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Up-Regulated Expression of Transforming Growth Factor-α in the Bronchiolar-Alveolar Duct Regions of Asbestos-Exposed Rats

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It has become apparent that numerous growth factors and cytokines are produced during the development of fibroproliferative lung disease. Investigators must sort out which combinations of these factors are playing mechanistic roles in the disease process. Here we demonstrate that transforming growth factor (TGF)-α, a potent epithelial and mesenchymal cell mitogen, is up-regulated specifically at the sites of asbestos fiber deposition in the lungs of rats exposed for 5 hours. Unexposed animals and those exposed to high concentrations of iron spheres exhibited no increase in TGF-α expression at any time during the experiment. Inhaled asbestos fibers deposit initially at the bronchiolar-alveolar duct regions and alveolar macrophages accumulate at these sites within hours. Non-isotopic in situ hybridization and immunohistochemistry were used to show that the mRNA that codes for TGF-α along with the peptide were clearly up-regulated at the bronchiolar-alveolar duct regions by 24 hours after the single asbestos exposure. The numbers of labeled cells demonstrated that expression of the mRNA and protein remained significantly above background for at least 2 weeks after exposure along with increased cell proliferation assessed by staining for proliferating cell nuclear antigen. This, to our knowledge, is the first demonstration of TGF-α expression at sites of lung injury in developing fibroproliferative disease. This finding supports the hypothesis that the growth factor is involved in the dramatic epithelial and mesenchymal proliferation we documented previously, although additional experiments will be essential to establish the precise role of TGF-α. (Am J Pathol 1996, 149:205–217)

It is well known that inhaling asbestos fibers causes interstitial pulmonary fibrosis (IPF).[1] We have been using chrysotile asbestos inhalation in rats and mice to better understand the temporal and anatomic details of IPF, a widespread disease that afflicts hundreds of thousands of individuals worldwide.[2,3] IPF exhibits several common features regardless of the etiological agent; these are epithelial hyperplasia, increased fibroblast proliferation, and elaboration of extracellular matrix components.[2,3] These features are accompanied by varying degrees of acute and chronic inflammation. The increased connective tissue is known to cause a stiff lung and dyspnea,[2,3] but it is not completely clear how the fibrogenic process is mediated at the biochemical and molecular levels. Thus, the physiological, anatomic, and cellular details of IPF are known from numerous studies carried out over the past 25 years.[2–4] Now investigators are in the process of elucidating the basic biochemical and molecular mechanisms that mediate lung fibrogenesis.[5]

Our model of asbestos-induced lung fibrosis (asbestosis) in rats and mice has provided an opportunity to approach fundamental questions.[6,7] For example, we learned that the inhaled fibers deposit initially on the surfaces of alveolar duct bifurcations.[8,9] Rapid activation of alveolar complement proteins at these deposition sites produce chemotactic peptides that attract macrophages as part of a hypertrophic, hyperplastic interstitial fibrogenic lesion at the bronchiolar-alveolar duct (BAD) re-

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gions.[10–13] Tantamount to the development of these lesions is a 5- to 40-fold increase in the percentages of proliferating cells as assessed by tritiated thymidine or bromodeoxyuridine uptake[13–15] and ultrastructural morphometry.[12,16] Now we must ask which genes, coding for their specific proteins, are responsible for the fibroproliferative lesions in the animal models and in humans with IPF. We, along with many other investigators, have postulated that a series of peptide growth factors operates in concert to mediate the development of pulmonary fibrosis.[3,5,17,18] In an attempt to simplify an astounding panoply of growth factors that could be participating in the fibrogenic process, we propose that three could be largely responsible. These are platelet-derived growth factor, the most potent inducer of mesenchymal cell proliferation yet described;[19] transforming growth factor (TGF)-β, a powerful stimulator of extracellular matrix production;[20] and TGF-α, a well characterized potent mitogen for epithelial and mesenchymal cells.[21] There is a rapidly expanding literature on the biological activities of these factors, and results in several animal models, including bleomycin[22] and silica-induced fibrosis,[23] support the view that peptide growth factors are playing a major role in disease development. Recently, we showed by immunohistochemistry that TGF-β is stained in the macrophages and epithelium in developing asbestos-induced lesions.[24]

In the work presented here, we test the growth factor hypothesis, in part, by demonstrating with *in situ* hybridization that the mRNA coding for TGF-α is up-regulated in the developing asbestos-induced lesions and by immunohistochemistry that the peptide is distributed concomitantly. In addition, we provide additional immunohistochemical information on the distribution of alveolar and interstitial macrophages in the asbestotic lesions. Finally, we show that expression of proliferating cell nuclear antigen (PCNA) corresponds anatomically and temporally with the up-regulation of TGF-α.

Materials and Methods

Animals, Exposure, and Tissue Preparation

Pathogen-free, male Sprague-Dawley rats 8 to 10 weeks old weighing 200 to 250 g were exposed (thirty-five rats per group) to an aerosol of chrysotile asbestos (10 mg/m^3 respirable mass), to an aerosol of carbonyl iron spheres (50 mg/m^3), or to room air (sham) for 5 hours in exposure chambers as previously described.[9,12,13,15] Five animals per group were sacrificed by intraperitoneal injection of 0.5 ml of 100 mg/ml ketamine hydrochloride at periods of 0, 24, 48, and 96 hours and 1, 2, and 4 weeks after the single 5-hour exposure. After cutting the renal artery, the lungs of the rats were perfused with fresh 4% paraformaldehyde in phosphate buffer, pH 7.4, through the trachea, at a pressure of 15 cm H_2O for 30 minutes. After perfusion, the trachea was clamped and the lungs were removed from the chest cavity and placed in fresh fixative for 16 hours at 4°C. After fixation, the lungs were embedded in paraffin and 5-μm-thick sections were placed on positively charged slides (SuperfrostR, CMS) for immunohistochemistry and *in situ* hybridization. The same fixation method was used for preparation of normal rat kidney and small intestine for use, respectively, as a positive control for the immunohistochemistry of TGF-α and PCNA and *in situ* hybridization of TGF-α. The positive control sections were placed on the same slide along with the experimental sections for simultaneous staining. The general histopathological appearance of tissues was assessed after routine hematoxylin and eosin staining. Before starting any exposures, five animals were sacrificed and the fixed lungs were processed for routine histopathology to be sure that the rats were healthy. The exposure and tissue preparation protocols were carried out two separate times several months apart, with no apparent differences between the two experiments for any of the parameters studied (see Results).

Immunohistochemistry

Immunohistochemistry staining for TGF-α protein and macrophages was performed using the immunoperoxidase technique described by Kaartinen et al.[25] Briefly, the deparaffinized tissue sections were preincubated in 0.3% hydrogen peroxide (H_2O_2) in methanol for 20 minutes to inhibit endogenous peroxidase activity. After washing with phosphate-buffered saline (PBS), nonspecific antibody binding was blocked by a 30-minute incubation in 2.5% fish gelatin/5% normal goat serum in 1% bovine serum albumin/PBS, pH 7.4. The slides were incubated at room temperature with Ab-2, a mouse anti-human TGF-α monoclonal antibody (2 μg/ml; Oncogene Science, Uniondale, NY) that reacts with human and rat TGF-α or with ED1, a mouse anti-rat macrophage monoclonal antibody (10 μg/ml; Harlan Bioproducts, Indianapolis, IN), in 0.1% gelatin/1%BSA/PBS for 1 hour at room temperature and washed with 0.1% gelatin/1%BSA/PBS. The slides were then incubated with biotinylated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) diluted 1:4000 in 0.1% gelatin/1%BSA/PBS for 1 hour, washed again,

and incubated for 1 hour in streptavidin-horseradish peroxidase (1:2000, Jackson Immunoresearch). Peroxidase activity was visualized with a 10-minute incubation in 50 ml of 0.05 mol/L Tris-HCl, pH 7.6, containing 10 mg of diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and 100 μl of 3% H_2O_2. The slides were counterstained with Lerner-3 hematoxylin (Lerner, Pittsburgh, PA). Normal rat kidney was used as a positive control for TGF-α. For negative controls, an equivalent dilution of normal mouse IgG replaced the primary antibodies for all immunostaining.

