# Immunohistochemical Localization of Transforming Growth Factor Beta Isoforms in Asbestos-Related Diseases

Jaishree Jagirdar,[1] Theodore C. Lee,[2] Joan Reibman,[2] Leslie I. Gold,[1] Christopher Aston,[2] Raymond Bégin,[3] and William N. Rom[2]

[1]Department of Pathology; [2]Department of Medicine, New York University Medical Center, New York, New York; [3]Unité de Recherche Pulmonaire, Université de Sherbrooke, Québec, Canada

Transforming growth factor beta (TGF-β), a multifunctional cytokine and growth factor, plays a key role in scarring and fibrotic processes because of its ability to induce extracellular matrix proteins and modulate the growth and immune function of many cell types. These effects are important in inflammatory disorders with fibrosis and cancer. The asbestos-related diseases are characterized by fibrosis in the lower respiratory tract and pleura and increased occurrence of lung cancer and mesothelioma. We performed immunohistochemistry with isoform-specific antibodies to the three TGF-β isoforms on 16 autopsy lungs from Quebec, Canada, asbestos miners and millers. There was increased immunolocalization of all three TGF-β isoforms in the fibrotic lesions of asbestosis and pleural fibrosis. The hyperplastic type II pneumocytes contained all three isoforms. By contrast, there was differential spatial immunostaining for the TGF-β isoforms in malignant mesothelioma, with TGF-β1 in the stroma but TGF-β2 in the tumor cells. These data are consistent with an important role for TGF-β in accumulation of extracellular matrix and cell proliferation in asbestos-related diseases. — *Environ Health Perspect* 105(Suppl 5):1197–1203 (1997)

Key words: transforming growth factor-β, asbestosis, pleural fibrosis, mesothelioma

## Introduction

Transforming growth factor beta (TGF-β) is a multifunctional cytokine that enhances extracellular matrix formation, inhibits cellular proliferation, and suppresses immune processes (*1,2*). The three TGF-β mammalian isoforms (TGF-β1, TGF-β2, and TGF-β3) have a different distribution and function *in vivo* (*3,4*). A variety of oncogenes including *jun*, *fos*, and *ras* upregulate TGF-β1 through activator protein-1 binding sites on the promoter (*5*). The retinoblastoma gene product activates TGF-β2 gene expression by stimulating nuclear proteins that bind to the promoter (*6*). TGF-β1 enhances expression of extracellular matrix components including fibronectin, αvβ1 fibronectin receptor, collagen, and decorin, a TGF-β1 binding proteoglycan (*1,7,8*). Furthermore, TGF-β inhibits metalloproteinases and stimulates protease inhibitors, which contributes to matrix accumulation (*9*).

Extracellular matrix accumulation is the hallmark of asbestos-related pulmonary and pleural fibrosis and some types of malignant mesothelioma (*10*). Increased amounts of collagen deposit in alveolar walls and terminal respiratory bronchioles, near the pleural lymphatics (plaque formation), and in the stroma of mesothelioma, where thick collagen fibers are intermixed with tumor cells. Therefore, we propose that TGF-β may be present to stimulate collagen production. We previously showed that TGF-β1, -β2, and -β3 are intensely localized to extracellular matrix zones of early and developing fibrotic lesions in the lungs of sheep treated intratracheally with chrysotile asbestos (*11*). In the nodular lesions of silicosis, we reported that central hyalinized areas showed the maximum immunostaining for TGF-β (*12*). In acute silicosis, we observed marked staining of hyperplastic alveolar epithelium, as well as intense staining of alveolar macrophage(s) (AM). In this study we hypothesized that TGF-β would promote matrix accumulation in asbestosis, pleural plaques, and mesothelioma, and that the TGF-β isoforms may have differential roles in this process.

## Methods

### Study Population

Autopsy lung tissue from 16 individuals exposed to chrysotile asbestos in the mines and mills in Thetford, Quebec, Canada, were evaluated. Autopsies were performed as part of the Quebec Workers' Compensation Board inquiries. Subjects were 71 ± 8 years old (mean ± SD) at the time of death and had worked for 38 ± 5 years in the asbestos industry. A review of their work histories revealed that all had significant chrysotile exposure, and that none had worked in areas where there may have been crocidolite exposures. All had histologic evidence of pulmonary fibrosis. Five individuals also had lung carcinoma and four had malignant mesothelioma. All study subjects were male and all had a history of cigarette smoking. There were two control autopsies from individuals killed in traffic accidents. (These were both smokers who smoked 36 and 48 pack-years, respectively). The control lungs had no histologic evidence of pulmonary fibrosis.

