Transforming Growth Factor-β (TGF-β) in Silicosis

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Silicosis is characterized by fibrosing nodular lesions that may eventually develop into progressive massive fibrosis (PMF). Cytokines (interleukin-1β [IL-1β], tumor necrosis factor-α [TNF-α] and growth factors insulin-like growth factor-1 [IGF-1] platelet-derived growth factor [PDGF]) have been implicated in the formation of these lesions. TGF-β promotes extracellular matrix accumulation by upregulating collagen and fibronectin gene expression, and inhibits matrix degradation by decreasing secretion of proteases and increasing secretion of protease inhibitors. We hypothesized that TGF-β is associated with matrix deposition and fibrosis in silicosis. To test this hypothesis we studied early and late nodular lesions and PMF (11 cases and two controls) with immunohistochemistry, using rabbit polyclonal antibody to the purified whole molecule of TGF-β in Bouin's fixed lung tissue. This antibody is reactive with both intra- and extracellular forms of TGF-β. In the control lungs, small amounts of TGF-β were present in the bronchial epithelium, macrophages, bronchial and vascular smooth muscle, and bronchial glands. There was minimal to moderate staining in the early silicotic peribronchiolar lesions. In the nodular lesions of silicosis, central hyalinized areas contained the maximum staining for TGF-β. Fibroblasts in the periphery of the nodular lesions were also positive. In acute silicosis, there was marked staining of hyperplastic alveolar epithelium. Macrophages were markedly positive. In the PMF lesions, large areas of scar tissue contained TGF-β. These data suggest a major role for TGF-β in silicosis, particularly in the formation of silicotic nodules and the development of PMF. Jagirdar J, Begín R, Dufresne A, Goswami S, Lee TC, Rom WN. Transforming growth factor-β (TGF-β) in silicosis.

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Interstitial pulmonary fibrosis due to inorganic particulates, especially silica, asbestos, and coal, continues to be an important cause of interstitial lung disease (1, 2). Silicosis remains prevalent among underground miners, foundry workers, sandblasters, quarry workers, and other trades (3). The pathology of silicosis is characterized by hyalinized, fibrotic nodules; thickening of the alveolar interstitium; and accumulation of alveolar macrophages and chronic inflammatory cells. Large, conglomerate fibrotic lesions may occur in progressive massive fibrosis (PMF), and massive exposures may result in acute silicosis with alveolar epithelial Type II cell hyperplasia and large amounts of intraalveolar proteinaceous material (4). The pathogenesis of these lesions has been thought be be related to activated inflammatory cells, especially alveolar macrophages recovered by broncheoalveolar lavage (1, 5). These cells may release increased amounts of oxidants (superoxide anion and hydrogen peroxide) spontaneously *in vitro*, and various mesenchymal growth factors (insulin-like growth factor-1 [IGF-1], interleukin-1β [IL-1β], tumor necrosis factor-α [TNF-α]) and matrix glycoproteins such as fibronectin (1, 6, 7). In idiopathic pulmonary fibrosis (IPF), immunohistochemical studies have shown platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) to be localized to alveolar macrophages and alveolar epithelial Type II cells (8, 9). Broekelmann and colleagues localized TGF-β_1 by *in situ* hybridization to activated alveolar macrophages, and intraalveolar fibroblastic foci stained intensely for antibody to the mature form of TGF-β_1 (10). They also found codistribution of TGF-β_1 staining with procollagen Type I and fibronectin expression by fibroblasts, and suggested that macrophage secretion of TGF-β_1 into extracellular matrix could stimulate extracellular matrix gene expression by fibroblasts. In this regard, TGF-β inhibits the production of proteases, and therefore can stabilize the newly formed matrix proteins (11). Khalil and associates found anti-TGF-β staining in four IPF biopsies in bronchioles, alveolar epithelial cells, and honeycomb cysts (12).