The procedure for monoclonal mouse anti-PCNA (PC-10, Dako, Glostrup, Denmark) immunohistochemistry staining is the same as above except for the following steps of antigen retrieval as described by Shi et al[26] and Siitonen et al.[27] After incubation with 0.3% H_2O_2 and washing with PBS, the mounted tissue sections were put in a Coplin jar containing 10 mmol/L citrate buffer (citric acid), pH 6.0, and heated in a microwave oven at the power setting of 700 W at which energy the buffer reaches 100°C and boils for 5 minutes. At the end of the 5 minutes, slides were placed into a fresh change of the same citrate buffer and microwaved for an additional 5 minutes. The Coplin jar is removed from the oven and the slides were cooled for 15 minutes at room temperature. After washing with PBS (twice for 5 minutes each) and blocking with 2.5% gelatin/5% normal goat serum, the tissue sections were incubated with the PCNA PC-10 antibody (1:75) for 1 hour at room temperature. Rat small intestine was used as a positive control each time.

In Situ *Hybridization*

Tissue and Probe Preparation

Tissue sections for *in situ* hybridization were kept at 4°C until used. The localization of TGF-α mRNA by *in situ* hybridization was performed on deparaffinized and rehydrated sections at 0, 24, and 48 hours and 14 days after exposure. The cRNA probe was transcribed from a pGEM vector containing an ~2.0 kb *Eco*RI/*Sal*I fragment derived from the 5′ end of the cloned rat TGF-α cDNA, kindly provided by Dr. David Lee (University of North Carolina at Chapel Hill, Chapel Hill, NC).[28] This fragment includes the 5′ untranslated sequence, the pro-TGF-α coding region and 1.5 kb of 3′ untranslated sequence. The 3′ terminus (*Sal*I site) of this fragment was chosen so as to exclude the short antisense transcript encoded on the opposite strand of the rat TGF-α gene in the 3′ untranslated region. Linearized plasmids were used in *in vitro* transcription for synthesis of both digoxigenin-11-UTP-labeled antisense and sense riboprobes with T7 and SP6 RNA polymerase, respectively (Genius 4 RNA labeling Kit, Boehringer Mannheim Corp., Indianapolis, IN).

Hybridization

After sections were permeabilized, with acetylation and prehybridization, the hybridization was conducted in 4× standard saline citrate (SSC)/50% formamide at 43°C for 16 hours with the digoxigenin-labeled cRNAs at a concentration of 150 ng/section. During hybridization incubation, the sections were covered with parafilm and placed in a humid chamber. Post-hybridization steps included removal of parafilm from the hybridized sections in two washes with 0.15 mol/L sodium chloride, 0.15 mol/L sodium citrate (2× SSC), one wash in 50% formamide/2× SSC, in 50% formamide/1× SSC, and in 0.2× SSC at 43°C for 15 minutes. After digesting with RNAse A (100 μg/ml, Sigma) at 37°C for 30 minutes, the sections were finally incubated in 50% formamide/0.2× SSC at 43°C for 15 minutes.

Immunological Detection

After washing with digoxigenin (DIG) buffer 1 (100 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl) for 10 minutes at room temperature, the sections were incubated in buffer 1 containing 0.05% Triton X-100 and 2% normal sheep serum for 4 hours at room temperature. After two washes with DIG buffer 1 (pH 7.5) for 10 minutes, the slides then were incubated with alkaline-phosphatase-conjugated sheep anti-digoxigenin antibody (1:500, Boehringer Mannheim) in DIG buffer 1 in a humid chamber for 16 hours at 4°C. Then the slides were washed with DIG buffer twice for 10 minutes each and DIG buffer 2 (100 mmol/L Tris-HCl, pH 9.5, 100 mmol/L NaCl, 50 mmol/L $MgCl_2$) once for 10 minutes, followed by visualization with a NBT/NCIP chromogen containing 1 mmol/L levamisole in 10 ml of buffer 2 at room temperature in darkness for 6 hours. The chromogen reaction was halted by rinsing the slides in DIG buffer 3 (10 mmol/L Tris-HCl, pH 8.1, 1.0 mmol/L EDTA), followed by a quick wash in double-deionized H_2O. The slides were then counterstained in 0.02% fast green and FCF (Fisher, Pittsburgh, PA) mounted in an aqueous solution with a coverslip.

Controls

Controls for *in situ* hybridization consisted of 1) replacing the antisense riboprobe with a sense probe or only hybridization buffer without probe, 2) using the antisense riboprobe on the sections after predigestion with 100 μg/ml RNAse A (Sigma) at 37°C for 30 minutes, and 3) using normal rat kidney as a positive control. No positive signal was observed with an antisense probe on RNAse A predigested sections or with sense probe.

Quantitative Analysis

Two histological sections from each lung were prepared and analyzed from six different animals of the two inhalation experiments and the three treatment groups (unexposed control, iron particle-exposed control, and asbestos exposed) at six time points (0, 24, 48, and 96 hours and 2 and 4 weeks) after a single 5-hour exposure. An average of six fields per section were analyzed. This number is dependent on the total bronchiolar-alveolar units available on a given section. These units were selected by a random method that required a Z-shaped movement across the section, as dictated by the stage micrometer. A total of 1500 epithelial cells and 1000 interstitial cells (ie, mesenchymal cells or macrophages) were counted per animal. Four to six bronchiolar/alveolar units were analyzed per section consisting of 1) a terminal bronchiole, 2) alveolar duct walls between the terminal bronchiole and first alveolar duct bifurcation, and 3) a first alveolar duct bifurcation. Stained cells were counted by light microscopy at $\times$400 magnification for TGF-α and PCNA immunohistochemistry to generate the percentages of positive cells. The cells that lined bronchiolar and alveolar lumens and clearly were above basement membranes were considered to be epithelial cells, whereas cells under basement membranes were interstitial cells. Three-way analysis of variance was carried out to determine differences among independent variables (ie, trial and exposure time). One-way analysis of variance was performed to establish differences between exposures at each time point.

Results

Macrophage Distribution

Our previous studies have quantified by scanning electron microscopy and ultrastructural morphometry the cellular details and volume of the macrophage population that responds to asbestos inhalation.[10–12] In Figure 1, a unique perspective on macrophage distribution is provided by the ED1 antibody. We see that numerous ED1-positive cells gather on the bifurcation surfaces during the first 24 hours after exposure (Figure 1, D and E). The interstitial macrophage population builds in the lesions through the following 96 hours, and although the alveolar macrophages slowly clear over the ensuing weeks, the interstitial population remains prominent (Figure 1I). Our previous studies show that a population of normal interstitial lung macrophages are efficient producers of platelet-derived growth factor.[29]

Exposure to high concentrations of iron particles induced the accumulation of few macrophages for a brief period (Figure 1B).

