RNase-free DNase (Promega M6101) to digest the template and purified with phenol/chloroform, 1:1 (vol/vol), and chloroform extractions followed by two ethanol precipitations. All probes used had >50% incorporation of ^{35}S-labeled UTP into RNA, and the specific activities ranged from 100 Ci/μmol, for TGF-β_1, to 250 Ci/μmol, for procollagen type I. For *in situ* hybridization, 300,000 cpm were added per slide.

The probes used for *in situ* hybridization were prepared as follows. The plasmid pSP64 (Promega) containing clone λBC1 for TGF-β_1 (10) was linearized with *Kpn* I and antisense RNA was generated with SP6 RNA polymerase, yielding a 325-base-pair (bp) fragment containing nucleotides 950–1274. A partial cDNA for human fibronectin was subcloned from the plasmid pFH-6 (11) using *Hin*dIII and *Xba* I, yielding a 599-bp fragment that was cloned into the Bluescript KS expression vector (Stratagene). The resulting plasmid was linearized with *Hin*dIII and antisense RNA transcribed with T3 RNA polymerase. Hf677, a 1.5-kilobase cDNA including 800 bp of mature α-helical portion and 700 bp of carboxyl-terminal propeptide of the human procollagen α1(I) cDNA was subcloned into the *Eco*RI site of pSP65 (Promega) (12). This plasmid was linearized using *Ava* I, which cleaved an internal site of the insert resulting in a 700-bp antisense RNA from the carboxyl-terminal propeptide region.

Fixation, Sectioning, and Immunohistochemistry. The methods used for staining lung tissue have been described (2–4). Briefly, sections were deparaffinized, rehydrated, di-

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Abbreviations: IPF, idiopathic pulmonary fibrosis; TGF-β_1, transforming growth factor β_1.

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gested briefly with trypsin, and incubated with primary antibodies at working dilutions established in preliminary experiments. After rinsing, bound immunoglobulin was detected using the ABC technique (Vector Laboratories). The insoluble reaction product was intensified using nickel. Sections were counterstained with methyl green. Because a wider range of probes were utilized than reported herein, in some cases we could not present truly serial sections hybridized with probes to procollagen, fibronectin, and TGF-β_1. However, in all cases we studied the same fibroblastic foci and refer to the sections as "semi-serial."

Patient Selection. The patients were selected for study by an experienced lung pathologist (T.V.C.) because of their characteristic clinical courses and lung histopathology. One patient (number TV83-71) received high-dose corticosteroids for 1 week prior to the open-lung biopsy being obtained; no other patients were treated prior to biopsy. Lung samples from five of the eight patients studied contained active foci of organizing fibroblasts, whereas samples from the remaining three represented end-stage fibrosis. The findings in these five patients were similar, and images from one representative patient are shown below.

RESULTS

Localization of Type I Procollagen, Fibronectin, TGF-β_1 mRNA, and TGF-β_1 Protein in IPF Lung Tissue. Fig. 1 shows a series of paired images of semi-serial sections from a region of involved lung parenchyma removed from a patient with IPF and hybridized with ^{35}S-radiolabeled antisense RNA probes to procollagen type I (*A* and *B*), fibronectin (*C* and *D*), or TGF-β_1 (*E* and *F*) or stained with the CC synthetic peptide antibody (13), specific for the mature form of TGF-β_1 (*G* and *H*). Several characteristic buds of intraalveolar organizing fibroblastic tissue (two lesions are indicated by the asterisks) and macrophage-rich exudates (arrows) are present. *In situ* hybridization reveals that the fibroblasts within the organizing foci contain very high levels of mRNA encoding procollagen type I (*A* and *B*) and fibronectin (*C* and *D*). Fibronectin mRNA is expressed by alveolar macrophages and by other parenchymal cells in the alveolar walls, in keeping with its synthesis by fibroblasts, type II epithelial cells, and endothelial cells (14). TGF-β_1 mRNA is present in the macrophages adjacent to the fibrotic foci, but there is much less hybridization signal within the fibroblastic foci indicated by the asterisks (*E* and *F*). In contrast, immunohistochemical staining for TGF-β_1 reveals intense staining within and around the fibroblastic foci (*G*), whereas the adjacent macrophages are barely visible. The distinctive fibrillar-appearing staining pattern is thought to represent TGF-β_1 associated with extracellular matrix (13). Staining is virtually abolished by preincubation of the synthetic peptide antibody with its peptide antigen (*H*); the specificity of the CC antibody for TGF-β_1 has been established (13). There is striking codistribution of the TGF-β_1 staining (*G*) with procollagen type I (*A*) and fibronectin (*B*) expression by fibroblasts. The presence of extracellular TGF-β_1 in fibroblastic foci plus the abundant TGF-β_1 mRNA expression by alveolar macrophages suggests that TGF-β_1 made by macrophages is concentrated in the extracellular matrix where it drives matrix gene expression. Of course, there are other possible sources for TGF-β_1 in fibrotic lung as discussed below.