In rat models of pulmonary fibrosis 7 d after bleomycin administration, TGF-β was localized by immunohistochemistry almost exclusively in alveolar macrophages (13). The distribution of matrix-associated TGF-β_1 was seen later in the course of injury and coincided with maximal collagen synthesis. Following a single intratracheal instillation of silica into rats, murine TGF-β peptide has been demonstrated in fibroblasts and macrophages located at the periphery of silicotic granulomas and in fibroblasts adjacent to hyperplastic Type II cells (14). In the present study

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TABLE 1

DEMOGRAPHIC DATA AND OCCUPATIONAL HISTORIES

Patient No.	Age (yr)	Smoking	Occupation	Duration of Exposure (yr)	Duration Since Onset of Exposure (yr)
1	55	45 pack-yr	Gold miner	29	32
2	63	Ex-smoker	Nonferrous foundry	36	39
3	73	24 pack-yr	Shops for asbestos mining and milling company (foundry worker)	44	52
4	44	50 pack-yr	Welder	30	N/A
5	76	Ex-smoker	Ferrous foundry	20	N/A
6	73	Ex-smoker	Shops for asbestos mining and milling company (foundry worker)	31	43
7	72	Ex-smoker	Shops for asbestos mining and milling company (laborer)	38	45
8	73	Ex-smoker	Ferrous foundry	39	55
9	44	50 pack-yr	Sandblaster	10	10
10	56	37 pack-yr	Underground miner and mill laborer	37	37
11	51	90 pack-yr	Carboselector in silicon carbide foundry	4	29

we addressed the potential role of TGF-β in human silicosis. In autopsy tissue from 11 individuals with all forms of silicosis, we evaluated TGF-β immunolocalization, using a pan-specific TGF-β antibody.

METHODS

Study Population

We evaluated autopsy lung tissue from 11 individuals exposed to crystalline silica during their working lifetime. Autopsies were performed as part of the Quebec Workers' Compensation Board inquiries. Table 1 describes demographic data and available occupational histories of the subjects. All study subjects were male, and all had a history of smoking (mean: 49 pack-yr). There were two underground miners in gold mines, five foundry workers, one silicon carbide foundry worker, one sandblaster, one welder, and one construction laborer. There were two control autopsies from individuals killed in traffic accidents (both were smokers having smoked 36 and 48 pack-yr, respectively).

Immunohistochemistry

Lung tissues were fixed in Bouin's fixative and embedded in paraffin. Sections 5 μm thick were cut on poly-L-lysine-coated slides. Adjacent sections were stained with hematoxylin and eosin (H & E) for histology, Masson's trichrome for collagen distribution, and avidin-biotin complex immunohistochemistry with anti-TGF-β antibody for localization and distribution of TGF-β. We used a pathology grading system suggested by Craighead and colleagues (4) for the American College of Pathologists.

The anti-TGF-β antibody used in this study is a panspecific TGF-β-neutralizing antibody obtained from R & D Systems, Minneapolis, MN. This polyclonal antibody was produced in rabbits immunized with a mixture of recombinant human TGF-β_1, porcine TGF-β_2, and recombinant amphibian TGF-β_5. This antibody had no cross-reactivity with other growth factors, interleukins, colony stimulating factors, or other cytokines. The antibody had previously been reported to stain myofibroblasts in the capsules surrounding gel-filled, smooth-surface silicone mammary implants (15). The specificity of the anti-TGF-β antibody has been verified by incubating 2.5 ml of the antibody (20 μg/ml) with recombinant human TGF-β_1 (100 μg) overnight at 4° C prior to staining (R & D Systems, Minneapolis, MN).

All sections were deparaffinized and treated with 0.3% hydrogen peroxide in methanol for 30 min. Prior treatment with hydrogen peroxide is required to inhibit the activity of endogenous peroxidase. Tissue sections were permeabilized with hyaluronidase and blocked with 1.5% goat serum. Tissue sections were then incubated overnight at 4° C in a 1:100 dilution of primary rabbit purified pan-TGF-β antibody. Slides were then washed extensively and incubated with biotinylated mouse anti-rabbit IgG at a 1:50 dilution for 30 min. Optimal reaction with 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and hydrogen peroxide was achieved. For control incubations, rabbit anti-TGF-β IgG was replaced by nonimmune goat serum. All microscopic analysis was done on a light photomicroscope (Olympus VaNox; New York Scientific, New York, NY). TGF-β staining intensity and extent were scored as mild (1+), moderate (2+), or intense (3+) as compared with a reference by one observer (J. J.) blinded as to the silica burden.