PCNA Expression

Immunohistochemistry of the PCNA protein revealed a rapid increase in labeling of both epithelial and interstitial cells in the developing lesions (Figures 2 and 5). Unexposed animals and those exposed to iron exhibited typical low numbers of PCNA-positive cells of approximately 1%. As expected, the asbestos fibers caused highly significant ($P < 0.001$) increases (~20-fold) in labeling. The degree of difference between asbestos-exposed and control animals remained significant for 2 weeks after exposure (Figures 2 and 5) and labeling returned to normal low levels by the 1-month time period.

In Situ *Hybridization for TGF-α*

In situ hybridization is necessary to identify the cells in the developing lesions that express TGF-α mRNA. Unexposed and iron-exposed animals showed no evidence of increased TGF-α mRNA at any point in time (Figure 3A). Immediately after the 5-hour exposure to asbestos, TGF-α mRNA could be detected at the alveolar duct bifurcations (Figure 3B). However, within the first 48 hours after exposure, there was a striking degree of hybridization in bronchiolar Clara cells and epithelial cells lining the alveolar duct walls and bifurcations (Figure 3, C–F). *In situ* hybridization of adjacent tissue sections with a sense TGF-α probe was completely negative (Figure 3, D and J). Alveolar macrophages and a few interstitial cells also hybridized for TGF-α message at the 48- and 96-hour time period (Figure 3). The hybridization signal was reduced by 2 weeks after exposure but still remained obvious in every animal (Figure 3K).

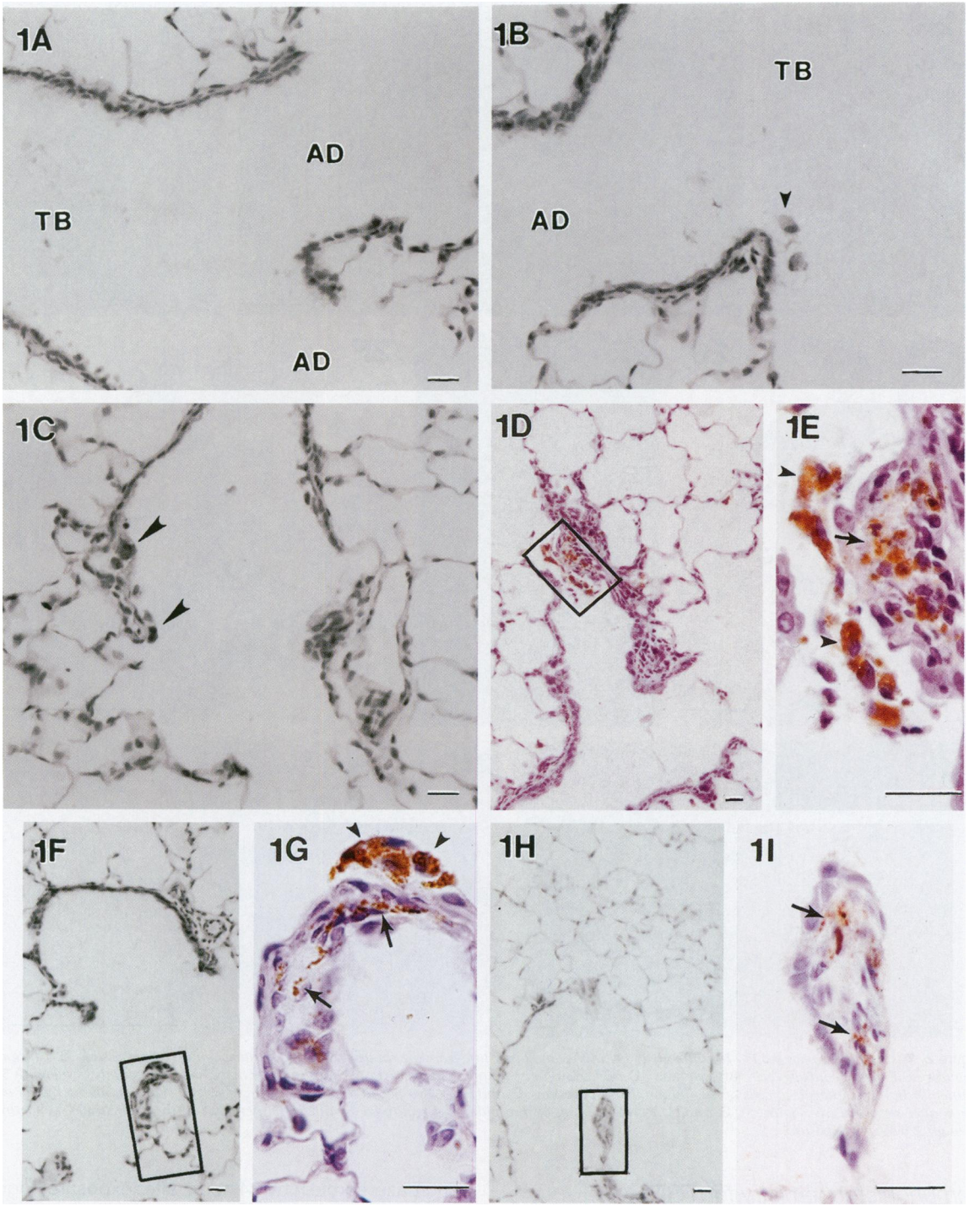

Figure 1. *Distribution of macrophages stained with the ED1 antibody. TB, terminal bronchiole; AD, alveolar duct. Alveolar macrophages are shown by* arrowheads, *and interstitial macrophages are shown by* arrows. A: *Normal tissue from unexposed animals showed no lesions and rare macrophages.* B: *Occasional alveolar macrophages were noted in the lungs of iron-exposed rats.* C: *Immediately after a 5-hour asbestos exposure, the ED1 antibody shows that few alveolar macrophages have migrated to the alveolar duct regions.* D *and* E: *A typical bronchiolar-alveolar duct lesion (enlarged in* E*) seen 48 hours after asbestos exposure. Numerous alveolar and interstitial macrophages have accumulated around and within this fibrotic lesion.* F *and* G: *Alveolar and interstitial macrophages remain around the lesions through 96 hours of exposure.* H *and* I: *The fibrogenic lesions persisted for at least 2 weeks after the single exposure, and the interstitial macrophage populations remained prominent.*