### Antibodies

The isoform-specific antibodies to TGF-β1, -β2, and -β3 were previously described and characterized (*13*). Briefly, peptides of each TGF-β isoform were synthesized using a 430A peptide synthesizer (Applied Biosystems, Foster City, CA). The following amino acid residues were

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Address correspondence to Dr. W.N. Rom, Division of Pulmonary and Critical Care Medicine, New York University Medical Center, 550 First Avenue, New York, NY 10016. Telephone: (212) 263-6479. Fax: (212) 263-8442. E-mail: romw01@mcgc.16.med.nyu.edu

Abrreviations used: AM, alveolar macrophage(s); BSA, bovine serum albumin; CDK, cyclin-dependent kinase; IGF, insulinlike growth factor; IPF, idiopathic pulmonary fibrosis; Rb, retinoblastoma; TBS, tris-buffered saline; TGF-β, transforming growth factor beta.

used: TGF-β1 and -β2, residues 4 to 19; TGF-β3, residues 9 to 20. The peptides were coupled to keyhole limpet hemocyanin prior to injection. Each rabbit polyclonal antiserum was purified by peptide affinity chromatography and was shown by Western blot analysis to be specific for the corresponding mature TGF-β isoform and noncross-reactive with the other TGF-β isoforms (*13*). The antibodies recognize both the latent and active forms of TGF-β isoforms; because TGF-β exists as a latent molecule, the epitopes on the mature molecule are available.

### Immunohistochemistry

Paraffin sections (5 μm) were incubated in tris-buffered saline ([TBS] 0.01 M Tris, 0.15 M NaCl, pH 7.4)/0.3% Triton X-100 for 15 min; TBS for 5 min; absolute methanol for 2 min; and methanol/0.6% hydrogen peroxide (v/v) for 30 min to quench endogenous peroxidase. The slides were then rinsed briefly in methanol, followed by three washes in TBS/0.1% (wt/v) bovine serum albumin (BSA) for 5 min each. The sections were treated with hyaluronidase (1 mg/ml in 100 mM sodium acetate buffer, pH 5.5, with 0.85% [wt/v] NaCl) for 30 min at 37°C, then rinsed three times in TBS/0.1% BSA. Nonspecific protein staining was blocked by 1.5% goat serum for 20 min. The sections were incubated overnight at 4°C with 100 to 200 ml of primary antibody to final concentration of 2.5 mg/ml in blocking solution. The sections were rinsed at room temperature with TBS and incubated with biotinylated goat antirabbit immunoglobulin for 1 hr in a humid staining box at room temperature (Elite Vectastain Kit, Vector Laboratories, Burlingame, CA). After extensive washes, the sections were incubated with avidin–biotin–peroxidase complex for 1 hr at room temperature and developed according to the manufacturer's recommendations. The slides were counterstained with Gill's hematoxylin #2 and viewed under a light microscope.

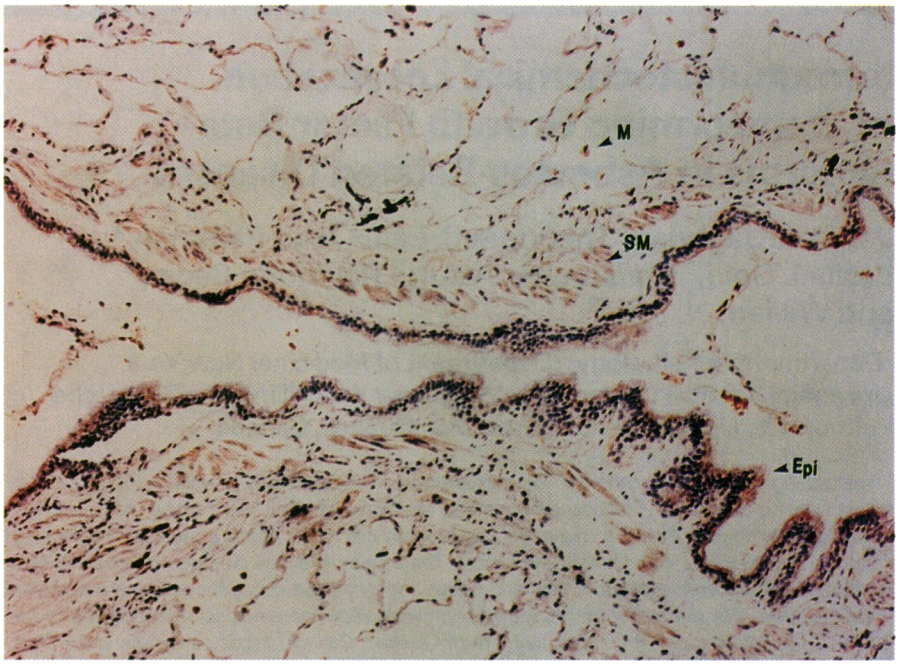


**Figure 1.** Control lung (TGF-β1). TGF-β1 immunostaining is detected in macrophages (M), smooth muscle cells (SM), and bronchial epithelial cells (Epi). Magnification ×100.