Mineralogic Analysis

The tissue samples were prepared and analyzed by transmission electron microscopy (TEM) and X-ray energy-dispersive spectroscopy (EDS). Lung samples were placed in xylene dewaxing cells for 7 d to remove paraffin from the tissues. The tissues were then dried, and from 1 to 5 mg was digested in sodium hypochlorite solution (fresh commercial bleach). The digestates were filtered through 25-mm-diameter, 0.45-μm pore-size membrane filters (mixed esters of cellulose; Millipore®, Bedford, MA) and subsequently ashed in a low-temperature asher. Ashes were suspended in distilled water, redeposited onto 0.2-μm pore-size polycarbonate Nuclepore® membranes from which grids were prepared using a replica technique, and then analyzed by TEM at a magnification of ×10,000). Particles having a diameter larger than 0.1 μm were counted, sized, and analyzed by EDS. For every 10 lung samples prepared, one blank sample was prepared as a control to detect contamination of sample during sample preparation.

RESULTS

The demographic and occupational histories of the 11 individuals exposed to silica are listed in Table 1. They had a mean age of 62 yr (range: 44 to 76 yr) and had a mean of 29 yr of exposure in dusty trades. They were a mean of 38 yr from the onset of exposure, and had thus been exposed to silica in the 1950s and 1960s, when dust exposure was considerably higher than at present.

There were seven individuals with simple silicosis, three with PMF, and one (a sandblaster) with acute or accelerated silicosis (Table 2). The individuals with simple silicosis included two with mild changes that were predominantly peribronchiolar fibrosis, one individual with three silicotic nodules per lower-power field, and four individuals with from 5 to > 8 silicotic nodules per low-power field. There were three individuals with large nodules > 2 cm in size consistent with complicated silicosis of PMF. Anthracotic pigment was prominent in peribronchial lesions and silicotic nodules, reflecting exposures in foundries and mills. Silica exposure was analyzed by determining the number of silicon-positive particles > 0.3 μm per milligram of dry lung (Table 2), using TEM with EDS analysis. All of the study subjects had a striking increase in silica particles, with 2 of 3 PMF patients having the highest number (4.723×10^6 and 9.204×10^6 particles, respectively). The lowest number of particles was found in the welder (Subject 4), but this individual had 5.151×10^6 metal-rich particles, and the two control subjects had fewer than 0.3×10^6 silica particles per milligram of dry lung.

Immunohistochemical staining for the presence and distribution of TGF-β in silicotic lungs demonstrated staining in macro-

TABLE 2

PATHOLOGY OF TGF-β IN HUMAN SILICOSIS

Patient No.	Number of Nodules	Grade of Fibrosis	No. of Particles of Silicon > 0.3 μm per mg Dry Lung × 10^6	TGF-β Localization and Staining Intensity				
				Peribronchiolar Fibrosis	Silicotic Nodule	Honeycombing*	Macrophages	Epithelium†
1	Peribronchiolar fibrosis	2	2.43	Mild				
2	N/A	Progressive massive fibrosis	4.723	Moderate	Moderate			
3	8	3	0.965		Moderate			Intense
4	> 7	3	0.212 *5.151 metal-rich particles		Moderate	Moderate	Intense	Intense
5	3	2	3.184	Mild	Moderate		Intense	
6	> 10	3	0.37	Mild	Moderate			
7	N/A	Progressive massive fibrosis	1.814	Moderate	Intense	Intense		
8	> 8 (Necrosis)	Progressive massive fibrosis	9.204	Moderate	Intense	Intense	Intense	Intense
9	Peribronchiolar fibrosis	2	1.131	Moderate		Mild		Intense
10	5	2	1.931	Moderate	Moderate			
11	Peribronchiolar fibrosis	1	0.761	Mild	Moderate			

* Staining in areas of honeycombing included metaplastic epithelium, macrophages, smooth muscle cells, and foci of fibrosis.
† Staining in normal bronchial cells and Type 2 pneumocytes.
Blank spaces refer to absence of that particular pathology on the tissue section.