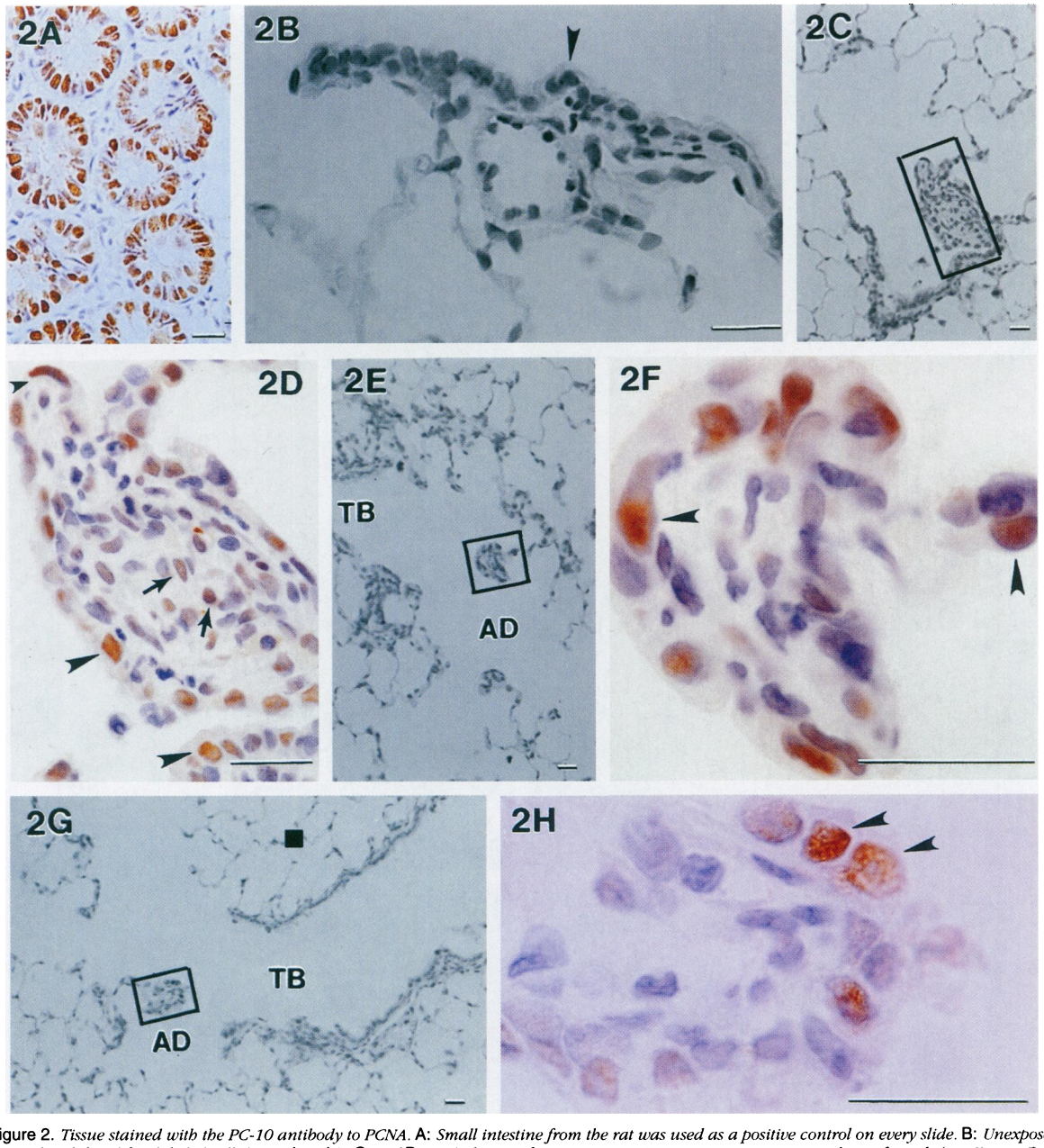

Figure 2. *Tissue stained with the PC-10 antibody to PCNA.* A: *Small intestine from the rat was used as a positive control on every slide.* B: *Unexposed animals exhibited few labeled cells* (arrowheads). C *and* D: *At 48 hours after exposure, PCNA staining was increased significantly* (*see Figure 5*) *in both epithelial and interstitial cells of the fibroproliferative lesions.* E *and* F: *PCNA-specific immunostaining was still significantly increased at 96 hours after exposure* (*see Figure 5*). G *and* H: *PCNA staining of the epithelium remained significantly increased in the epithelium* (*see Figure 5*) *through 2 weeks of exposure.*

Immunohistochemistry for TGF-α

The pattern of TGF-α protein expression followed that of the *in situ* hybridization, ie, bronchiolar Clara cells, alveolar duct epithelium, macrophages, and a few interstitial cells clearly were positive for TGF-α immunostaining (Figure 4). Again, unexposed and iron-exposed animals exhibited little TGF-α labeling (Figure 4C). A quantitative analysis of the percentages of stained cells showed the first evidence of increased protein by 24 hours after exposure (Figure 5). Clara cells of the bronchioles, cuboidal epithelium of the duct walls, and type II cells of the alveoli stained most prominently through the 48- to 96-hour time periods (Figure 4, D–I), although alveolar macrophages stained clearly as well (Figure 4F). In a temporal pattern similar to that of PCNA staining, TGF-α expression remained significantly elevated through the 2-week period after exposure and returned to normal by 1 month after exposure (Figure 5).