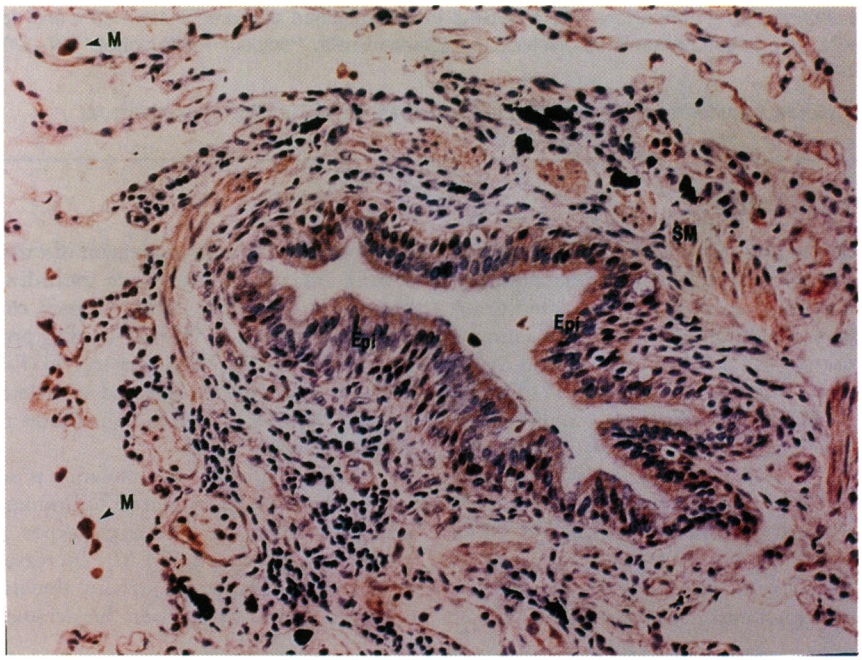


**Figure 2.** Control lung (TGF-β2). TGF-β2 immunostaining with M, SM, and Epi identified. Magnification ×400.

## Results

### Immunohistochemical Localization of TGF-β Isoforms in Asbestosis

TGF-β isoform-specific antibodies applied to sections of lung from two unexposed controls demonstrated the minimal presence of TGF-β1, -β2, and -β3 in bronchi, bronchiolar epithelium, AM, bronchial and vascular smooth muscle, but not in cartilage or immune cells. There were no consistent differences in the staining pattern of each isoform (Figures 1 to 3, TGF-β1, -β2, and -β3, respectively). The presence of all isoforms in control lungs suggests a physiologic role for TGF-β in normal tissue homeostasis. No immunostaining was detected in fibrotic lung sections using nonimmune sera or saline followed by secondary antibody.

In contrast, fibrotic lungs demonstrated intense staining for all three TGF-β isoforms in the extracellular matrix or early peribronchial fibrosis and in metaplastic proliferating epithelium of far advanced

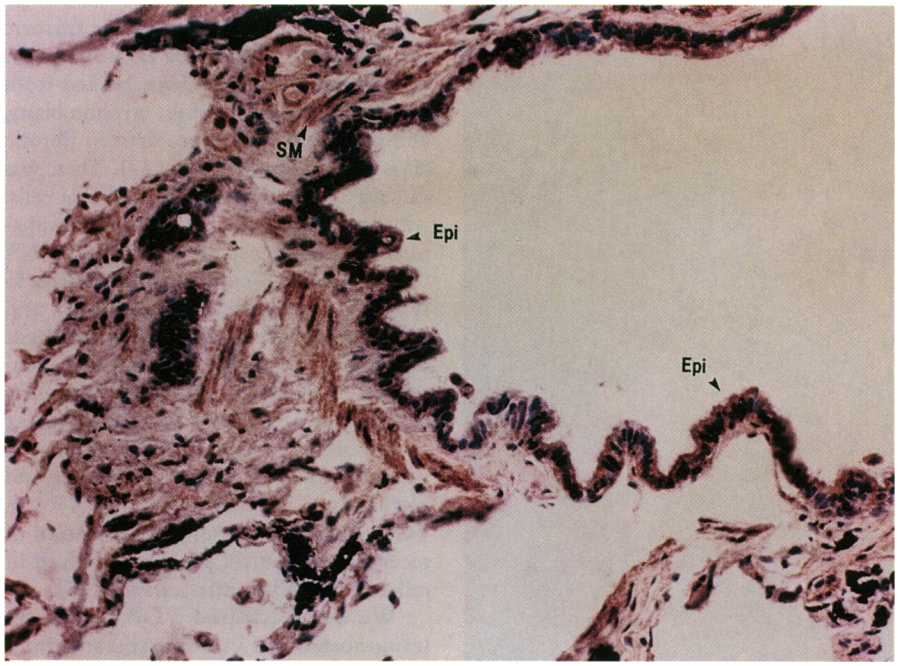


**Figure 3.** Control lung (TGF-β3). TGF-β3 immunostaining is noted in Epi and SM. Anthracotic pigment is also noted. Magnification ×400.