phages, silicotic nodules, peribronchiolar fibrosis, honeycombing, and bronchial epithelium (Table 2). Minimal lesions were characterized by peribronchiolar thickening with collections of anthracosilicotic pigment. TGF-β staining was noted in the fibrosed area as well as the bronchial epithelium. Alveolar macrophages from silicotic patients were intensely stained with pan-TGF-β (Figure 1). A classic silicotic nodule (Case 10) with a whorled, onion-skin appearance is shown in Figure 2a, and stained markedly for mature collagen with Masson's trichrome (Figure 2b). TGF-β stained extracellularly in the central hyalinizing area, with minimal staining of the peripheral, anthracotic, pigmented area (Figure 2c). Simple silicosis with peribronchiolar fibrosis had mild to moderate TGF-β immunostaining, whereas the silicotic nodules were more heavily stained, followed by PMF lesions, in which the matrix was most intensely stained (Table 2). Patients 7 and 8 had PMF with intense staining of fibrotic areas in the silicotic nodules and PMF lesions. Patient 2 had PMF by chest radiography, but only silicotic nodules were available for evaluation, and these stained moderately. Areas of honeycombing showed intense staining of metaplastic epithelium. The sandblaster (Patient 9) had markedly thickened alveolar walls, with airspaces filled with a loosely granular proteinaceous material (Figure 3a). Staining for TGF-β revealed intense staining of hyperplastic alveolar epithelium (Figure 3b). The staining was intracellular, and most cells shared the cuboidal features of alveolar epithelial Type II cells. There was only minimal staining of thickened interstitium. Patients 3, 4, 8, and 9 had intense staining of alveolar epithelium, and all except Patient 3 had honeycombing. A PMF lesion stained intensely for TGF-β (Figure 4). Extracellular staining was observed in hyalinized collagenous areas; interstitial macrophages stained intensely; and fibroblasts located in the periphery of the PMF lesions showed intense intracellular staining for TGF-β.

TGF-β staining of the two normal controls was minimal in both cases; minimal staining could be detected in bronchial epithelium, in a few scattered macrophages, bronchial and vascular smooth muscle cells, and bronchial glands (Figure 5). The control nonimmune antibody sections had no staining.

DISCUSSION

We used immunohistochemistry to localize a pan-TGF-β antibody in human silicosis to sites of peribronchiolar fibrosis, hyalinized central areas of silicotic nodules, extracellularly in PMF lesions, intracellularly in fibroblasts at the periphery of PMF lesions, to normal and metaplastic epithelium, and within alveolar macrophages. There was a gradation in extent and intensity of staining from peribronchiolar lesions to nodules of simple silicosis to the PMF lesion, which had the most widespread and intense staining. Similarly, there was a gradation in pathology and number of respirable silica particles from simple silicosis to PMF. The patient with acute silicosis had extensive staining of hyperplastic alveolar epithelial Type II cells that had replaced Type I alveolar epithelial lining cells. In silicosis, particle deposition occurs in respiratory bronchioles, leading to peribronchiolar

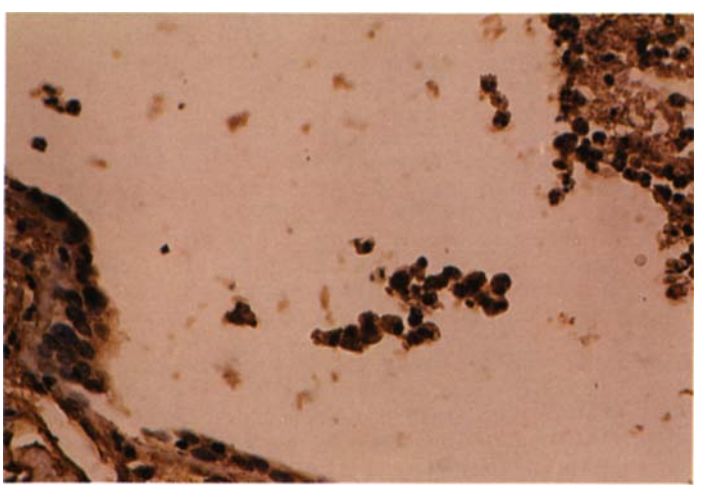

Figure 1. Alveolar macrophages stained for pan-TGF-β in simple silicosis. Note intense brown staining of cytoplasm. (×400).

