Expression and Activity of Urokinase and its receptor in Endothelial Cells Exposed to Asbestos.

Melinda D. Treadwell*, Roy A. Fava**, Jane A. Hunt**, Ronald J. Krieser*, and Aaron Barchowsky*

*Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH.

**Department of Medicine, Veterans Hospital White River Junction, VT.

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send correspondence to:

Aaron Barchowsky, Ph.D.

Department of Pharmacology and Toxicology

Dartmouth Medical School

7650 Remsen

Hanover, NH 03755-3835

email: Barchowsky@Dartmouth.edu

phone: (603) 650-1673

fax: (603) 650-1673

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16. Abstract (Limit: 200 words) Endothelial cells were exposed to chrysotile (12001295), crocidolite (12001284), or refractory-ceramic-fiber-1 (RCF-1) in an effort to determine whether asbestos induced endothelial cell activation is associated with altered proteolysis and expression of urokinase-like-plasminogen-activator (uPA) and urokinase-like-plasminogen-activator-receptor (uPAR). Second or third passage porcine aortic endothelial cells grown to confluence were incubated with the fibers for up to 24 hours. In-situ zymography for uPA activity in exposed cultures demonstrated that these fibers produce localized increases in proteolysis. Within 8 hours, exposure to chrysotile or crocidolite increased uPA expression. The increases in proteolysis correlate well with increases in steady state uPA mRNA levels and with increased levels of uPAR protein. In contrast, rRCF-1 has a lower fibrogenic potential than asbestos and it failed to elicit endothelial cell activation or affect uPA/uPAR. The authors suggest that asbestos fibers directly activate vascular endothelial cells to express proteins that facilitate tissue remodeling.

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Abstract

Non-cytotoxic concentrations of asbestos induce an elongated endothelial cell phenotype, which expresses increased ICAM-1-dependent neutrophil adherence (39). To examine whether these changes are associated with increased activity of the protease, urokinase-like plasminogen activator (uPA), the present study used an *in situ* zymography assay to compare the effects of non-cytotoxic concentrations of asbestos or a non-asbestos ceramic fiber on second passage porcine aortic endothelial cells. Cultures incubated with chrysotile (10 $\mu g/cm^2$) or crocidolite asbestos (1 $\mu g/cm^2$) demonstrate localized cleavage of plasminogen that is inhibited by amiloride. Solution or *in situ* hybridization of steady-state uPA mRNA demonstrated that exposure to either type of asbestos caused increased uPA expression within eight hours. Exposure of cells to chrysotile asbestos also results in a time-dependent, increased expression of uPA receptor (uPAR). In contrast, exposure to refractory ceramic fiber 1 (RCF-1) (10 $\mu g/cm^2$) for up to 24 hours had no effect on levels of uPA message nor enzymatic activity. These data suggest that asbestos causes fiber-specific activation of endothelial cells, resulting in a phenotype capable of facilitating tissue remodeling.

Index terms: urokinase-like plasminogen activator, urokinase receptor, asbestos, endothelial cell.

List of abbreviations: uPA, urokinase-like plasminogen activator; uPAR, urokinase-like plasminogen activator receptor; ICAM-1, intercellular adhesion molecule 1; RCF-1, refractory ceramic fiber 1.

Introduction

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 (22,27,32). Hallmarks of asbestosis include increased fibrinolysis (16), expansion of interstitial matrix components (28), and angiogenesis (7). These events are indicative of activated pulmonary cells, which require enhanced pericellular proteolytic activity and matrix interactions for cell motility and proliferation (19,26,34). There have been many studies of the pathogenesis of pneumoconiosis related to asbestos or other fibers; yet, few have investigated the cellular and molecular activation in vascular cells contacted by fibers (28,32). However, it has recently been demonstrated that non-cytotoxic concentrations of asbestos cause endothelial cells to elongate and increase expression of adhesion molecules for phagocytes (39).