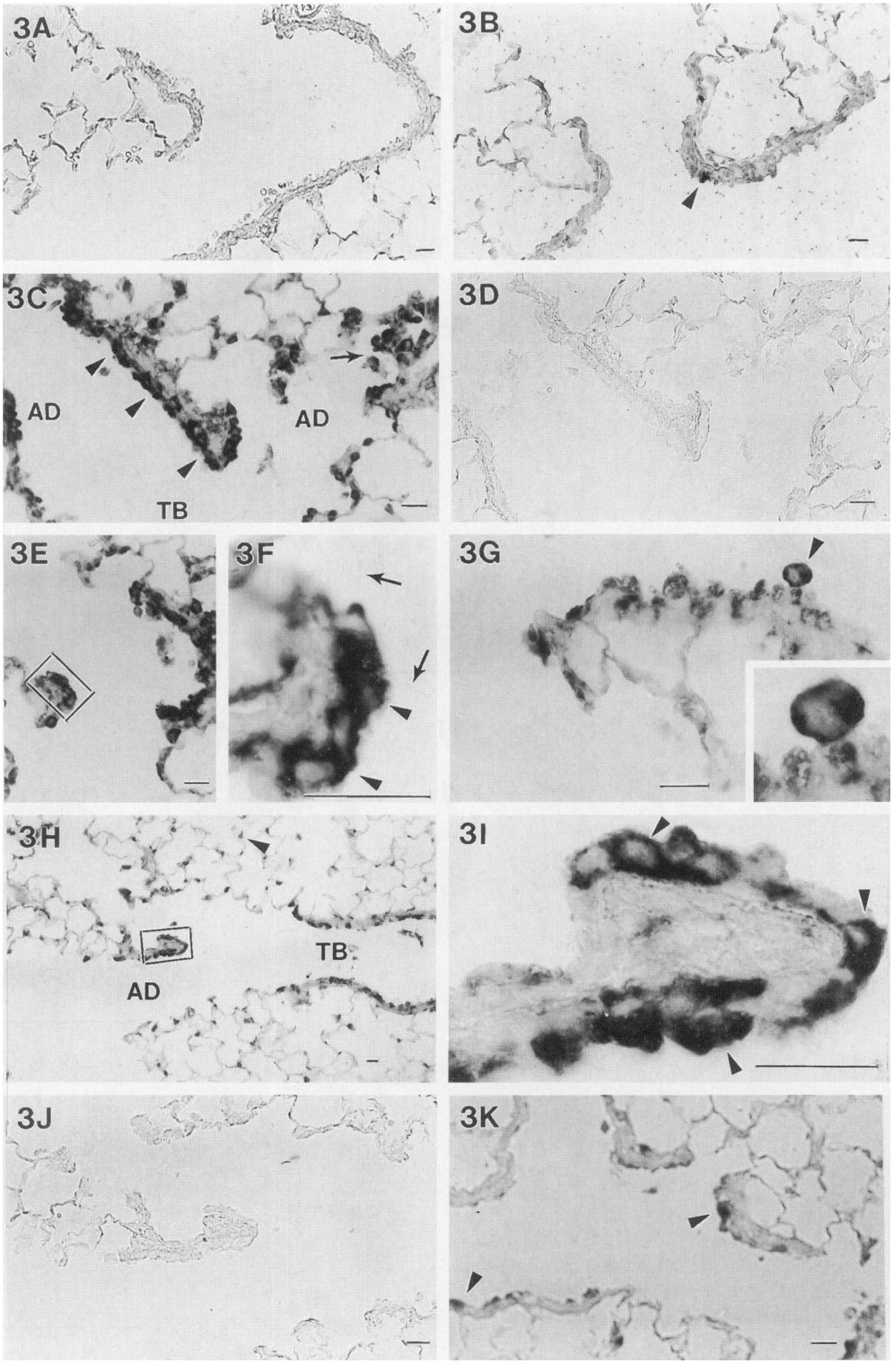

Figure 3. In situ *hybridization of the TGF-α probe in the terminal bronchiolar (TB)-alveolar duct (AD) regions.* **A:** *Unexposed control animals showed little hybridization.* **B:** *Immediately after the 5-hour asbestos exposure, some evidence of hybridization* (arrowhead) *was seen at bifurcations.* **C:** *By 48 hours after exposure, the hybridization signal was intense in the bronchiolar epithelium* (arrowheads) *and macrophages* (arrow). **D:** *The signal in a serial section (to that in* C) *was completely abrogated by using the sense RNA for TGF-α.* **E** *and* **F:** *The epithelium* (arrowheads) *of an alveolar duct bifurcation (enlarged in* F) *exhibits strong hybridization 48 hours after exposure.* **G:** *At 96 hours after exposure, the hybridization signal is still strong in epithelium and macrophages* (arrowheads), *one of which is enlarged in the* inset. **H** *and* **I:** *The TB-AD regions (bifurcation enlarged in* I) *exhibited clear hybridization at 96 hours after exposure.* **J:** *A section adjacent to that shown in* H *is negative when sense RNA is used.* **K:** *By 2 weeks after exposure, the number of hybridized cells is reduced, but the signal is still clear.*

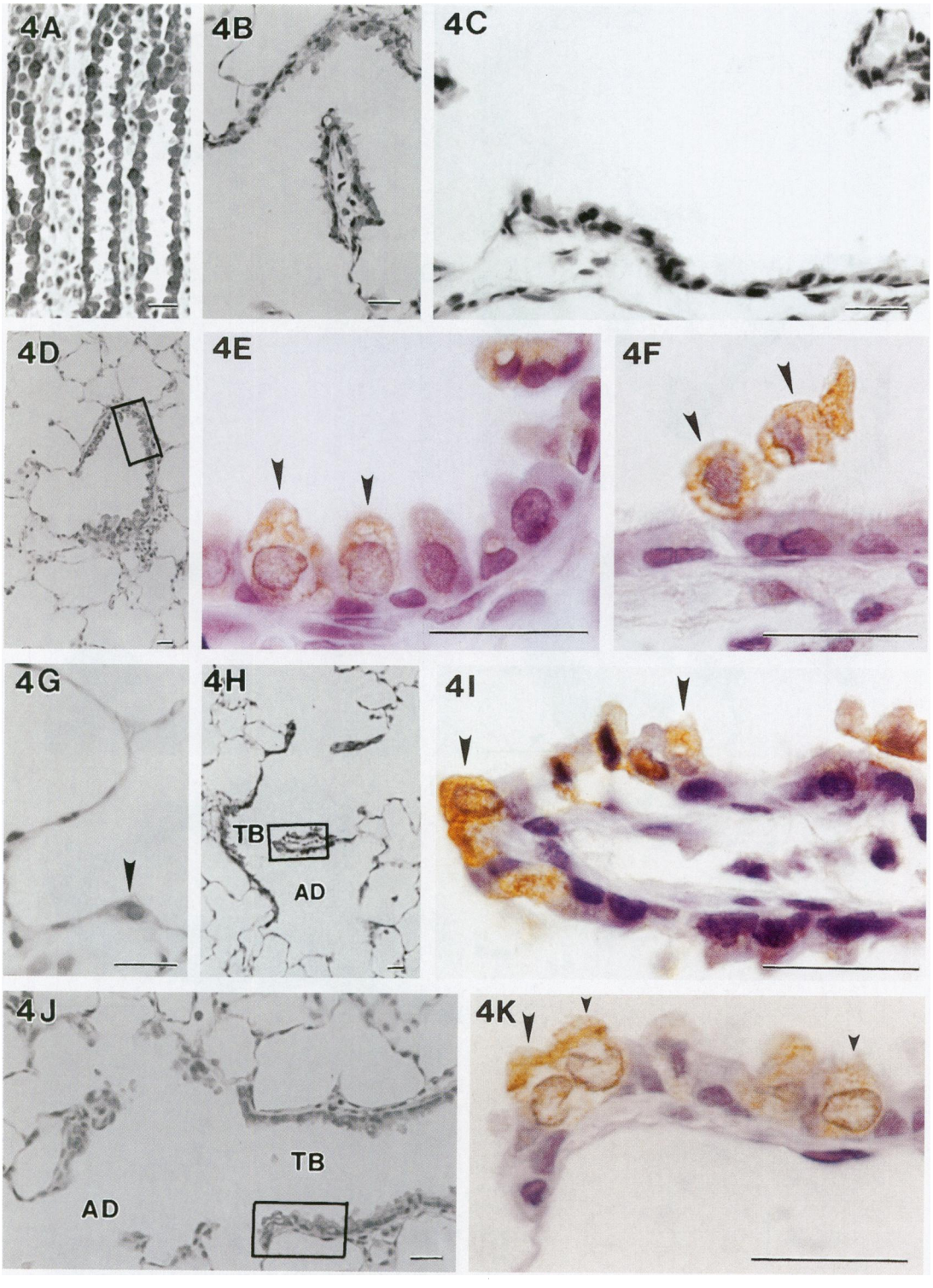

Figure 4. *Light micrographs of immunohistochemistry for TGF-α protein.* A: *Normal rat kidney sections are placed on every slide and here the tubules are clearly stained.* B: *Non-immune mouse IgG does not stain lung (shown here) or kidney.* C: *Unexposed control animals rarely show any stained cells.* D *and* E: *At 48 hours after exposure, numerous Clara cells* (arrowheads) *of the bronchiolar epithelium (enlarged in* E) *are stained by the TGF-α antibody.* F: *Alveolar macrophages* (arrowheads) *are obviously stained 48 hours after exposure.* G: *Type II epithelial cells* (arrowheads) *also stain positively for TGF-α in asbestos-exposed animals.* H *and* I: *The terminal bronchiolar (TB) and alveolar duct (AD) bifurcations (enlarged in* I) *exhibit numerous stained epithelial cells* (arrowheads) *at 96 hours after exposure (see Figure 5).* J *and* K: *The bronchiolar epithelium* (arrowheads) *remained significantly elevated in numbers of stained cells (see Figure 5) even 2 weeks after exposure.*