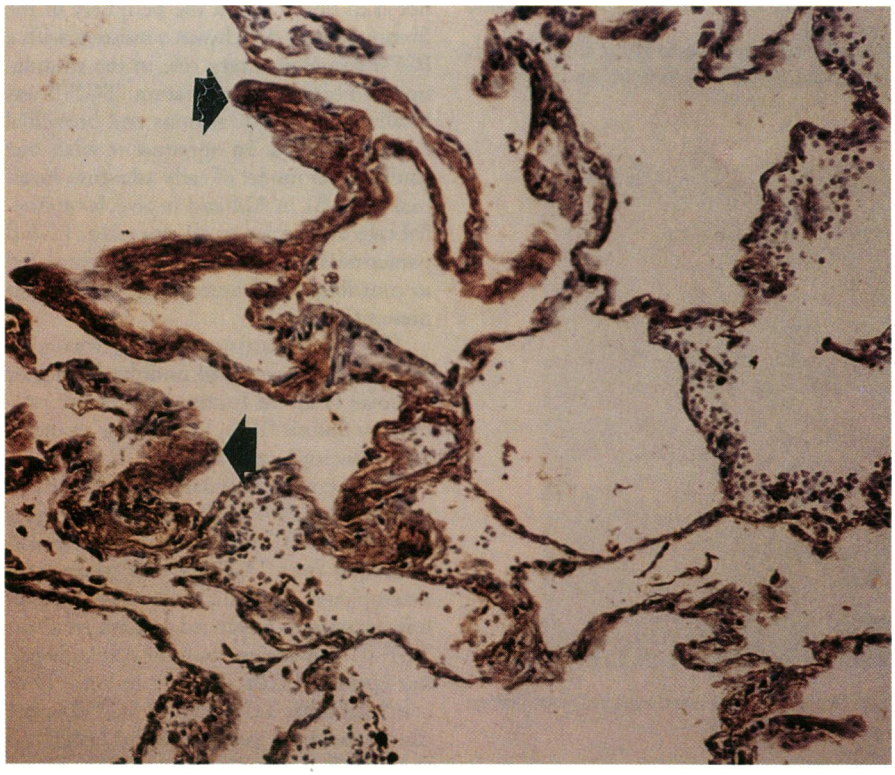

**Figure 4.** Asbestosis. TGF-β1 immunostaining of hyperplastic type II pneumocytes (arrows) and extracellular matrix in thickened alveolar walls. Magnification ×10.

lesions of honeycomb lung. In contrast to normal lung, fibrotic peribronchiolar lesions had intense immunostaining for all three isoforms in type II pneumocytes, smooth muscle cells, and macrophages (Figure 4). The TGF-β1 immunostaining increased in these cells with an increase in the number of these cells per unit area. In the advanced lesions characterized by honeycombing and marked architectural derangement, all three TGF-β isoforms were detected in hyperplastic type II pneumocytes and epithelial cells that lined cystic spaces (Figure 5). The extracellular matrix within fibrotic areas demonstrated intense immunostaining for all three TGF-β isoforms (Figure 5). Pleural plaque is characteristically a basket-weave pattern of collagen fibrils. All three TGF-β isoforms stained the matrix in the pleural fibrosis intensely (Figure 6).

### Immunohistochemical Localization of TGF-β Isoforms in Malignant Mesothelioma

Three distinct spatial distributions for the TGF-β isoforms were observed. TGF-β1 immunostaining was detected in the desmoplastic extracellular matrix between mesothelial cells of mesothelioma (Figure 7). Stromal cells stained more intensely for TGF-β2 than TGF-β1, and staining of the tumor cells was markedly less (Figure 8). TGF-β3 was detected in the stroma, and many of the tumor cells stained positively (Figure 9).

## Discussion

We used immunohistochemistry to demonstrate TGF-β isoform staining in fibrotic lesions of asbestosis and pleural plaques in autopsy samples of Quebec chrysotile miners and millers. All three antibodies to TGF-β isoforms stained early peribronchiolar fibrosis as well as bronchial epithelial cells, AM, and smooth muscle cells. As the fibrosis progressed, TGF-β isoform immunoreactivity markedly increased parallel to the increase of extracellular matrix, reactive hyperplastic type II pneumocytes, and macrophages. Normal lungs had minimal TGF-β immunostaining but immunostaining was present in the same lung structures (e.g., macrophages and bronchial epithelial cells), and we previously demonstrated no staining with nonimmune sera (*11*). In malignant mesothelioma, TGF-β1 localized more to the stroma, whereas TGF-β2 localized more to the mesothelioma cells than to the stroma. TGF-β3 had minimal immunostaining of both stroma and mesothelioma cells. These results suggest that TGF-β isoforms are important for the accumulation of extracellular matrix in asbestosis, pleural fibrosis, and malignant mesothelioma.