Inhalation of asbestos, especially chrysotile asbestos, causes fibers to accumulate along capillaries and to penetrate into the capillary lumen (15,32). Asbestos has been demonstrated not only to affect proliferative rates of the pulmonary endothelium (7,25), but also to increase endothelial cell permeability and synthesis of proteins associated with wound repair or fibrosis (8,14,15). Several weeks after a single, one hour exposure to chrysotile asbestos, aberrant pulmonary endothelial and smooth muscle cell proliferation occurs in the small arterioles and venules of animals (1). Asbestos also increases fibrinolytic activity in the lung (8) and uPA activity in cultured pulmonary epithelial cells (16). Increased uPA activity stimulates endothelial cell motility and the binding of uPA to its receptor and its proteolytic activity are central to the role of endothelial cells in tissue remodeling (9,24,26,38).

uPA is released from cells as a single chain pro-enzyme that binds uPAR and is converted to a two chain active form by limited proteolysis (13,26). 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 (bFGF) release from the matrix. The system is negatively regulated by plasminogen activator inhibitor-1 (PAI-1) expression. PAI-1 binds uPA/uPAR and causes internalization of the entire complex (13,26). Thus, uPA activity is regulated by levels of active uPA, uPAR binding, and the levels and localization of PAI-1. 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 (26). Increased uPAR expression is also associated with these events and can be induced by cytokines, hormones, and tumor promoters (26,37). However, the effects of uPA on cell morphology, motility and proliferation require receptor occupancy, but often not uPA proteolytic activity (17,31,35). In fact, uPA may suppress uPAR-mediated adhesion of monocytes (35).

Previous studies in this laboratory have demonstrated that asbestos, but not RCF-1, alters endothelial cell morphology and gene expression (39). Therefore, endothelial cells were exposed to either chrysotile or crocidolite asbestos to investigate whether asbestos-induced endothelial cell activation is associated with altered proteolysis and expression of uPA and uPAR. *In situ* zymography for uPA activity in exposed cultures demonstrated that these fibers produce localized increases in proteolysis. These increases correlate well with increases in steady-state uPA mRNA levels and with increased levels of uPAR protein. In contrast, RCF-1, which has a lower fibrogenic potential than asbestos (20), failed to elicit endothelial cell activation or affect uPA/uPAR. These data demonstrate that asbestos fibers directly activate vascular endothelial cells to express proteins that facilitate tissue remodeling.

Materials and Methods

Materials. Culture media, balanced salts solutions, culture supplements, and trypsin were from Life Technologies (Gaithersburg, MD) and characterized fetal calf serum was from Hyclone Laboratories (Logan, UT). Protease inhibitors, casein, porcine plasminogen, and amiloride were from Sigma (St. Louis, MO). All other reagents were of the highest purity available.

Cell Culture. Second or third passage porcine aortic endothelial cells were used for the following experiments and were cultured essentially as described previously (4,39). Cells were grown to confluence in gelatin (Difco, Detroit, MI) coated 25 cm^2 flasks (Costar, Cambridge, MA) containing Dulbecco's modified Eagle's medium (DMEM) plus 10% FBS and were maintained at 37°C under an atmosphere of 5% CO_2 / 95% air. The cells were subcultured using 0.1% Trypsin-EDTA and plated in different sized tissue culture plates, depending upon the nature of the experiment. Post-confluent monolayers were used in all experiments, and greater than 95% of the 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 (4). The culture medium was replaced with fresh DMEM plus 10% FBS at least 8 hours 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. Working preparations of fibers were baked at 200°C for 12 hours to remove endotoxin or other biological contaminants. The fibers were then diluted in sterile DMEM and tested for endotoxin using the E-toxate® assay (Sigma, St. Louis, MO.)

In situ zymography Casein gel overlays were used to demonstrate localized uPA production

essentially as described (29,33,36). Briefly, cells, grown to confluence on gelatin-coated glass slides, were exposed to fibers for 24 hours. The cells were then rinsed with PBS and overlaid with a mixture of 0.5 ml 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 (Sigma Chemical, St. Louis, MO). The slides were coverslipped and placed in a 37°C, humid reaction chamber for 6-24 hours, 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.

Solution hybridization for steady state levels of uPA mRNA . Confluent cells, grown in 25 cm^2 flasks, were exposed for up to 24 hours to control medium or medium containing fibers. To terminate exposure, the cells were rinsed twice with HBSS and total cellular RNA was isolated using RNA Stat-60 (Leedo Medical Laboratories, Houston, TX), according to the manufacturer's instructions. The resulting RNA pellet was suspended in 100 µl of diethylpyrocarbonate (DEPC)-treated water by heating for 5 minutes at 60°C. Absorbance from 320-240 nm of each preparation was scanned in a spectrophotometer to determine RNA quality and quantity. Triplicate 10 µg aliquots of total RNA from each sample were analyzed for levels of uPA mRNA by solution hybridization, according to the method of Hamilton *et al.* (18,39). Total RNA was hybridized with a 5'-[^{32}P]end-labeled 24 base cDNA probe complementary to porcine uPA mRNA (base 6340: 5' GCC CTT CCC TCA AAT CAT TAT TGT-3'). The specificity of the probe for hybridization to uPA was determined by comparing the sequence against known sequences in Genebank by using NCBI network Blast software version 1.8 (3). This probe demonstrated linear hybridization to increasing amounts of total porcine mRNA. Non-hybridized probe and RNA were digested with S1 nuclease. The hybridized duplex was precipitated onto glass filters and counted by liquid scintillation. Data are expressed as fmol mRNA per mg total cellular RNA.