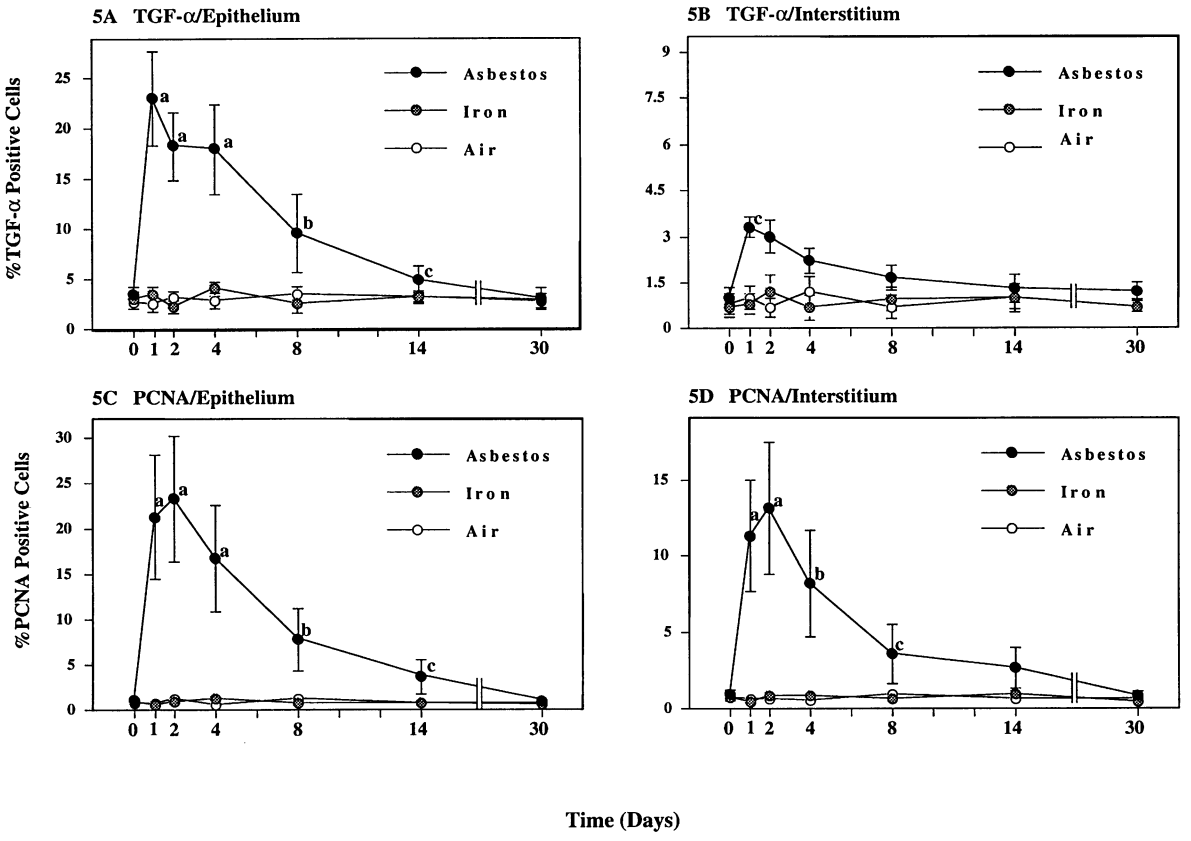

Figure 5. *The single, brief exposure to asbestos induced clear increases in the numbers of cells that stain for TGF-α protein and that express PCNA as a measure of cell proliferation. The data here are means and standard deviations of the stained cells counted in 6 exposed animals (at least 2500 cells per animal) compared with 6 animals from the unexposed and 6 from the iron-exposed groups through the 1-month period after exposure. The standard deviations reflect variations among animals. The small letters indicate degree of statistical significance compared with the corresponding control animals at each time point: a, P < 0.001; b, P < 0.01; c, P < 0.05. Note that both TGF-α and PCNA expression remained significantly elevated in the epithelium of the asbestos-exposed animals for 2 weeks after exposure. PCNA staining, but not TGF-α, was significantly increased in lung interstitial cells for 2 weeks after exposure.*

Discussion

The studies presented here are, to our knowledge, the first to describe the temporal and anatomic expression of TGF-α mRNA and its protein in developing fibrogenic lesions of the lung. These observations support the hypothesis that TGF-α plays some role in the process, but additional studies obviously are necessary to prove this is the case. The fact that TGF-α appears within the first 24 hours after exposure in the asbestos-exposed animals but not in the iron-exposed group provides additional evidence for an important role for TGF-α. Iron particles are deposited in large numbers at the same sites as the asbestos fibers,[30] but they induce no measurable proliferative response.[13,15] In accord with the known mitogenic effects of TGF-α on epithelial cells, we propose that this factor promotes the dramatic proliferative response in the bronchiolar and alveolar duct epithelium. In addition, it seems reasonable to speculate that the TGF-α synthesized by the epithelium could be released to the underlying interstitium where it might act as a mesenchymal cell mitogen in a paracrine fashion. If the gene expression and protein staining had not appeared in concert with the proliferative response, it might have been possible to dismiss TGF-α as a significant factor in the disease process.

Other model systems have been studied in attempts to understand the role of TGF-α in lung fibrosis. Madtes et al[22] reported that bleomycin induces the up-regulation of TGF-α along with its cell surface binding site, the epidermal growth factor receptor. In direct agreement with our findings, these authors showed immunochemical localization in macrophages as well as airway and alveolar epithelial cells. Madtes et al[31] had shown previously that human alveolar macrophages produce TGF-α. In the animal model,[22] they used whole lung extraction of RNA to study gene expression and suggested that "*in situ* hybridization should unambiguously define

the cellular sources of [TGF-α] in the injured lung." Madtes et al[22] speculated that they were unsuccessful in doing so because of the low levels of TGF-α mRNA. We suspect this may be true and suggest that the strong hybridization signal we demonstrated here in Figure 3 likely reflects the potent effects of asbestos on cells of the developing lesions. The patterns of disease development are quite different between bleomycin- and asbestos-induced fibrosis. Bleomycin causes a widespread process involving all aspects of the parenchyma,[32] whereas the initial lesions of asbestosis are focal, concentrated at the ends of bronchioles and along the alveolar duct walls where the fibers first deposit and the macrophages accumulate.[8,10,12,15] Thus, it is appropriate to study gene expression by whole lung extraction after bleomycin treatment and by *in situ* hybridization when studying more focal lesions. Of course, if asbestos exposure is continued for weeks or months, the disease becomes diffuse in both animals[33] and man,[1] and whole-lung RNA extraction might prove to be useful.

TGF-α expression has been studied in two other models of lung fibrosis. Vivekananda et al[34] showed that fibroblasts from the lungs of hamsters exposed to 100% oxygen expressed the mRNA for TGF-α and produced the mitogenic protein *in vitro*. Normal lung fibroblasts exhibited no evidence of TGF-α production. Similarly, Absher et al[23] showed that macrophages and epithelial cells from rats inhaling crystalline silica released substantial quantities of bioactive TGF-α, whereas the cells from normal animals produced no detectable activity. These findings are consistent with our results reported here inasmuch as the lungs of normal unexposed animals exhibited no TGF-α message by *in situ* hybridization and little protein by immunohistochemistry. In addition, the pattern of expression in the exposed animals was predominantly epithelial, and there was little evidence of interstitial TGF-α message (Figures 3 and 4).