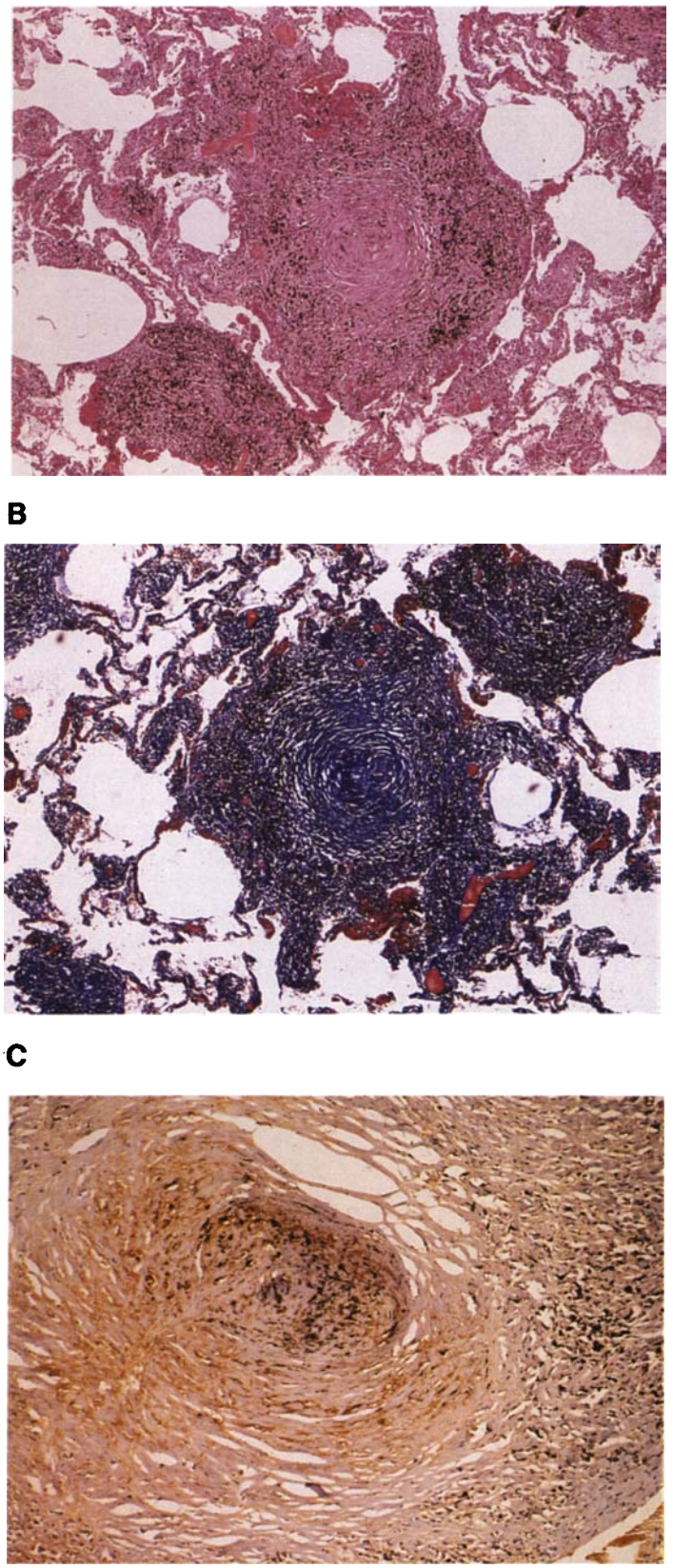

Figure 2. Silicotic nodule. (*A*) H & E-stained section of an anthracosilicotic nodule. (×200). (*B*) Same area as in (*A*), stained with Masson's trichrome for mature collagen. (×200). (*C*) Adjacent area as in (*A*), stained for TGF-β. Note predominant extracellular staining of central hyalinized area and minimal staining of peripheral pigmented area. (×400).

fibrosis followed by alveolar wall thickening and silicotic nodule formation. TGF-β localization in alveolar epithelial cells may play a paracrine role in attracting fibroblasts and serving as a mitogen for immature fibroblasts (10). TGF-β then serves to expand the interstitium by attracting both mesenchymal and inflammatory cells and enhancing the accumulation of extracellular matrix (16, 17). TGF-β upregulates genes for collagen and fibronectin that contribute to matrix production in the center of a collection of inflammatory cells that develops concentrically into the nodular lesion (18–21). Our immunohistochemical staining results suggest that continued TGF-β release, in concert with other cytokines, may lead to the extraordinary large lesions of centimeters in size, that are typical of PMF.