To date, examination of lung biopsies from these eight patients with IPF reveal similar findings. The macrophage-rich inflammatory exudates always express high levels of fibronectin and TGF-β_1 mRNA. This is compatible with an activated macrophage phenotype in the lower respiratory tract of patients with IPF (15–17). When organizing fibroblastic foci expressing high levels of procollagen and fibronectin mRNA and protein are present, they are always associated with abundant extracellular TGF-β_1 protein. TGF-β_1 augments the expression of both matrix proteins *in vitro* and stimulates formation of granulation tissue *in vivo* and in model systems (18–25). However, in areas of dense fibrosis as found in end-stage IPF, TGF-β_1 protein may be present without evidence of procollagen or fibronectin mRNA expression (data not shown). This may be related to removal of effete fibroblasts from the scarred tissue by apoptosis or counter-regulatory mechanisms that may inhibit the expression of matrix components in response to chronic TGF-β_1 stimulation (26).

To ensure that these results were not the result of methodological artifacts, we performed several control experiments. For each probe used, areas exhibiting positive hybridization with antisense probes were either treated with RNase or hybridized with sense probes. RNase treatment abolished specific hybridization with antisense probes, and the sense probes gave no cellular hybridization signal (unpublished data). The regional expression of procollagen and fibronectin mRNAs using *in situ* analysis and their immunohistochemical localization in serial sections (2–4) revealed excellent congruity between mRNA and protein expression, although the *in situ* analysis appeared to be slightly more sensitive for detecting collagen expression than immunohistochemistry (data not shown). As shown in Fig. 1, the hybridization signals display appropriate cell specificity; i.e., the procollagen type I complementary RNA probe hybridizes only with fibroblasts, not with alveolar macrophages. Finally, preincubation of the anti-TGF-β_1 antiserum with the peptide immunogen reduced staining of IPF lungs to that found with control serum.

Localization of Type I Procollagen, Fibronectin, TGF-β_1 mRNA, and TGF-β_1 Protein in Normal Lung Parenchyma. We also evaluated lung removed from patients with disorders that do not affect the lung parenchyma, including lung removed from a patient with a localized lung adenocarcinoma and from a patient with primary pulmonary hypertension. Utilizing the same methods, procollagen type I mRNA was not detectable in these lungs (Fig. 2 *A* and *B*). The mRNAs for TGF-β_1 and fibronectin were only weakly detectable in the alveolar macrophages present in the normal lung parenchyma (compare Fig. 1 *C–F* with Fig. 2 *C–F*). Similarly, immunohistochemical staining for TGF-β_1 in lung parenchyma removed from a patient with primary pulmonary hypertension was completely negative (Fig. 2 *G* and *H*).

TGF-β_1 Protein Is Codistributed with Foci of Extracellular Matrix Gene Expression. An even more compelling argument for a relationship between TGF-β_1 and matrix gene activation is provided by examination of minimally involved areas of lungs from patients with IPF. The fibrosis in IPF is patchy with uninvolved regions adjacent to active fibrotic lesions. Strikingly, the regions of uninvolved lung (based upon normal tissue architecture and low expression of procollagen and fibronectin) contained virtually undetectable TGF-β_1 protein (Fig. 3) or mRNA (data not shown). Thus, lungs from patients with disorders with localized carcinoma and idiopathic pulmonary hypertension with no parenchymal scarring did not contain detectable TGF-β_1 and there is an excellent correlation between TGF-β_1 staining and sites of matrix gene expression in lungs of patients with IPF.

