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Expression and Activity of Urokinase and Its Receptor in Endothelial and Pulmonary Epithelial Cells Exposed to Asbestos

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An elongated endothelial cell phenotype, which demonstrated increased ICAM-1-dependent neutrophil adherence, was induced when these cells were exposed to noncytotoxic concentrations of asbestos (Treadwell *et al., Toxicol. Appl. Pharmacol.* 139, 62–70, 1996). The present study examined mechanisms underlying this phenotypic change by investigating the effects of asbestos on transcription factor activation and expression of urokinase-type plasminogen activator (uPA) and its receptor uPAR. *In situ* zymography was used to compare the effects of these fibers on the activity of uPA. Cultures incubated with chrysotile or crocidolite asbestos, but not refractory ceramic fiber 1 (RCF-1), demonstrate localized cleavage of plasminogen, which was inhibited by amiloride. Immunocytochemistry showed that chrysotile-stimulated uPA activity was associated with a time-dependent augmentation of uPAR protein levels. RT–PCR analysis was used to investigate molecular mechanisms for these increases. Chrysotile asbestos, but not RCF-1, increased endothelial cell uPA message, relative to changes in β-actin mRNA. This response to asbestos was not limited to endothelial cells, since both uPA and uPAR mRNA levels increase in human bronchial epithelial BEAS-2B cells exposed to chrysotile fibers. Finally, both types of asbestos, but not RCF-1, increased nuclear levels of nuclear factor-kappaB (NF-κB), a transcription factor common to increased expression of ICAM-1 and uPA. These data demonstrate that asbestos caused fiber-specific activation of endothelial and pulmonary epithelial cells, resulting in phenotypes capable of facilitating tissue remodeling.

Asbestos is a class of naturally occurring fibrous silicates used in many industrial applications. Inhalation of asbestos fibers is a major health risk causing pulmonary fibrosis (asbestosis), mesotheliomas, and lung carcinomas (Mossman *et al.,* 1996; Mossman and Gee, 1993; Rom *et al.,* 1991). Vascular effects of asbestos include increased procoagulant activity (Hamilton, 1983b; Callahan *et al.,* 1990), increased vascular permeability (Hamilton, 1983b), increased extravasation of leukocytes (Hamilton *et al.,* 1976), remodeling of small vessels (McGavran *et al.,* 1990), and angiogenesis (Branchaud *et al.,* 1989). In the intact lung, direct effects of asbestos on the vasculature are complicated by soluble factors elaborated by fiber-stimulated epithelial cells.

Noncytotoxic concentrations of chrysotile asbestos cause endothelial cells in culture to elongate and increase expression of adhesion molecules for phagocytes (Treadwell *et al.,* 1996). These events are indicative of activated endothelial cells, which often have enhanced pericellular proteolytic activity and matrix interactions (Mignatti and Rifkin, 1993; Lu *et al.,* 1996). Although asbestos has been demonstrated to increase procoagulant activity *in vivo* and *in vitro,* chrysotile asbestos stimulates fibrinolytic activity in pulmonary bronchial epithelial cells and extravasating macrophages (Gross *et al.,* 1993; Hamilton *et al.,* 1976). This stimulation results from elevated urokinase-type plasminogen activator (uPA) enzyme activity and similar increases in uPA activity in vascular cells may explain many asbestos-induced vascular responses. However, the cellular and molecular effects of asbestos on endothelial cell expression of uPA or its receptor have not been reported.

Inhalation of asbestos, especially chrysotile asbestos, causes fibers to accumulate along capillaries and to penetrate into the capillary lumen (Rom *et al.,* 1991; McGavran *et al.,* 1990). Asbestos activates coagulation, increases levels of vasoactive substances, and decreases plasma partial thromboplastin time (Hamilton, 1983b; Callahan *et al.,* 1990). Several weeks after a single, 1-h exposure to chrysotile asbestos, aberrant pulmonary endothelial and smooth muscle cell proliferation occurs in the small arterioles and venules of animals (McGavran *et al.,* 1990). Increased uPA activity and uPA binding to uPA receptor (uPAR) are central to endothelial cell participation in this type of tissue remodeling and are essential for endothelial cell motility and proliferation (Stetler-Stevenson *et al.,* 1993; Mignatti and Rifkin, 1993; Lu *et al.,* 1996). Asbestos-induced increases in uPA or its receptor may facilitate this tissue remodeling and tumor formation (Hamilton *et al.,* 1976; Hamilton, 1983a), as the plasminogen activator system has been

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proposed as an important component of inflammatory, fibrotic, and tumorogenic processes (Andreasen *et al.,* 1997; Hamilton, 1983b; Shetty *et al.,* 1996). In asbestosis, remodeling of the vasculature may be enhanced by poduction of cytokines and growth factors, such as platelet-derived growth factor or transforming growth factor-α, by airway epithelial cells (Hoyle and Brody, 1996).