A rat model of intratracheal instillation of silica demonstrated TGF-β in fibroblasts near hyperplastic epithelial cells and near the margins of granulomatous lesions, resembling our findings and consistent with a role for TGF-β and mesenchymal cells in enhancing extracellular matrix accumulation (14). In the rat silicosis model, TGF-β precursor was also localized in proliferating alveolar epithelial Type II cells, in accord with the production of TGF-β by these cells (14). However, silicotic nodules, the hallmark of human silicosis, were not observed in this animal model. In the bleomycin rat model, alveolar macrophages produced maximal amounts of TGF-β on Day 7 (13). In this rat model, the TGF-β_1 isoform was increased by 5- to 6-fold, constituting from 50% to 77% of total TGF-β, whereas isoforms TGF-β_2 and -β_3 remained unchanged (22). The pan-TGF-β antibody that we used should detect both TGF-β_1 and -β_2, as shown in our figures. Santana and colleagues found that all three mammalian TGF-β isoforms were increased in acutely injured areas in the rat bleomycin pulmonary fibrosis model, with prominent staining of macrophages and parenchymal cells (23). In the reparative stage there was continued TGF-β staining in the parenchyma, but the bronchial epithelium, previously not expressing TGF-β messenger ribonucleic acid (mRNA), showed strong expression of mRNA for the three isoforms concomitantly with increased immunoreactivity. In a more definitive study of the role of TGF-β in the bleomycin murine model, Giri and associates infused anti-TGF-β antibody into the tail vein at the same time as bleomycin was administered (24). Lung collagen was significantly reduced at 14 d, and results were similar with anti-TGF-β_1 or combined anti-TGF-β_1 and -β_2 antibodies.

Khalil and colleagues described the time course of alveolar epithelial Type II cell proliferation in the bleomycin injury model (25). The peak level of TGF-β release by alveolar epithelial cell explants occurred at 28 d, whereas peak proliferation was early when TGF-β levels were low, suggesting that TGF-β is more important in the reparative phase of the alveolar epithelium. Low doses of silica (2 to 20 μg/ml) stimulated enhanced [^{3}H]thymidine incorporation and replication of fetal Type II pneumocytes cultured *in vivo*, suggesting a direct inorganic dust effect on these cells (26). In our patient with acute silicosis, Type II alveolar epithelial cells had extensively replaced Type I cells, and stained intensely with TGF-β. In the human, TGF-β probably plays a role in the reparative process of injured alveolar epithelium, but it is unclear whether the cells are proliferating, differentiating into Type I cells, or both.

TGF-β stimulates fibroblasts to synthesize collagen and fibronectin, and smooth muscle cells to synthesize elastin (18–21, 27–30). In addition, it promotes matrix accumulation by decreasing collagenase synthesis, by repressing the stimulatory effects of growth factors on collagenase gene expression and increasing the production of collagenase inhibitors such as tissue inhibitor of metalloproteinase (TIMP) and alpha-2 macroglobulin (11). TGF-β also increases the transcription, translation, and processing of cellular receptors for matrix proteins (31). TGF-β has been