In the bleomycin rat model, AM produced TGF-β1 after 7 days. This TGF-β1 subsequently increased 5 to 6-fold,

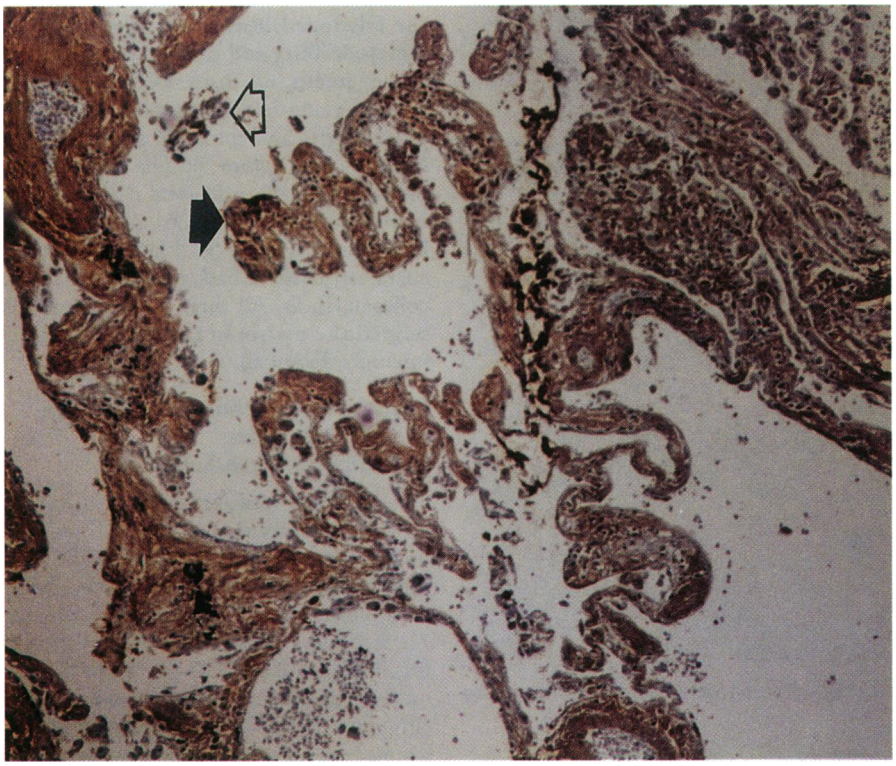

**Figure 5.** Advanced lesion of honeycomb lung. TGF-β3 was intense in the extracellular matrix of fibrotic areas. Immunostaining was prominent in alveolar macrophages (open arrow). Significant anthracotic pigment was also present. Closed arrow, type II pneumocytes. Magnification ×6.5.

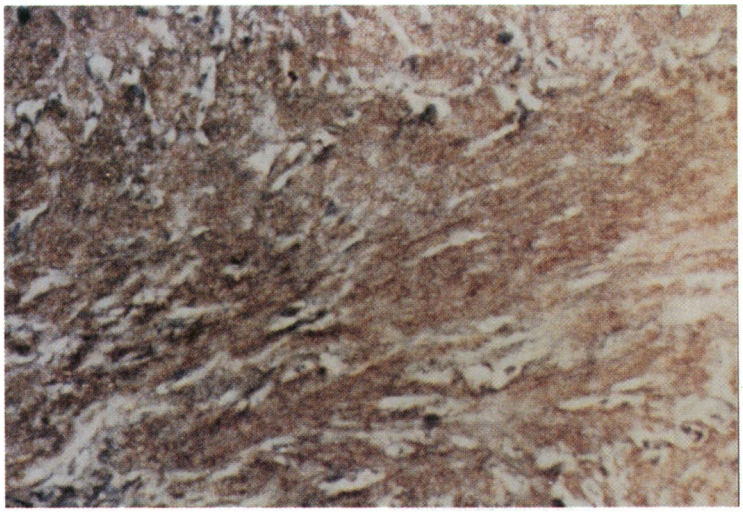

**Figure 6.** Pleural plaque. TGF-β1 immunostaining of collagen fibrils in parietal pleural plaque. Magnification ×400.

constituting 50 to 77% of total TGF-β. Isoforms TGF-β2 and -β3 remained unchanged (*14,15*). Santana and colleagues found that all three mammalian TGF-β isoforms increased in the acutely injured areas in the same rat pulmonary fibrosis model, with prominent staining of macrophages, parenchymal cells, bronchial epithelium, and type II pneumocytes (*3*). Infusion of anti-TGF-β1 antibody in the tail vein in the murine bleomycin model significantly reduced lung collagen at 14 days, which provided direct evidence for the presence of TGF-β as a cause of collagen overproduction in the fibrotic process (*16*). Zhang et al. noted that TGF-β mRNA and protein peaked from days 3 to 14 in eosinophils, myofibroblasts, and fibroblasts, with fibroblasts in fibrotic areas prominent thereafter (*17*). There was discordance in bronchiolar epithelial cells, with strong protein expression unaccompanied by a commensurate increase in mRNA (*16,17*). TGF-β stimulates fibroblasts to synthesize collagen and fibronectin, and stimulates smooth muscle cells to synthesize elastin (*18*). In addition, it promotes extracellular matrix accumulation by decreasing collagenase synthesis and increasing the production of protease inhibitors such as tissue inhibitor of metalloproteinase and plasminogen activator inhibitor-1 (*9,19*). TGF-β also modulates the expression of receptors for matrix proteins involved in cell–cell and cell–matrix adhesion (*20*).