A recent paper[35] describes an interesting set of experiments using exposures to a high concentration of chrysotile asbestos similar to that used here and to a lower concentration of fibers. In this model, high fiber concentrations induced bromodeoxyuridine incorporation and fibrogenesis, and the data presented on bromodeoxyuridine[35] compare favorably to those we show here using PCNA staining and to findings we presented previously using tritiated thymidine[13] and bromodeoxyuridine.[14,15] It would seem that this early proliferative response is relevant to the development of fibroproliferative lesions at the sites of fiber deposition. If no inflammatory or fibrogenic lesions ensue, one could conclude that a brief proliferative event was inconsequential or merely nonspecific. It is apparent that cell division provides the increased populations of epithelial and mesenchymal cells in such clearly defined asbestos-induced lesions as hyperplastic alveolar duct bifurcations[12] and smooth muscle cells in thickened hyperplastic vascular walls.[36] These fibroproliferative lesions develop during the month after exposure to a single, brief, high concentration of chrysotile asbestos.[12] Three days of such an exposure causes the proliferative response to remain significantly elevated for two weeks and the lesion to be maintained as hyperplastic for at least six months after exposure.[15] It seems highly likely that the proliferative responses shown by numerous investigators contribute to the accumulation of the cells that constitute these fibroproliferative lesions of asbestosis. This disease process occurs rapidly from brief, intense concentrations of fibers as used here or develops more slowly from low, chronic exposures, explaining the long latency periods experienced by most exposed humans.[1,6,7] If asbestos exposure ceases, the initial proliferative responses wane, but the lesions persist.[12,15,16] The key questions, of course, are which growth factors or cytokines are mediating this response and what are the molecular mechanisms involved. In our studies, it is clear that gene expression and protein elaboration take place focally at sites of developing lesions, and methods such as *in situ* hybridization and immunohistochemistry employed here are necessary to avoid dilution of TGF-α message and to study the fibroproliferative lesions. An advantage of the asbestos model we use is that the location of these lesions corresponds precisely to the sites of fiber deposition and macrophage accumulation.

A fascinating experiment relevant to the findings presented here has been reported by Korfhagen et al.[38] Transgenic mice that overexpressed human TGF-α message under control of the human surfactant protein promoter develop severe pulmonary fibrosis as adults. The rapid course of disease in the transgenic animals suggests that TGF-α plays a major role in the development of the pulmonary fibrogenesis, probably through its expression by alveolar and airway epithelial cells. The authors also clearly demonstrated TGF-α expression by *in situ* hybridization in the pleural region. This suggests a possible correlation with the development of pleural plaques and fibrosis so prevalent in the lungs of asbestos-exposed individuals.[1] If this potent fibrogenic factor were activated in the mesothelium and submesothelial connective

tissue by asbestos fibers, an explanation for this disease process would be forthcoming, although at this time there is no evidence that mesothelial cells are capable of synthesizing and secreting TGF-α. It is interesting to note that overexpression of TGF-α by epithelial cells causes fibrosis in the lung parenchyma of transgenic mice.[38] This is consistent with our postulate that TGF-α expressed in the alveolar and bronchiolar epithelium of asbestos-exposed rats plays some role in the fibroproliferative process that develops subsequently.

TGF-α clearly is a potent growth factor in a number of settings.[21–23] It promotes wound healing[21] and is a powerful angiogenic factor.[39] TGF-α is expressed in a wide variety of human tissue types,[40] and expression has been demonstrated in fetal human and rat lungs.[41,42] In accordance with these findings, Ryan et al[43] showed that TGF-α induces neonatal rabbit type II cells to proliferate. Thus, TGF-α is highly conserved and widespread among tissue types, attesting to its likely importance in development and repair of lung injury. However, the molecular mechanisms that lead to activation of TGF-α expression in the lung after exposure to asbestos have not been defined. Several observations suggest that asbestos induces a transcriptional response.[44–47] An increase in AP-1 activity and a concomitant increase in the mRNAs and proteins for *fos* and *jun*, which constitute AP-1 has been observed in animals and cells exposed to asbestos fibers.[44–46] Similarly, NF-κB, an additional transcription factor induced by stress, is activated after exposure of cells in culture to asbestos fibers.[47] Although asbestos activates expression of these two transcription factors, there is no evidence that either one mediates activation of TGF-α gene expression. However, we have new data demonstrating that asbestos activates expression of the p53 tumor suppressor protein at the sites of fiber deposition (unpublished observations). Furthermore, p53-mediated activation of the TGF-α promoter recently has been demonstrated.[48] Taken together, these observations suggest that a cellular response to asbestos could induce p53-mediated activation of TGF-α expression after exposure to asbestos.

In summary, we have shown by *in situ* hybridization and immunohistochemistry that TGF-α mRNA and protein are expressed in the epithelium and macrophages of developing asbestos-induced fibroproliferative lesions. Unexposed and nonfibrogenic iron-exposed animals exhibit little TGF-α labeling. The temporal and anatomic distribution of TGF-α expression are consistent with the appearance of PCNA, which can serve as a marker of cell proliferation although PCNA also functions as a DNA repair protein.[49] The findings reported here support the postulate that TGF-α contributes to the rapid mitogenic response that occurs at sites of asbestos deposition where fibrogenic lesions develop in the lungs of exposed rats. Additional studies using transgenic and knockout animals, along with administration of appropriate antibodies and antisense oligonucleotides will be necessary to determine the relative contribution of TGF-α expression to the development of interstitial pulmonary fibrosis.

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References

1. Selikoff IJ, Lee DH: Asbestos and Disease. Academic Press, New York, 1978
2. Crystal RG: Interstitial lung disease: current concepts of pathogenesis, staging and therapy. Am J Med 1981, 70:542–565
3. Crouch E: Pathobiology of pulmonary fibrosis. Am J Physiol 1990, 259:L159–L184
4. Kuhn C: An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. Am Rev Respir Dis 1989, 140: 1693–1703
5. Kelly J: Cytokines of the lung. Am Rev Respir Dis 1990, 141:765–788
6. Brody AR: Asbestos exposure as a model of inflammation inducing interstitial pulmonary fibrosis. Inflammation: Basic Principles and Clinical Correlates. New York, Raven Press, 1992, pp 1033–1049
7. Brody AR: Asbestos induced lung disease. Environ Health Perspect 1993, 100:21–30
8. Brody AR, Hill LH, Adkins B, O'Connor R: Chrysotile asbestos inhalation in rats: deposition pattern and reaction of alveolar epithelium and pulmonary macrophages. Am Rev Respir Dis 1981, 123:670–679
9. Brody AR, Roe MW: Deposition pattern of inorganic particles at the alveolar level in the lungs of rats and mice. Am Rev Respir Dis 1983, 128:724–729
10. Warheit DB, George G, Hill LH, Snyderman R, Brody AR: Inhaled asbestos activates a complement-dependent chemoattractant for macrophages. Lab Invest 1985, 52:505–514
11. Warheit DB, Hill LH, George G, Brody AR: Time course of chemotactic factor generation and the corresponding macrophage response to asbestos inhalation. Am Rev Respir Dis 1986, 134:128–133
12. Chang LY, Overby LH, Brody AR, Crapo JD: Progressive lung cell reactions and extracellular matrix pro-