uPA is released from cells as a single chain proenzyme that binds uPAR and is converted to a two-chain active form by limited proteolysis (Andreasen *et al.,* 1997). Pro-uPA localization to uPAR on the cell surface and cleavage of pro-uPA brings the active enzyme in close proximity to its major substrate, plasminogen. Cleavage of plasminogen to produce active plasmin initiates the fibrinolytic cascade. This, in turn, leads to the activation of other proteases, such as collagenases and stromolysin, and increases basic fibroblast growth factor release from the matrix. The system is negatively regulated by expression of plasminogen activator inhibitors, especially PAI-1 (Vassalli *et al.,* 1991). PAI-1 binds uPA/uPAR and causes internalization of the complex (Conese and Blasi, 1995; Vassalli *et al.,* 1991). Thus, uPA activity is regulated by levels of active uPA, uPAR binding, and the levels and localization of PAI-1. Expression of both uPA and uPAR increases in physiological and pathophysiological tissue remodeling. uPA activity is elevated in many physiological settings requiring vascular cell motility and proliferation, such as ovulation, angiogenesis, tumor metastasis, and smooth muscle or monocyte migration in atherosclerosis (Conese and Blasi, 1995; Stetler-Stevenson *et al.,* 1993). Increased uPAR expression is also associated with these events and can be induced by cytokines, hormones, and tumor promoters (Conese and Blasi, 1995; Andreasen *et al.,* 1997). Elevated fibrinolytic protein and message levels are negative prognostic indicators of lung, breast, colon, and other cancers (Andreasen *et al.,* 1997). Finally, the effects of uPA on cell morphology, motility, and proliferation require receptor occupancy, but often not uPA proteolytic activity (Rao *et al.,* 1995; Gyetko *et al.,* 1995; Sitrin *et al.,* 1996). In fact, uPA activity may suppress uPAR-mediated adhesion of monocytes (Sitrin *et al.,* 1996).

Exposure of pulmonary cells to asbestos, both *in vivo* (Janssen *et al.,* 1997) and *in vitro* (Janssen *et al.,* 1995; Simeonova and Luster, 1996), increases nuclear translocation of the transcription factor NF-κB. This translocation results from activating signaling cascades that culminate in degradation of the inhibitory subunit, IκB, which normally blocks the nuclear localization sequences of p50 and p65 subunits (Stancovski and Baltimore, 1997; Guerrini *et al.,* 1996). The genes regulated by NF-κB are diverse and are often associated with inflammatory responses, cell adhesion, and growth control (Stancovski and Baltimore, 1997). A partial list of NF-κB-regulated genes include both ICAM-1 (Collins *et al.,* 1995) and uPA (Guerrini *et al.,* 1996; Reuning *et al.,* 1995), as well as important inflammatory proteins, such as interleukin 8 and monocyte chemoattractant protein (Kilgore *et al.,* 1997), and regulators of cellular proliferation, such as c-*myc* (Janssen *et al.,* 1995; Mossman *et al.,* 1997). Thus, activation of this transcription factor has been proposed to play a crucial role in pulmonary responses to environmental stimuli.

Previous studies in this laboratory have demonstrated that asbestos, but not RCF-1, alters endothelial cell morphology and gene expression (Treadwell *et al.,* 1996). In the current studies, endothelial cells were exposed to either chrysotile or crocidolite asbestos to investigate whether activation of endothelial cells by asbestos is associated with altered proteolysis and expression of uPA/uPAR. *In situ* zymography for uPA activity in exposed cultures demonstrated that only asbestos increases pericellular proteolysis. These increases correlate well with increases in steady-state uPA mRNA levels and with increased levels of uPAR protein. This effect of asbestos is not limited to endothelial cells, since uPA and uPAR expression was also increased in human bronchial epithelial cells exposed to the chrysotile. In contrast, RCF-1, which has a lower fibrogenic potential than asbestos (Hesterberg *et al.,* 1993), failed to elicit endothelial cell activation or affect uPA/uPAR expression. Finally, only asbestos was demonstrated to increase nuclear levels of NF-κB, indicating selective activation of signaling pathways leading to transcriptional activation. These data demonstrate that asbestos fibers directly activate pulmonary cells to express proteins that facilitate tissue remodeling.

METHODS

Materials. Culture media, balanced salts solutions, culture supplements, and Trizol were from Life Technologies (Gaithersburg, MD). Characterized fetal calf serum was purchased from Hyclone Laboratories (Logan, UT). Protease inhibitors, casein, porcine plasminogen, E-toxate, and amiloride were obtained from Sigma (St. Louis, MO). Moloney murine leukemia virus (M-MLV) reverse transcriptase, dNTPs, and RNAse inhibitor were purchased from Ambion (Austin, TX). Taq polymerase was supplied by Promega (Madison WI). Oligo $dT_{12–18}$ was obtained from Pharmacia (Piscataway, NJ). All other reagents were of the highest purity available.

Cell culture. Porcine aortic endothelial cells were isolated from freshly harvested vessels and were cultured essentially as described previously (Barchowsky *et al.,* 1989; Treadwell *et al.,* 1996). Cells were grown to confluence in gelatin (Difco, Detroit, MI) -coated plates or flasks (Belco, Vineland, NJ) containing Dulbecco's modified Eagle's medium (DMEM) plus 10% FBS and 1% penicillin/streptomycin. BEAS-2B bronchial epithelial cells were obtained from American Type Tissue Culture (Rockville, MD). The cells were cultured on a matrix of 0.01 mg/ml human fibronectin, 0.03 mg/ml vitrogen 100, and 0.01 mg/ml bovine serum albumin in LHC9 medium (Biofluids, Inc, Rockville, MD). The cultures were maintained at 37°C under an atmosphere of 5% CO_2/95% air. The cells were subcultured using 0.1% Trypsin–EDTA with 0.5% PVP and plated in different-sized tissue culture plates, depending upon the experiment. Greater than 95% of the endothelial cells in confluent monolayers tested positive for endothelial cell-specific markers, such as antigenicity for anti-factor VIII antibody and for rapid uptake of di-I acetylated LDL (Barchowsky *et al.,* 1989). The culture medium for either cell type was replaced with fresh complete medium at least 8 h prior to the beginning of an experiment.