DISCUSSION

Our observations reveal that TGF-β_1 protein is apparently associated with the extracellular matrix of the lung in sites of active fibroblastic proliferation and matrix expression, possibly by binding to extracellular proteoglycans or fibronectin (27–29). Interestingly, although TGF-β_1 is not considered a mitogen for lung fibroblast proliferation, it does promote growth of fibroblasts recovered from lungs of patients with scleroderma, an autoimmune disorder associated with alve-

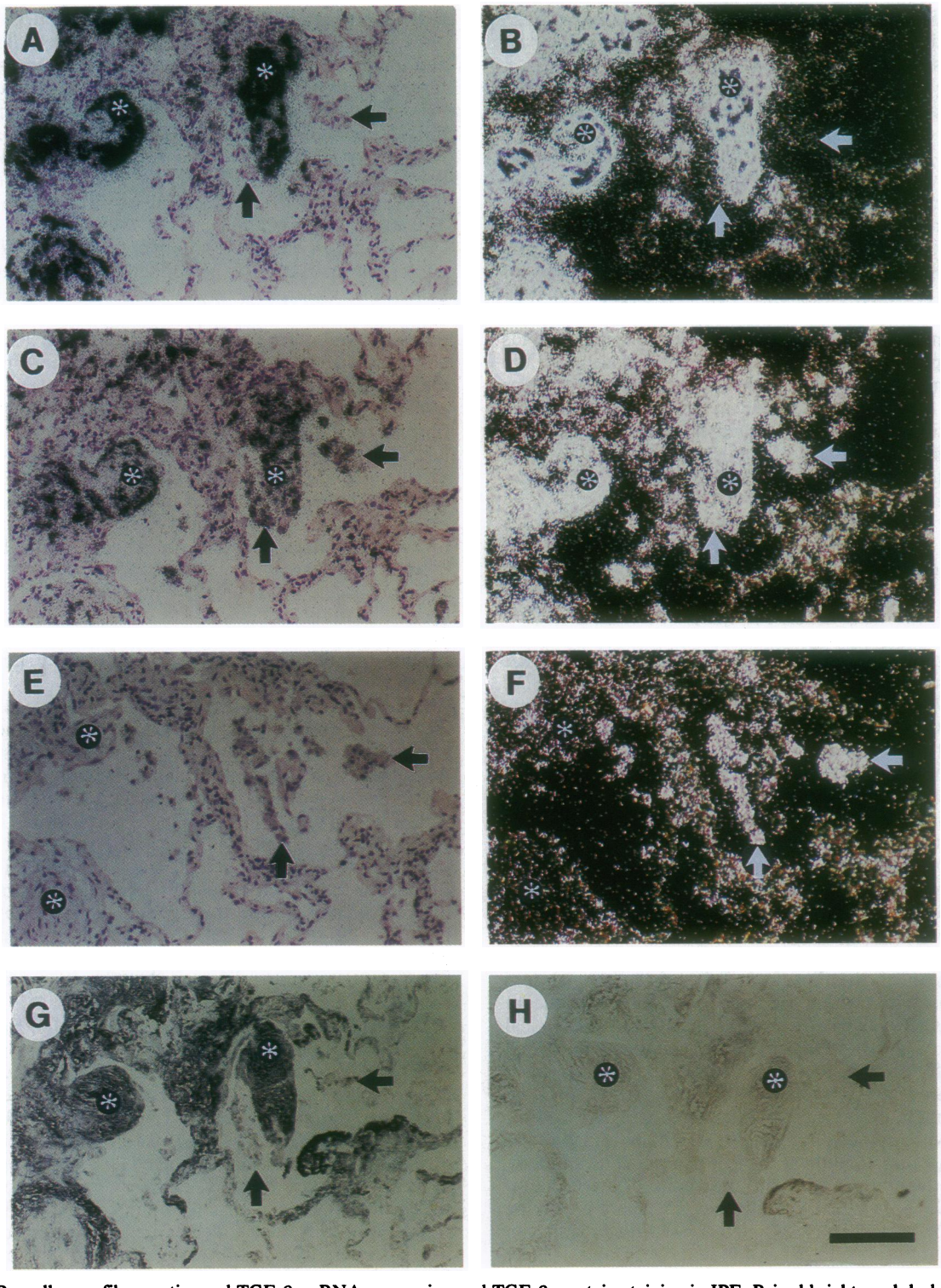

FIG. 1. Procollagen, fibronectin, and TGF-β_1 mRNA expression and TGF-β_1 protein staining in IPF. Paired bright- and dark-field images of semi-serial sections of the same region of lung are shown hybridized with complementary RNAs for procollagen type I (*A* and *B*), fibronectin (*C* and *D*), and TGF-β_1 (*E* and *F*). (*G* and *H*) Immunohistochemical stains for TGF-β_1 protein using the CC synthetic peptide antibody alone (*G*) or preincubated with the peptide antigen (*H*). Fibrogenic foci are indicated by the symbol *, and clusters of alveolar macrophages are indicated by arrows. Note the codistribution of procollagen type I and fibronectin mRNAs with extracellular TGF-β_1. (Bar = 100 μm.)

olar macrophage activation and lung fibrosis (30). In animal models of pulmonary fibrosis induced by bleomycin, a chemotherapeutic drug also associated with pulmonary fibrosis in humans, elevated levels of TGF-β_1 mRNA and protein, and focal matrix-associated staining for TGF-β_1 has been identified (31–34). However, the sites of collagen synthesis were neither identified nor correlated with TGF-β_1 localization in previous studies and, to our knowledge, no similar findings have been reported to date in humans. Therefore, our results strongly support some pathogenic role for TGF-β_1 in human pulmonary fibrosis.