duction after a brief exposure to asbestos. Am J Pathol 1988, 131:156–170
13. Brody AR, Overby LH: Incorporation of tritiated thymidine by epithelial and interstitial cells in bronchiolar-alveolar regions of asbestos-exposed rats. Am J Pathol 1989, 134:133–144
14. Gardner SY, Brody AR: Incorporation of bromodeoxyuridine as a method to quantify cell proliferation in bronchiolar-alveolar duct regions of asbestos-exposed mice. Inhal Toxicol 1995, 7:215–224
15. Coin PC, Osornio-Vargas A, Moore L, Brody AR: Three consecutive exposures to chrysotile asbestos induces prolonged cell proliferation and a fibrogenic lesion of the alveolar duct walls. Am J Respir Crit Care Med 1996 (in press)
16. McGavran PD, Butterick CJ, Brody AR: Tritiated thymidine incorporation and the development of an interstitial lesion in the bronchiolar-alveolar regions of the lungs of normal and complement-deficient mice after inhalation of chrysotile asbestos. J Environ Pathol Toxicol Oncol 1990, 9:377–392
17. Brody AR: Production of cytokines produced by fiber-activated rodent macrophages. Cellular and Molecular Fiber Carcinogenesis. Edited by W Brinkley, J Lechner, C Harris. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1991, pp 83–102
18. Sporn MB, Roberts AB (Eds): Peptide Growth Factors and Their Receptors, vol 1. Heidelberg, Springer-Verlag, 1991, p 794
19. Ross R, Raines EW, Bowen-Pope DF: The biology of platelet-derived growth factor. Cell 1986, 46:155–169
20. Pelton RW, Moses HL: The β-type transforming growth factors, mediators of cell regulation in the lung. Am Rev Respir Dis 1990, 142:31–35
21. Derynck R: The physiology of transforming growth factor-α. Adv Cancer Res 1992, 58:27–52
22. Madtes DK, Busky HK, Strandjord TP, Clark JG: Expression of transforming growth factor-α and epidermal growth factor receptor is increased following bleomycin-induced lung injury in rats. Am J Respir Cell Mol Biol 1994, 11:540–551
23. Absher M, Sjöstrand M, Baldor LC, Hemenway DR, Kelley J: Patterns of secretion of transforming growth factor-α (TGFα) in experimental silicosis: acute and subacute effects of cristobalite exposure in the rat. Reg Immunol 1993, 5:225–231
24. Perdue TD, Brody AR: Distribution of transforming growth factor β1, fibronectin, and smooth muscle actin in asbestos-induced pulmonary fibrosis in rats. J Histochem Cytochem 1994, 42:1061–1070
25. Kaartinen L, Nettesheim P, Adler KB, Randel SH: Rat tracheal epithelial cell differentiation *in vitro*. In Vitro Cell Dev Biol 1993, 29A:481–492
26. Shi SR, Iama SA, Young L, Cote RJ, Taylor CR: Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. J Histochem Cytochem 1995, 43(2):193–201
27. Siitonen SM, Kallioniemi OP, Isola JJ: Proliferating cell nuclear antigen immunohistochemistry using monoclonal 19A2 and a new antigen retrieval technique has prognostic impact in archival paraffin-embedded node-negative breast cancer. Am J Pathol 1993, 142: 1081–1089
28. Blasband AJ, Kathleen TR, Chen X, Azizkhan JC, Lee DC: Characterization of the rat transforming growth factor-α gene and identification of promoter sequences. Mol Cell Biol 1990, 10:2111–2121
29. Brody AR, Overby LH, Badgett A, Kalter V, Kumar RK, Bennett RA: Interstitial pulmonary macrophages produce platelet-derived growth factor which stimulates rat lung fibroblast proliferation *in vitro*. J Leukocyte Biol 1992, 51:640–648
30. Warheit DB, Overby LH, Brody AR: Pulmonary macrophages are attracted to inhaled particles through complement activation. Exp Lung Res 1988, 14:51–66
31. Madtes DK, Raines EW, Sakariassen KS, Assoian RK, Sporn MB, Bell GI, Ross R: Induction of transforming growth factor-α in activated human alveolar macrophages. Cell 1988, 53:285–293
32. Phan SH, Kunkel SL: Lung cytokine production in bleomycin-induced pulmonary fibrosis. Exp Lung Res 1992, 18:29–43
33. Wagner JC: The effects of the inhalation of asbestos in rats. Br J Cancer 1974, 29:252
34. Vivekanada J, Lin A, Coalson JJ, King RJ: Acute inflammatory injury in the lung precipitated by oxidant stress induces fibroblasts to synthesize and release transforming growth factor-α. J Biol Chem 1994, 269: 25057–25061
35. Quinlan TR, BéruBé KA, Marsh JP, Janssen YMW, Taisi P, Leslie KO, Hemenway D, O'Shaughnessy PT, Vacek P, Mossman BT: Patterns of inflammation, cell proliferation, and related gene expression in lung after inhalation of chrysotile asbestos. Am J Pathol 1995, 147: 728–739
36. McGavran PD, Moore LM, Brody AR: Inhalation of chrysotile asbestos induces rapid cellular proliferation in small pulmonary vessels of mice and rats. Am J Pathol 1990, 136:695–705
37. Liu J-Y, Brody AR: The BB isoform of PDGF is expressed at sites of fiber deposition in asbestos-exposed rats. Am J Respir Crit Care Med 1996, 153:A299
38. Korfhagen TR, Swantz RJ, Wert SE, McCarty JM, Kerlakian CB, Glasser SW, Whitsett JA: Respiratory epithelial cell expression of human transforming growth factor-α induces lung fibrosis in transgenic mice. J Clin Invest 1994, 93:1691–1699
39. Schreiber AB, Winkler ME, Derynck R: Transforming growth factor-α: a more potent angiogenic mediator than epidermal growth factor. Science 1986, 232:250–253
40. Yasui W, Zhong-Qiang J, Kuniyasu H, Ayhan A, Yokozaki H, Ito H, Tahara E: Expression of transforming growth factor-α in human tissues: immunohistochemical study and Northern blot analysis. Virchows Arch A Pathol Anat 1992, 421:513–519

41. Strandjord TP, Clark JG, Madtes DT: Expression of TGFα, EGF, and EGF receptor in fetal rat lung. Am J Physiol 1994, 267 (Lung Cell Mol Physiol 11):L384–L389
42. Strandjord TP, Clark JG, Hodson A, Schmidt RA, Madtes DK: Expression of transforming growth factor-α in mid-gestation human fetal lung. Am J Respir Cell Mol Biol 1993, 8:266–272
43. Ryan RM, Mineo-Kuhn MM, Kramer CM, Finkelstein JN: Growth factors alter neonatal type II alveolar epithelial cell proliferation. Am J Physiol 1994, 266 (Lung Cell Mol Physiol 10):L17–L22
44. Timblin CR, Janssen YW, Mossman BT: Transcriptional activation of the proto-oncogene c-*jun* by asbestos and H_2O_2 is directly related to increased proliferation and transformation of tracheal epithelial cells. Cancer Res 1995, 55:2723–2726
45. Quinlan TR, Marsh JP, Janssen YMW, Leslie KO, Hemenway D, Vacek P, Mossman BT: Dose-responsive increases in pulmonary fibrosis after inhalation of asbestos. Am J Respir Crit Care Med 1994, 150:200–206
46. Heintz NH, Janssen YM, Mossman BT: Persistent induction of c-*fos* and c-*jun* expression by asbestos. Proc Natl Acad Sci USA 1993, 90:3299–3303
47. Janssen YM, Barchowsky A, Treadwell M, Driscoll KE, Mossman BT: Asbestos induces nuclear factor kappa B (NF-kappa B) DNA-binding activity and NF-kappa B-dependent gene expression in tracheal epithelial cells. Proc Natl Acad Sci USA 1995, 92: 8458–8462
48. Shin TH, Paterson AJ, Kudlow JE: p53 stimulates transcription from the transforming growth factor α promoter: a potential growth-stimulatory role for p53. Mol Cell Biol 1995, 15:4694–4701
49. Shivji MKK, Kenny MK, Wood RD: Proliferating cell nuclear antigen is required for DNA excision repair. Cell 1992, 69:367–374