We demonstrated TGF-β isoform immunostaining in the peribronchiolar fibrotic lesions in a sheep model of asbestosis (*11*). Interestingly, insulinlike growth factor-I (IGF-I) immunolocalization was noted in fibroblasts at the periphery of the fibrotic lesions, which was consistent with a IGF-I complementary role in the stimulation of fibroblast proliferation. TGF-β was localized to fibrotic regions and bronchial epithelial cells. In agreement with our studies, a rat model of early asbestosis localized TGF-β1 in AM and in alveolar epithelial cells at alveolar septal tips (*21*). TGF-β paracrine/autocrine action was postulated to contribute to fibronectin deposition near these sites (*21*).

TGF-β immunostaining was also intense in the rounded nodular lesions of silicosis (*12*). In human idiopathic pulmonary fibrosis (IPF), bronchiolar epithelial cells, macrophages, and type II pneumocytes contained abundant TGF-β mRNA and protein (*22–24*). Khalil and colleagues (*25*) studied TGF-β isoforms in IPF and observed that TGF-β1 was present in macrophages, epithelial cells, and extracellular matrix in advanced lesions, whereas TGF-β1 was present only in macrophages, not epithelial cells, in early lesions. They concluded that TGF-β2 and TGF-β3 were ubiquitously expressed in the lung, and that TGF-β1 was an indication of the chronic lung injury. Two of the study's 12 patients had asbestosis; TGF-β staining was noted in bronchiolar epithelial cells, epithelial cells lining honeycomb cysts, AM, and fibrous connective tissue in the interstitium. The alveolar type II epithelium constitutes at least the main reservoir, if

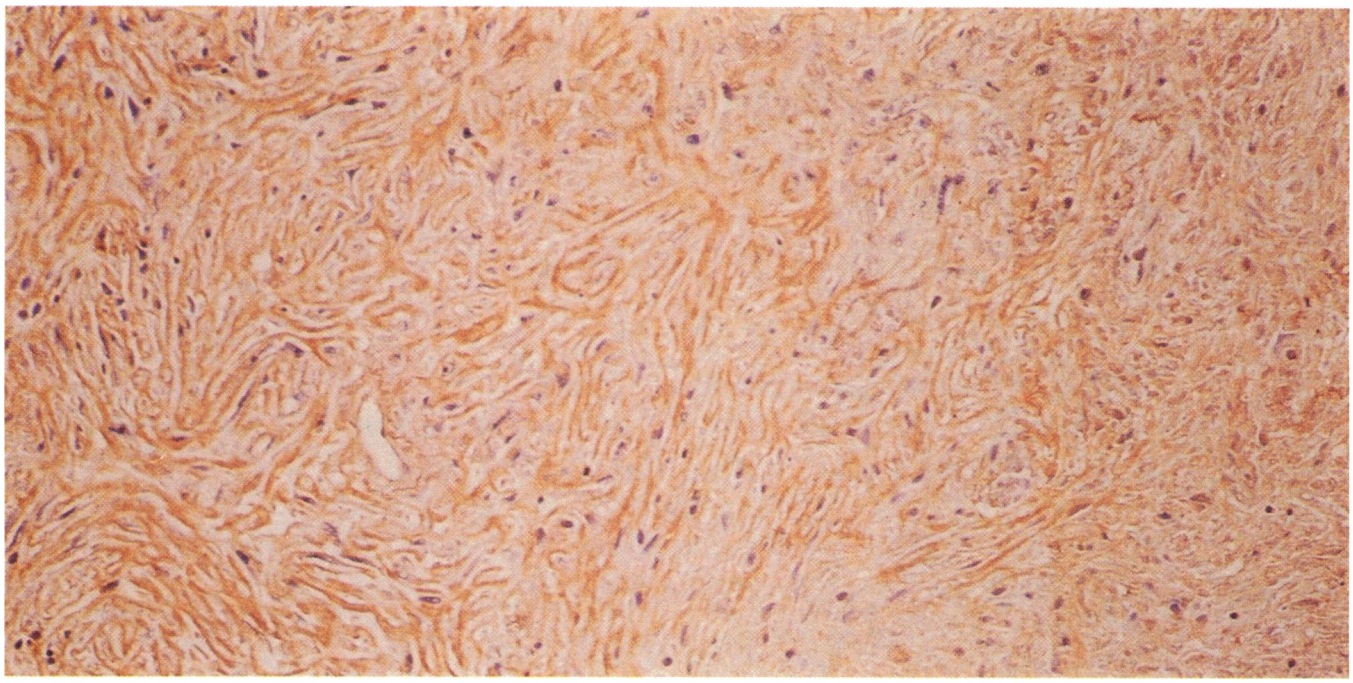

**Figure 7.** Malignant mesothelioma. TGF-β1 immunostaining was detected in the stroma of mesothelioma where thick collagen fibers contain clefts lined with tumor cells. Magnification ×10.