The source(s) of the TGF-β_1 are not known. This study demonstrates that alveolar macrophages express abundant TGF-β_1 mRNA in IPF, consistent with their activation (35). However, fibrin is also present in fibroblastic foci in IPF (2) and in bleomycin-injured lungs (36), so we cannot exclude the formal possibility that platelets, a rich source of TGF-β_1 (37, 38), also contribute to its deposition in the lung at sites of

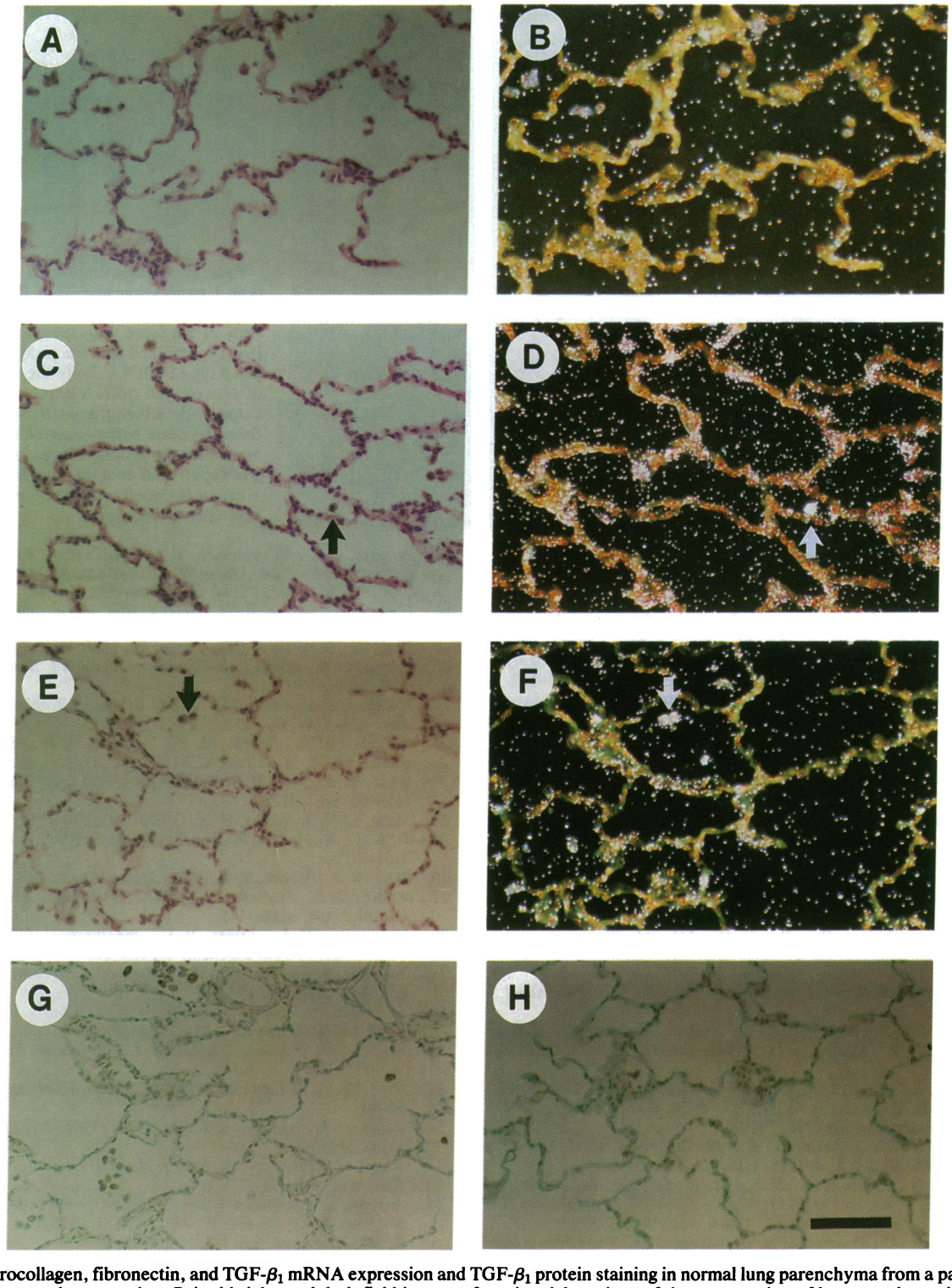

FIG. 2. Procollagen, fibronectin, and TGF-β_1 mRNA expression and TGF-β_1 protein staining in normal lung parenchyma from a patient with primary pulmonary hypertension. Paired bright- and dark-field images of semi-serial sections of the same region of lung are shown hybridized with antisense RNAs for procollagen type I (*A* and *B*), fibronectin (*C* and *D*), and TGF-β_1 (*E* and *F*). (*G* and *H*) Immunohistochemical stains for TGF-β_1 protein using the CC synthetic peptide antibody alone (*G*) or preincubated with peptide antigen (*H*). No cells hybridize with the procollagen type I probe, whereas cells within the alveolar walls and alveolar macrophages express fibronectin (*C* and *D*). There is some TGF-β_1 mRNA expression in alveolar macrophages as well (arrows). Note the complete absence of TGF-β_1 protein. (Bar = 100 μm.)