Fiber samples. Reference samples of National Institute of Environmental Health Sciences (NIEHS) chrysotile and crocidolite asbestos used in these studies were obtained from Dr. Brooke Mossman (University of Vermont,

Department of Pathology, Burlington, VT). Refractory Ceramic Fiber-1 (RCF-1) was obtained from the Thermal Insulation Manufacturer's Association, Fiber Repository, (TIMA, Littleton, CO). All fibers were characterized by scanning electron microscopy for fiber size dimensions and were greater than 10 μM in length. Working preparations of fibers were baked at 200°C for 12 h to remove endotoxin or other biological contaminants. The fibers were then diluted in sterile DMEM and tested for the absence of endotoxin using the E-toxate assay.

In situ zymography. Casein gel overlays were used to demonstrate localized uPA production essentially as described (Spiers *et al.,* 1994; Sappino *et al.,* 1991). Briefly, cells, grown to confluence on gelatin-coated glass slides, were exposed to fibers for 24 h. The cells were then rinsed with PBS and overlaid with a mixture of 0.5 ml of 8% milk in PBS, 0.75 ml of PBS, 0.7 ml of 2.5% agar in H_2O, and 20 μl of 4 mg/ml purified porcine plasminogen. The slides were coverslipped and placed in a 37°C, humid reaction chamber for 6 to 24 h, until clear borders of lysed casein were observed by dark field microscopy. In addition to cells that received no fibers, controls for this assay include incubation with overlay mixture containing 1 mM amiloride to inhibit uPA activity or overlay mixture containing no plasminogen. Five fields from each treatment were photographed and compared for width of the enzyme activity zones.

RT–PCR assays for steady-state mRNA levels. Postconfluent cells, grown in 35-mm wells, were exposed for up to 24 h to control medium or medium containing fibers. Exposures were terminated by rinsing the cells twice with HBSS. Total cellular RNA was then isolated with Trizol reagent, according to the manufacturer's instructions. The resulting RNA pellet was suspended in 50 μl of diethylpyrocarbonate (DEPC)-treated water by heating for 5 min at 60°C and quantified by measuring absorbance at 260 nm. Oligonucleotide primer sets used to amplify either porcine uPA (forward 5′ ggctgtctgaatggaggaaaatgt 3′; reverse, 5′ agcaccagggtcttctctgattgt 3′), human uPA (forward, 5′ aaaatgctgtgtgctgctgacc 3′; reverse, 5′ ccctgccctgaagtcgttagtg 3′), human uPAR (forward, 5′ gattgcctgtggaagagt 3′; reverse, 5′ gtttttcggttcgtgagtg 3′), or β-actin (forward, 5′ gggacctgaccgactacctc 3′; reverse, 5′ gggcgatgatcttgatcttc 3′). cDNAs were synthesized and HPLC purified in the Molecular Biology Core facility at Dartmouth. All primers amplify products that are of greater than 300 bases and that span introns in the respective genes. Specificity of primers and products was determined by comparing their sequences against known sequences in Genebank by using NCBI network Blast software version 1.8 (Altschul *et al.,* 1990). Total RNA (0.5 μg) was reverse transcribed in a 30-μl reaction mixture containing Ambion 10× first strand buffer, 10 U RNAse inhibitor, 0.33 mM each dNTP, 1.7 μM oligo dT, and 100 U M-MLV reverse transcriptase. The mixtures were incubated at 44°C for 60 min in a MJ Research PTC-100 thermocycler. After inactivating the enzymes at 95°C, separate 5-μl aliquots of the reaction mixtures were then mixed with either 15 μM of uPA or 5 μM of β-actin forward and reverse primers, 2.5 U of Taq polymerase, and 0.125 mM each dNTP in Ambion complete PCR buffer. The total reaction volume was 50 μl and cDNA was amplified by either 20 or 23 cycles of 20 s at 95°C, 30 s at 55°C, and 40 s at 72°C for β-actin or uPA and uPAR, respectively. The number of cycles was demonstrated to be within the linear amplification range for each product. Amplified products were separated on 2% agarose gels in 0.5× TBE and detected by ethidium bromide staining.

Immunocytochemistry. For these analyses, endothelial cells were grown on gelatin-coated chamber slides and incubated for up to 8 h in 10% FBS/DMEM with or without 5 μg/cm^2 of chrysotile asbestos. The cells were then rinsed and fixed in 3.7% paraformaldehyde, blocked with 2% bovine serum albumin, and incubated with monoclonal anti-human uPAR (clone 3936, American Diagnostica, Greenwich, CT). After rinsing, the slides were incubated with FITC-conjugated goat antimouse IgG in 2% goat serum for 60 min. The cells were rinsed, coverslipped in gelmount, and imaged in the Englert image facility of the Norris Cotton Cancer Center at Dartmouth. Western analysis was used to demonstrate that the anti-human uPAR antibody detected a 35- to 60-kDa band of glycosylated protein in total pig endothelial cell extracts, which is consistent with the expected molecular weight of uPAR.