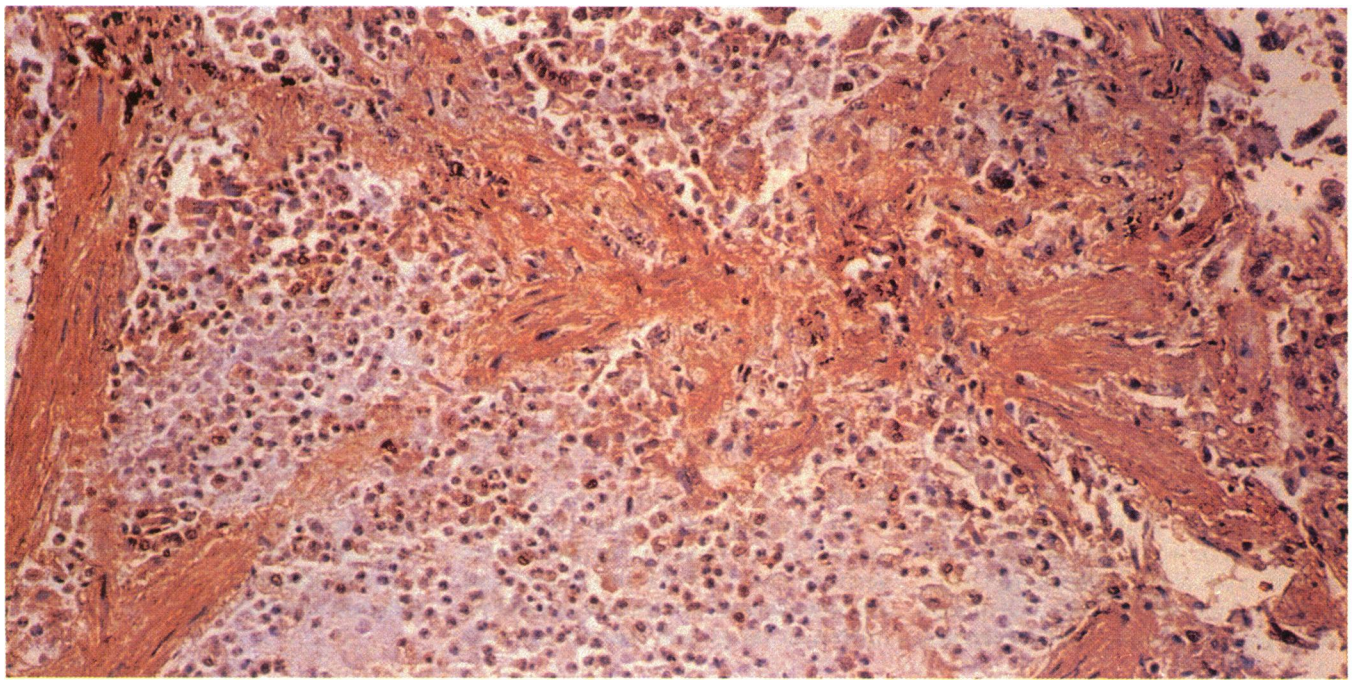

**Figure 8.** Malignant mesothelioma. Mesothelioma cells were not immunostained for TGF-β2. Extensive immunostaining was detected in the stroma. Magnification ×10.

not the site of synthesis, for TGF-β1 and tumor necrosis factor alpha (*26*). TGF-β immunostaining was largely found in the matrix, which is consistent with recent reports indicating that TGF-β is converted from a latent to an active molecule while bound to the extracellular matrix by the latent TGF-β binding protein (*27*).

Both TGF-β2 and -β3 immunolocalized to mesothelioma cells. TGF-β has been measured in the pleural fluid from mesothelioma patients and was three to six times higher than in primary lung cancer patients (*28*). TGF-β has a growth inhibitory effect in the G1 phase of the cell cycle (*29*). The