injury. Multiple other cell types also express TGF-β_1, including T lymphocytes, macrophages, monocytes, neutrophils, and fibroblasts, and the lower respiratory tract in healthy individuals contains high levels of latent TGF-β_1 (39). Our *in situ* hybridization results suggest that alveolar macrophages are a predominant cellular source in IPF. Regardless of its origin, our results strongly imply that TGF-β_1 associated with extracellular matrix in human lung alters the phenotype of resident fibroblasts by stimulating matrix gene expression. This information may eventually prove to be of therapeutic benefit, as antibodies inhibiting TGF-β_1 ameliorate the fibroproliferative response to injury in an animal model of glomerulonephritis (40, 41).

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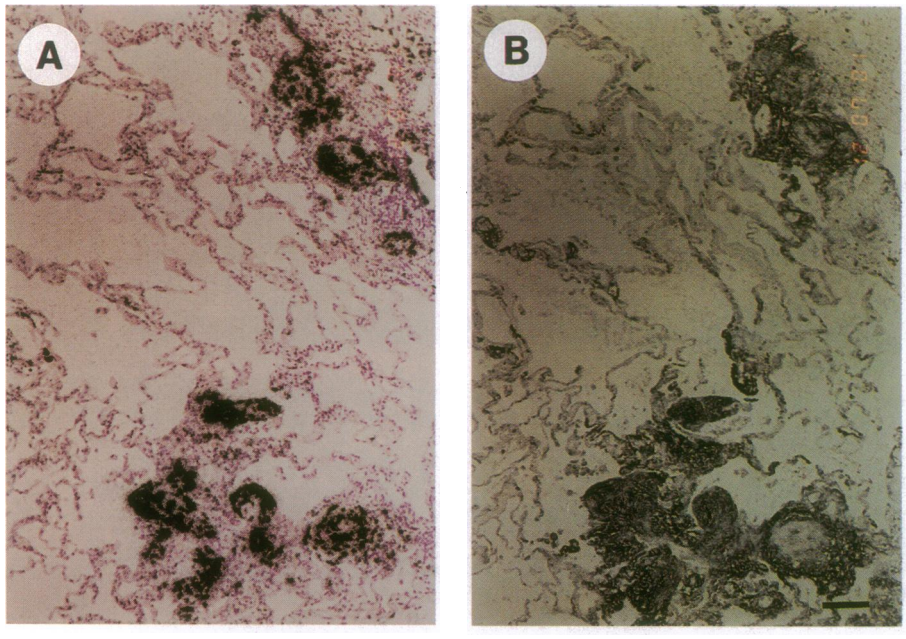

FIG. 3. Codistribution of procollagen type I expression and TGF-β_1 protein in IPF. (*A*) Bright-field low-magnification view of the same lung specimen shown in Fig. 1 hybridized with the probe for procollagen type I mRNA. Note the discrete foci of procollagen mRNA expression demarcated by the silver grains. (*B*) Serial section from the same biopsy stained for TGF-β_1. Note that the lesions expressing procollagen type I also contain abundant TGF-β_1, whereas the noninvolved regions of lung do not. (Bar = 100 μm.)

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