Electrophoretic mobility shift assays. EMSA were used to demonstrate activation and translocation of proteins that bind to specific consensus DNA sequences for NF-κB, essentially as previously described (Barchowsky *et al.,* 1995; Janssen *et al.,* 1995; Barchowsky *et al.,* 1996). Briefly, after exposure to fibers, cells were rinsed twice with ice-cold 10 mM Tris saline/5 mM EDTA (pH 7.5). Nuclear extracts were prepared as described for endothelial cell cultures (Barchowsky *et al.,* 1996). Protein content of the extracts was determined using Pierce BCA reagents (Pierce, Rockford, IL) and bovine serum albumin as a standard. Equal amounts of nuclear proteins were incubated under high ionic conditions with a 5′-[^{32}P]labeled double-strand oligomer, which contains the consensus binding sequence for NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′). The incubation mixtures were separated on 4% non-denaturing PAGE using high ionic conditions (Barchowsky *et al.,* 1996) and radioactive bands were detected by autoradiography. Supershift assays were performed with affinity-purified, polyclonal antibodies to p65 or p50 (Santa Cruz Biotechnology, Santa Cruz, CA). Nuclear extracts were incubated with labeled probe as above, and then incubated for an additional hour with 1.0 μg of the appropriate antibody.

RESULTS

Localized Increases in uPA Proteolytic Activity Following Exposure to Asbestos

We previously demonstrated that concentrations of asbestos fibers between 1 and 10 μg/cm^2 maximally stimulate endothelial cell activation, induce expression of adhesion molecules, and promote cell motility (Treadwell *et al.,* 1996). However, 48 h of exposure to concentrations of greater than 20 μg/cm^2 are required for significant loss of cell viability or attachment. RCF-1 fibers, even at more than 20 μg/cm^2, failed to affect endothelial cell activation or viability. Therefore, the current studies were performed with fiber concentrations that were maximally effective in activating the cells but were below levels that affected cell viability. Since endothelial cell motility is facilitated by expression of matrix degrading proteases (Stetler-Stevenson *et al.,* 1993; Lu *et al.,* 1996), *in situ* zymography was used to examine whether stimulation of uPA activity could explain motility changes induced by asbestos. The data in Fig. 1 support this hypothesis, since cultures exposed to either chrysotile or crocidolite asbestos for 24 h expressed increased pericellular proteolytic activity that required cleavage of plasminogen to plasmin. Consistent with our earlier findings, RCF-1 fibers did not activate the cells and had no effect on this protease activity.

The specificity of the proteolytic activity observed in Fig. 1 was demonstrated by adding 1 mM amiloride to the overlay gels (Fig. 2). This concentration of amiloride has previously been shown to selectively inhibit uPA-dependent proteolytic activity relative to other plasminogen activators or proteases (Crowley *et al.,* 1993). There was little cleavage of plasminogen in the amiloride-treated gels (Fig. 2), even though the cells had been exposed to five times more crocidolite than in Fig. 1.

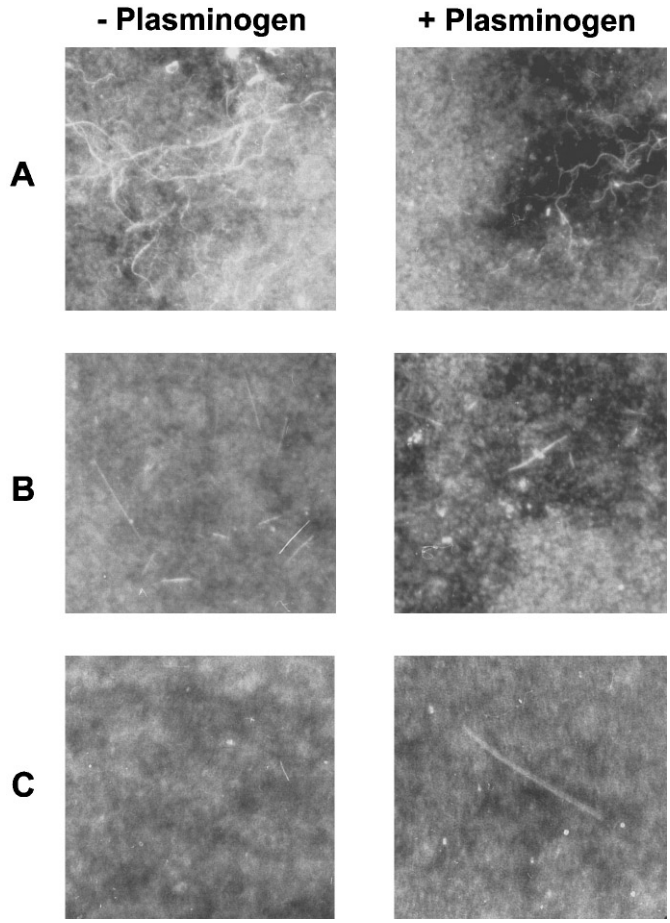

FIG. 1. Localized activity of uPA. Postconfluent endothelial cells, grown on gelatin-coated glass chamber slides, were exposed to 10 μg/cm^2 of chrysotile asbestos (A) or RCF-1 (C) or to 1 μg/cm^2 of crocidolite asbestos (B) for 24 h. Following this exposure period, the slides were rinsed and an indicating casein overlay gel, with (+) or without (−) plasminogen, was added. The slide was coverslipped and incubated in a humid chamber for up to 24 h to allow proteolytic clearance. Slides were evaluated by dark field microscopy for dark zones of proteolytic clearance. Data represent dark field photomicrographs at 200× magnification and are representative of at least five fields.

Increased Expression of uPAR in Cells Exposed to Chrysotile Asbestos

Immunocytochemistry was used to test the hypothesis that increased expression of uPAR might also contribute to the asbestos-induced increases in uPA activity. Porcine cells were exposed to chrysotile asbestos for 4 or 8 h, fixed, and then incubated with monoclonal antibody against human uPAR. As seen in Fig. 3, control uPAR expression and expression 4 h after adding chrysotile was low and limited to punctate extranuclear staining. However, 8 h of chrysotile exposure results in a large increase in antigenicity and appeared to correlate with changes in morphology. Careful examination of the dichroic image indicated that all cells in the field were in contact with fibers. The chrysotile-induced increase in uPAR expression was sustained for more than 24 h (data not shown).