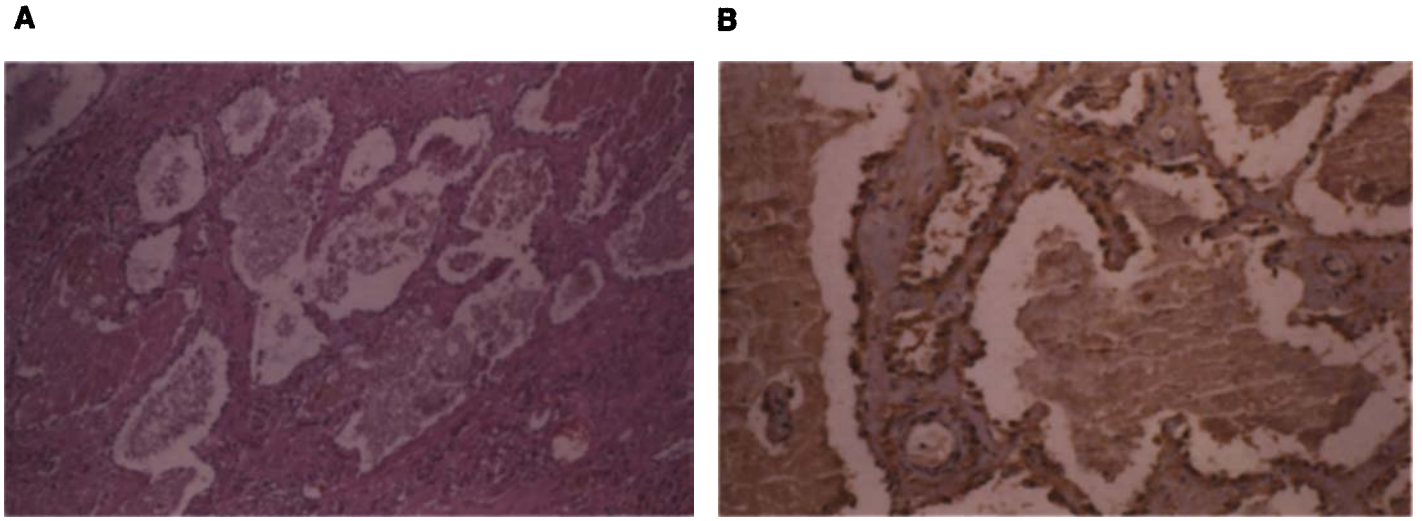

Figure 3. Sandblaster with acute silicosis. (*A*) H & E-stained section demonstrating thickening of alveolar walls and hyperplastic alveolar epithelium. (×200). (*B*) Same area as in (*A*), stained for TGF-β. Note strong TGF-β staining of epithelium lining cystic spaces. (×400).

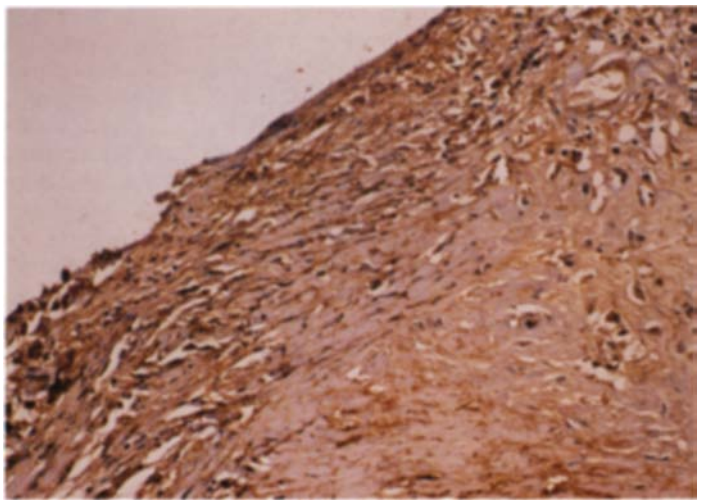

Figure 4. PMF lesion with anti-TGF-β. Note intracellular staining of fibroblasts and extracellular staining in foci of fibrosis. (×400).

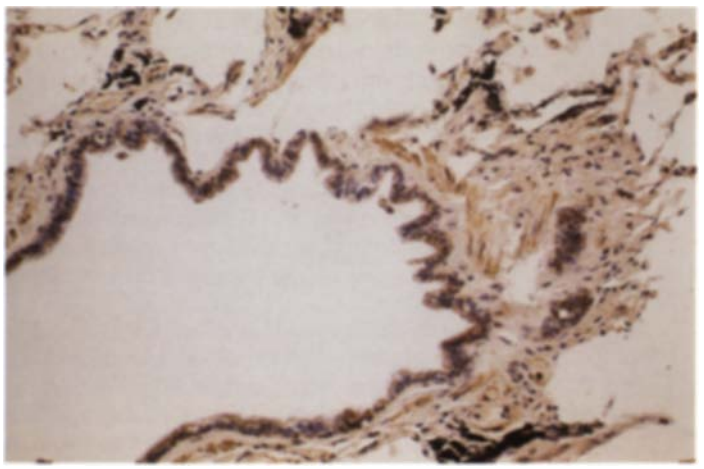

Figure 5. Control lung tissue. There is TGF-β staining of peribronchial smooth muscle, bronchial epithelium, and a few alveolar macrophages.

shown to rapidly accumulate in epithelial and mesenchymal cells following injury, in the process of normal wound healing (32). We could explain the cellular and matrix accumulation of TGF-β in our study through a chemotactic effect on fibroblasts, monocytes, and epithelial cells; and since TGF-β has an inhibitory effect on the proliferation of several cell types *in vitro*, increased numbers of cells observed *in vivo* may be due to chemotaxis (33). TGF-β also stimulates c-*sis* or PDGF-B-chain gene expression, and addition of PDGF-AA antisera can neutralize TGF-β-induced deoxyribonucleic acid (DNA) synthesis in aortic smooth muscle cells (34). Thus, TGF-β contributes indirectly to human silicosis by stimulating profibrotic growth factors, cytokines, and their receptors, and stimulates collagen and fibronectin gene expression and inhibits their breakdown. TGF-β contributes to the development of silicosis by macrophage activation, and as our results demonstrate, by release of TGF-β in silicotic nodules, PMF matrix, and hyperplastic and metaplastic alveolar epithelium.

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