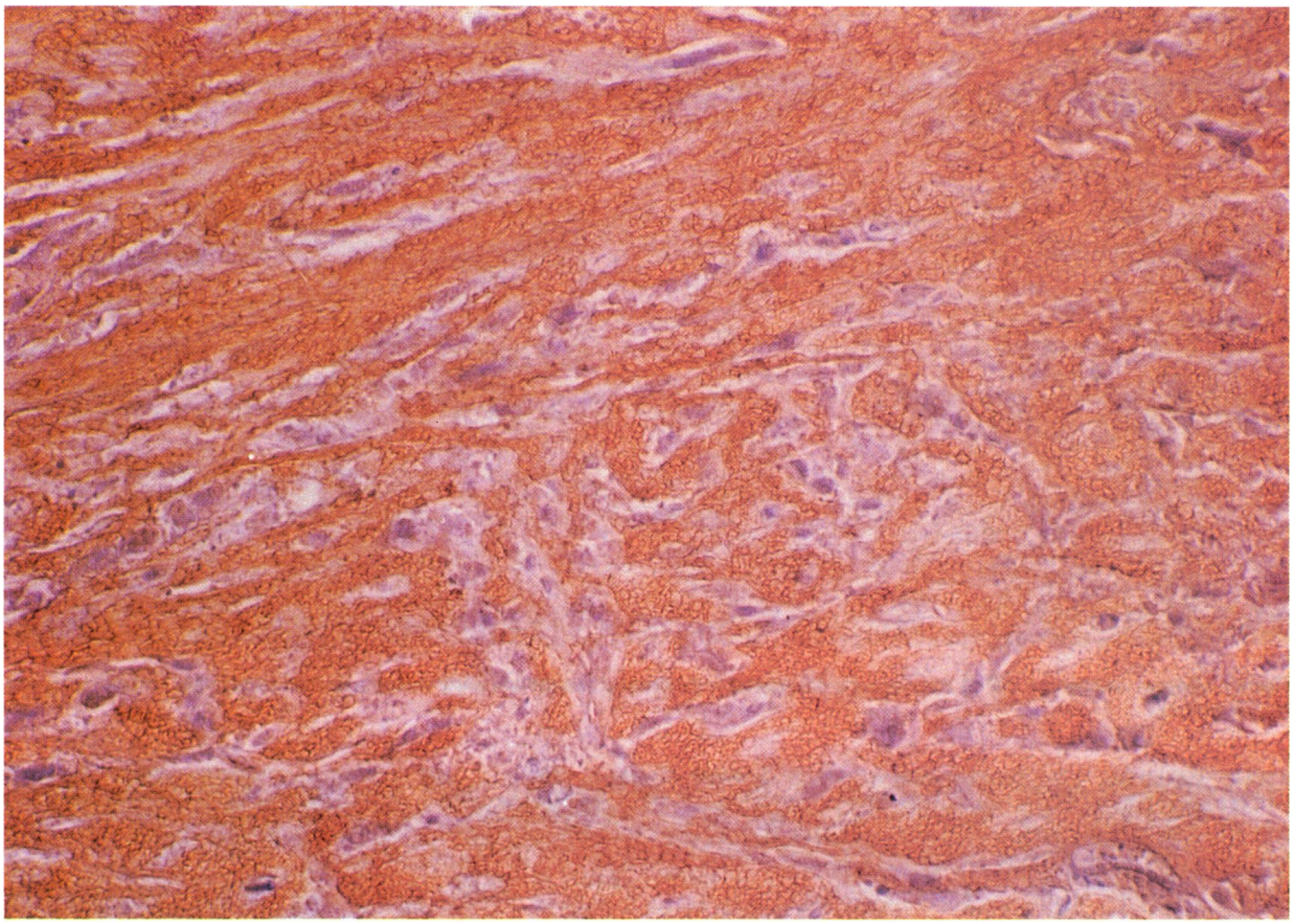

**Figure 9**. Malignant mesothelioma. TGF-β3 was detected both in stromal elements and mesothelioma cells. Magnification ×25.

growth inhibitory effect is mediated through cyclins and cyclin-dependent kinases. TGF-β inhibitory effects may be mediated through two different families of cell-cycle inhibitors. First, TGF-β induced p15 activity 30-fold in human keratinocytes, and p15 blocks cyclin-dependent kinases (CDK)4 and CDK6, which bind cyclin D early in the G1 phase (*30*). Second, TGF-β induces p21 (WAF1/Cip1), which acts as a cell-cycle checkpoint late in G1 by inhibiting CDK2 complexes with cyclin E (*31*). TGF-β suppresses phosphorylation of retinoblastoma (Rb) protein, favoring formation of a complex between Rb/E2F that blocks cells from moving beyond the G1 restriction point (*6*). The protein product of Rb can transcriptionally activate the TGF-β1 and -β2 genes, which may be a novel mechanism for constraining cellular proliferation (*5*). TGF-β also suppresses c-*myc*, which functions, at least in part, in the early half of G1 as a progression factor (*32*). Both *jun* and *fos* are transcription factors that activate the TGF-β1 promoter, and asbestos exposure upregulates *jun* and *fos* mRNA in rat pleural mesothelial cells within 4 hr (*33*). Other cytokines, including IL-6, growth factor IGF-I, and platelet-derived growth factor, have autocrine growth regulatory loops in mesothelioma (*34,35*). The role of TGF-β in malignant mesothelioma may be to inhibit the cell proliferation that is consistent with the bulkiness and lack of metastases by this tumor, and to enhance stroma. TGF-β isoforms are induced in asbestos-related diseases and are associated with extracellular matrix in asbestosis, pleural fibrosis, and malignant mesothelioma.

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