Asbestos Increases Steady-State uPA mRNA Levels in Endothelial Cells

Steady-state uPA mRNA levels in control and fiber-exposed cells were compared to investigate whether increased proteolytic activity correlated with increased expression of uPA. The data in Fig. 4 demonstrated that none of the fibers altered levels of β-actin message, relative to control levels. However, chrysotile asbestos significantly increased uPA message following 24 h of exposure. In other experiments, message levels appeared elevated as early as 8 h following addition of either chrysotile or crocidolite to the cultures (data not shown). This time course for induction was similar to the time- and dose-dependent increases in ICAM-1 expression observed following exposure of endothelial cells to asbestos (Treadwell *et al.*, 1996). In contrast, exposure of these cells to RCF-1 does not affect either ICAM-1 (Treadwell *et al.*, 1996) or uPA (Fig. 4) message levels. This indicated that asbestos caused fiber-specific induction of gene expression in endothelial cells.

The data in Fig. 4 demonstrated that induction of uPA message does not require the presence of serum. This was consistent with earlier observations regarding asbestos-induced alterations in endothelial cell morphology, ICAM-1 expression, and neutrophil adherence (Treadwell *et al.*, 1996). This suggests that the majority of the chrysotile actions were dependent on direct interactions of the fibers with cells and are independent of growth factors or other proteins contained in serum. Serum was included in the majority of experiments, since it is unlikely that endothelial cells would ever be serum free *in vivo.*

Asbestos Induces uPA and uPAR Expression in Bronchial Epithelial Cells

BEAS-2B cells were exposed to chrysotile asbestos to determine whether the effect of asbestos on fibrinolytic protein expression was limited to the vascular cells. This is important, since bronchial epithelial cells would be presented with much higher fiber concentrations. In addition, primers for amplifying porcine uPAR cDNAs were not available, but were available to examine the effects of chrysotile fibers on human uPAR message levels. The data in Fig. 5 demonstrated that following 3-h exposures of BEAS-2B cells to 5 μg/cm^2 of chrysotile, there was a significant increase in both uPA and uPAR message relative to β-actin mRNA. Again, it is important to note that these exposures were in serum-free conditions, indicating that the chrysotile interacts directly with the cells to increase message levels.

Chrysotile Increases Nuclear Levels of NF-κB in Endothelial Cells

Asbestos increases epithelial cell activation and nuclear translocation of NF-κB (Janssen *et al.*, 1995, 1997; Simeonova and Luster, 1996). Therefore, we examined whether asbestos

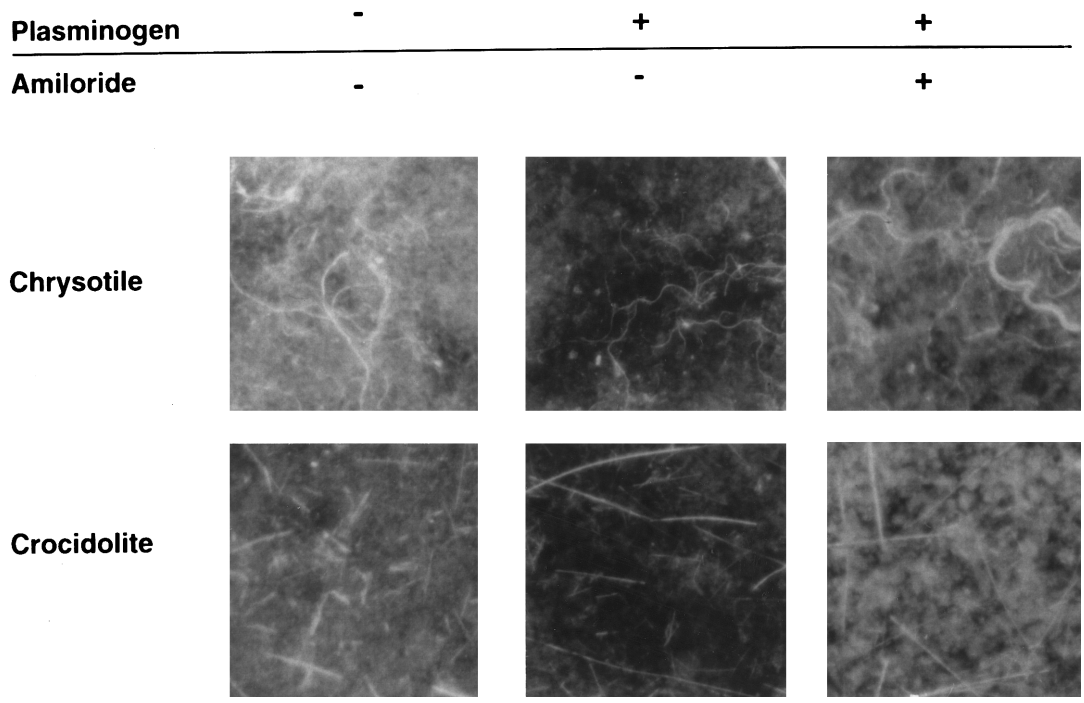

FIG. 2. Specificity of asbestos-induced uPA proteolytic activity. The experiment in Fig. 1 was repeated, with the exception that the amount of crocidolite added during the 24-h exposure period was increased to 5 $\mu g/cm^2$. Also groups of control and fiber-exposed cultures were overlayed with gels containing 1 mM amiloride to selectively inhibit uPA-dependent proteolytic activity. After a 24-h incubation with the overlay gels, the slides were evaluated by dark field microscopy. Data represent dark field photomicrographs at 200× magnification and are representative of at least five fields.

has similar actions in endothelial cells. The experiments presented in Fig. 6 demonstrated that both chrysotile and crocidolite, but not RCF-1, fibers increase nuclear translocation of p50/p65 heterodimers that bind specifically to the consensus NF-κB probe. While these data were qualitatively similar to our earlier observations in epithelial cells (Janssen *et al.*, 1995, 1997), the time course for activation of NF-κB in endothelial cells was more rapid. Increased nuclear NF-κB levels occur as early as 30 min in the endothelial cells and are maximal by 1 h. Experiments not shown demonstrated that chrysotile exposure significantly increases nuclear NF-κB levels in BEAS-2B cells, which was consistent with the changes we observed in crocidolite-exposed rat and hamster epithelial cells (Janssen *et al.*, 1995, 1997).

DISCUSSION

Extravasation of phagocytes and pulmonary fibrosis are hallmarks of asbestosis. In developing fibrotic lesions, there is increased expression and coordination of proteolytic activity of uPA, increased expression of uPAR, increased expression of and adhesivity to ICAM-1, and increased content of extracellular matrix components such as fibronectin (Eddy and Giachelli, 1995; Hauser *et al.*, 1993). Wounded or activated endothelial cells increase surface expression of ICAM-1, uPAR, and release of pro-uPA (Washington *et al.*, 1994; Hauser *et al.*, 1993; Almenar-Queralt *et al.*, 1995). Asbestos may cause similar endothelial cell wounding and activation of a proinflammatory phenotype. Previous studies in this laboratory demonstrated that asbestos altered cell morphology, increased ICAM-1 expression, and promoted localized increases in neutrophil adherence to fiber-contacted cells (Treadwell *et al.*, 1996). The data in the present studies demonstrated that asbestos causes fiber-specific stimulation of uPA activity and increases the expression of both uPA and uPAR. While it is difficult to directly correlate the exposures used in this *in vitro* model to the amount of fibers affecting cells in inhalation studies, it is clear that our data may explain asbestos-induced changes in endothelial and epithelial cells observed *in vivo*. Endothelial and epithelial cell activation and elaboration of proteases and adhesion molecules could promote the vascular remodeling (McGavran *et al.*, 1990; Branchaud *et al.*, 1989; Rom *et al.*, 1991), development of vascularized granular tissue (Raghow, 1991), increased matrix turnover (Chang *et al.*, 1988), and leukocyte extravasation (Rom *et al.*, 1991; Hamilton, 1983a) that occur in rodents following inhalation of asbestos.

uPA and uPAR are central to cell adhesion and motility in a variety of cell types (Sitrin *et al.*, 1996; Rao *et al.*, 1995; Wei *et al.*, 1994; Blasi, 1996), including endothelial cells (Lu *et al.*, 1996). The increased expression of uPA and uPAR following

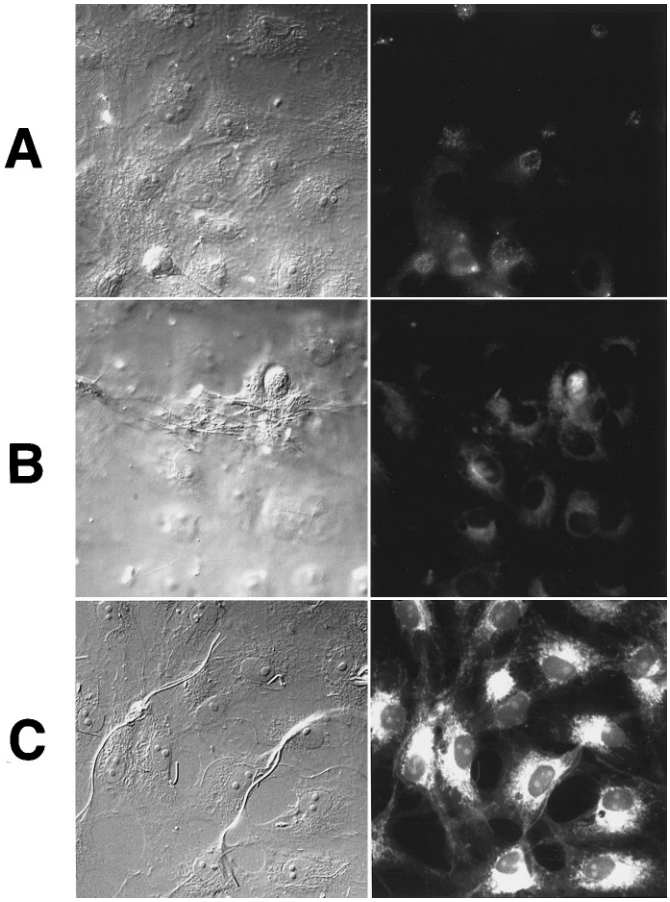

FIG. 3. Time-dependent expression of uPAR in cells exposed to chrysotile asbestos. Cells, grown on gelatin-coated glass slides, were exposed to control medium (A) or medium containing chrysotile asbestos (5 μg/cm^2). Chrysotile exposures were for either 4 (B) or 8 (C) h. At the end of the 8-h period, all cultures were fixed in 3.7% formaldehyde and blocked. The samples were incubated with monoclonal antibody to human uPAR (clone 3936) and then rinsed. Goat anti-mouse antibody conjugated to FITC was used to detect immune complexes. The exposures are of identical fields photographed with either dichroic (left) or epifluorescent illumination (right). These fields were highly representative of the entire cultures.

exposure of endothelial cells to asbestos, observed in the present study, may facilitate the profound alteration of cell shape that occurs as these fibers contact the endothelial cell monolayer (Treadwell *et al.,* 1996). The increased proteolytic activity shown in Figs. 1 and 2 would reinforce cell motility by weakening matrix interactions. However, only the binding of uPA to uPAR, not proteolytic activity, is required for endothelial cells to deform and move (Lu *et al.,* 1996). Motility mediated by uPA/uPAR has also been shown to be required for angiogenic factor-induced endothelial cell migration (Lu *et al.,* 1996). This suggests a role for uPA/uPAR in asbestos-induced angiogenesis in the peritoneum of animals exposed to intraperitoneal injections of asbestos (Branchaud *et al.,* 1989) and in the vascular remodeling seen after inhalation of chrysotile fibers (McGavran *et al.,* 1990).

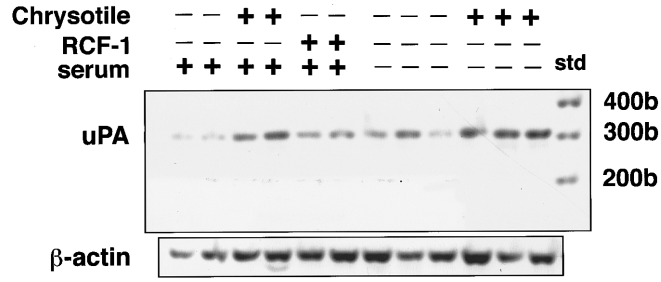

FIG. 4. Increased uPA steady-state mRNA levels following asbestos exposure. Postconfluent endothelial cells were exposed to either buffer alone or 10 μg/cm^2 chrysotile or RCF-1 for 24 h, in the presence or absence of FBS. The serum-free DMEM was supplemented with 2% BSA. Following exposure, the monolayers were rinsed with HBSS and total cellular RNA was isolated. The RNA was reverse transcribed and separate aliquots were amplified by PCR with primers specific for either uPA or β-actin.

Increased expression of uPA and uPAR following exposure to asbestos may be a global mechanism for pulmonary toxicity or fibrosis caused by crystalline fibers. The data in Figs. 1 and 2 demonstrated, in a primary culture model, that chrysotile asbestos increases uPA proteolytic activity. This was consistent with previous observations made in transformed pulmonary epithelial cells (Gross *et al.,* 1993) and in extravasating or isolated macrophages (Hamilton, 1983a; Hamilton *et al.,* 1976). The data in Fig. 5 provided mechanistic support for these previous observations by showing increases in both uPA and uPAR expression in bronchial epithelial cells exposed to chrysotile asbestos. However, asbestos-induced expression of uPA/uPAR *in vivo* may be a complex integration of primary effects of the fibers on target cells and secondary effects of cytokines elaborated from these stimulated targets (Mossman and Gee, 1993; Hamilton, 1983b). For example, uPAR expression in malignant mesotheliomas is induced by a variety of cytokines and uPA binding to its receptor has been suggested as a mechanism for proliferation of these tumors (Shetty *et al.,* 1995, 1996). That uPA/uPAR is induced in a variety of pulmonary cells, including macrophages, endothelial, mesothelial, and epithelial cells, following inhalation of asbestos suggests

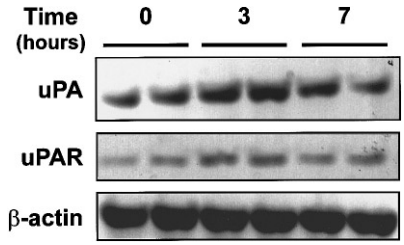

FIG. 5. Increased uPA and uPAR message in bronchial epithelial cells exposed to asbestos. Duplicate wells of confluent BEAS-2B cells were exposed to 5 μg/cm^2 of chrysotile asbestos in serum-free conditions for up to 7 h. Total RNA was extracted from duplicate flasks and reverse transcribed. Separate aliquots were amplified by PCR using primer sets specific to human uPA, uPAR, and β-actin.

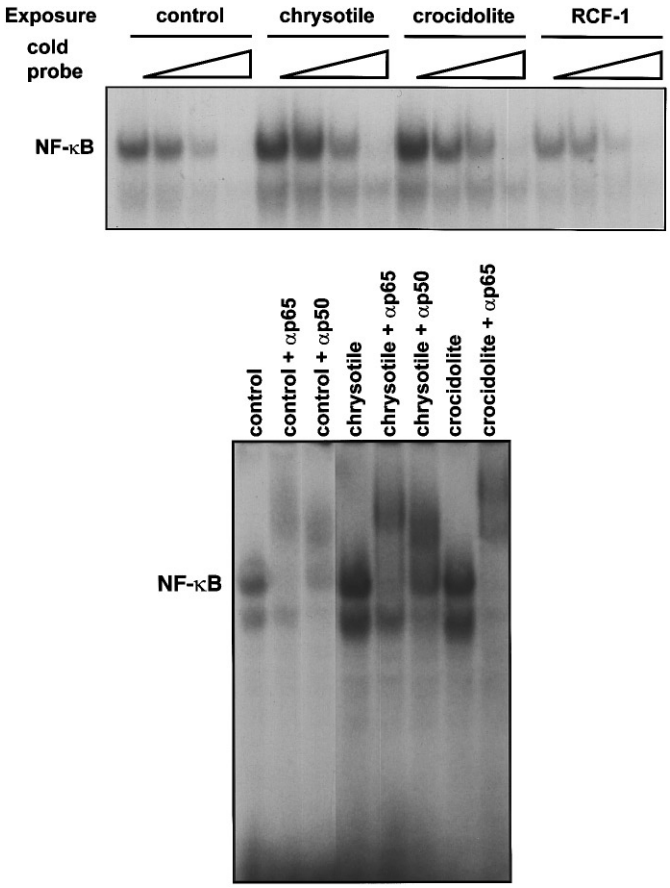

FIG. 6. Increased nuclear levels of NF-κB in endothelial cells exposed to asbestos. (A) Endothelial cells were exposed to either chrysotile (10 μg/cm^2), crocidolite (5 μg/cm^2), or RCF-1 (10 μg/cm^2) fibers for 1 h. Nuclear extracts were prepared and levels of NF-κB were compared by EMSA, as described in Methods. The specificity of binding was demonstrated by competing with 2.5-, 25-, or 250-fold excess of cold probe, respectively. (B) The composition of the specific NF-κB bands was demonstrated by supershift analysis. The mobility of the major bands were further retarded by antibodies to p65 and p50 indicating the presence of heterodimers.

that this a global response that plays a central role in the etiology of diseases induced by these fibers.

Direct asbestos-induced stimulation of uPA/uPAR expression could be explained by either mechanical stimulation of the cells or by interactions between the fibers and proteins on the cell surface. Alterations of cell shape or cytoskeletal reorganization increase expression of uPA in porcine epithelial cells (Irigoyen *et al.,* 1997) and uPAR in human fibroblasts (Bayraktutan and Jones, 1995). While mechanisms for changes in uPAR expression are less well characterized, transcriptional activation of the uPA gene following cytoskeletal reorganization depends on increased signaling through the Ras/extracellular signal-regulated kinase (ERK) pathway (Irigoyen *et al.,* 1997). Although the initial signals feeding into this pathway have not been defined, it is clear that Sos is required and it has been suggested that Src and Grb2 play important roles (Irigoyen *et al.,* 1997). Asbestos activates ERK signaling in mesothelial and pulmonary epithelial cells through an epidermal growth factor-associated pathway (Zanella *et al.,* 1996; Mossman *et al.,* 1997). Further, we have demonstrated that chrysotile asbestos activates Src and focal adhesion kinase (FAK) activities in endothelial cells (Barchowsky *et al.,* 1997). This activation and increased tyrosine phosphorylation of cellular proteins associated with uPAR complexes occurs with a time course that precedes the increases in nuclear NF-κB levels shown in Fig. 6 of the present study. While it is clear that surface receptors can link to Ras activation through pathways that require Src and FAK activity, further studies are needed to demonstrate causal relationships between the signals elicited by asbestos and the activation of transcription factors regulating specific gene promoters, such as those for uPA and uPAR.

Altered FAK activity and changes in cell shape imply interactions between asbestos fibers and integrins. Internalization of vitronectin-coated asbestos fibers by mesothelial cells is mediated by interactions with integrins (Boylan *et al.,* 1995) and integrins induce functional uPAR in lymphocytes (Bianchi *et al.,* 1996). However, the relevance of the mesothelial vitronectin-dependent internalization pathway to the activation of endothelial or epithelial cells by chrysotile asbestos in the present study is questionable. The data in Figs. 4 and 5 indicate that chrysotile efficiently induces uPA and uPAR expression in the absence of added serum or vitronectin. These data imply that chrysotile must initiate signals by binding integrins or other surface proteins directly. It is interesting to note that removal of surface glycophosphatidylinositol-anchored (GPI) proteins, but not blockade of integrin binding sites, prevents asbestos from eliciting changes in endothelial cell shape (Barchowsky *et al.,* 1997). The identities of the GPI anchored receptors or proteins complexed with these receptors required for activation of cell signaling remain uncharacterized.

Asbestos activates transcription through multiple pathways (Mossman *et al.,* 1997). Enhanced nuclear levels and transcriptional activities of both NF-κB and AP-1 binding proteins are observed in cultured epithelial cells exposed to asbestos (Janssen *et al.,* 1995; Simeonova and Luster, 1996; Mossman *et al.,* 1997). *In vivo,* the content of NF-κB-binding proteins in rat airway epithelial cells increases over 5 days following exposure for 6 h/day to chrysotile asbestos (approximately 8.25 mg/m^3 air). The increased nuclear NF-κB levels observed in Fig. 6 demonstrate that chrysotile asbestos has similar affects on vascular endothelial cells, as well. While NF-κB contributes significantly to transcriptional activation of the uPA gene, changes in this factor may not completely explain the asbestos-stimulated uPA and uPAR message levels observed in Figs. 4 and 5. The inducible regions of the uPA and uPAR promoters are complex and contain multiple *cis* elements in addition to their κB sites (Irigoyen *et al.,* 1997; Reuning *et al.,* 1995; Soravia *et al.,* 1995; Novak *et al.,* 1991). The expression of these genes is not necessarily coordinated, however. Disruption of NF-κB signaling in ovarian cancer cells decreases basal uPA, but not uPAR, expression (Reuning *et al.,* 1995). Further,

stability of uPAR message can be induced in lung cells, which could further contribute to the enhanced levels observed in Fig. 5 (Shetty *et al.*, 1997). Since there is cross-talk between the signal cascades leading to NF-κB and AP-1 activation, it is likely that both of these factors, and perhaps other proteins, contribute to the full transcriptional induction of uPA and uPAR observed in the present study. Further studies will be required to determine the essential roles played by these factors and the signaling pathways that activate them in transactivation of genes in pulmonary cells exposed to chrysotile asbestos.

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