In situ hybridization. Cells were grown to confluence in gelatin coated glass chamber slides,

changed to DMEM plus 10% FBS, incubated overnight, and then exposed to chrysotile or crocidolite asbestos for the time period of maximal uPA expression. Following exposure to various fibers, cells were rinsed twice in Hank's Balanced Salt Solution (HBSS), fixed in 3.7% formaldehyde in PBS, covered with sterile 100% glycerol, and frozen at -80°C until performance of *in situ* hybridization. Cells were thawed at room temperature, rinsed several times in DEPC treated water to remove any residual glycerol. Hybridization was performed using the combined methods of Larsson *et al.* (23) and Hoffman *et al.*(21) with modifications for cell monolayers. The slides were rinsed three times with HBSS, dehydrated through graded ethanol baths for five minutes each (50,70,95,100%), and then hybridized to 2 ng/ml (1 X 10^6 cpm/ml) of a synthetic, 307 base cDNA antisense sequence complementary to porcine uPA (5' base 204 - 3' base 511). The probe was either 5'-[^{33}P]end-labeled or radom prime-labeled with ^{35}S to reduce non-specific background. The hybridization buffer contained 50% formamide in 3xSSC and 10% Denhardt's. Hybridization was performed for 16-18 hours in a 53°C water bath. The slides were then rinsed 2 X 15 minutes in 2 X SSC at room temperature, 2 X 15 minutes 0.5 X SSC at room temperature, and 2 X 15 minutes in 0.1 X SSC at 37°C. The slides were then dehydrated in graded ethanol baths for five minutes each (50,70,95,100%), immersed in Kodak K.5 Emulsion, placed in light protected boxes, and stored for up to two weeks at 4°C. Following the development period, slides were immersed in Kodak developer for 8 minutes, rinsed in water for 2 minutes, immersed in Kodak fixer for 5 minutes, and rinsed in water for 10 minutes. Slides were then counter-stained with a 2 minute immersion in hematoxolin and a 1 minute eosin soak, followed by coverslipping with Permount. To control for the specificity of hybridization, sense 307 cDNA sequence complementary to the antisense probe was also hybridized with matched treated monolayers.

Immunocytochemistry. For these analyses, endothelial cells were grown on gelatin coated chamber slides and incubated for up to 8 hours in 10% FBS/DMEM with or without 5 $\mu g/cm^2$ 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 minutes. 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 35-60 kDA band of glycosylated protein in total pig endothelial cell extracts, which is consistent with the expected molecular weight of uPA.

Statistics. Data were analyzed by analysis of variance to test for significant differences. Significant differences between groups of data were determined using the Student-Newman-Keuls test. Data are presented as mean $\pm$ standard deviation.

Results

Localized increases in uPA proteolytic activity following exposure to asbestos. Non-cytotoxic concentrations of asbestos promote endothelial cell motility (39). Since endothelial cell motility is facilitated by expression of matrix degrading proteases (26,38), *in situ* zymography was used to examine whether fiber contact increases uPA activity. Cultures exposed to either chrysotile or crocidolite asbestos for 24 hours expressed increased pericellular proteolytic activity that was dependent on the cleavage of plasmin to plasminogen (Figure 1). In contrast, there was no effect of RCF-1 fibers on protease activity. The specificity of the proteolytic activity observed in Figure 1 was demonstrated by adding 1 mM amiloride to the overlay gels (Figure 2). This concentration of amiloride has previously been shown to selectively inhibit uPA-dependent proteolytic activity relative to other plasminogen activators or proteases (40). There was little cleavage of plasminogen in the amiloride treated gels (Figure 2), even though the cells had been exposed to 5 times more crocidolite than in Figure 1.

Time course and fiber-specific nature of increased uPA steady state mRNA. Solution hybridization was used to determine whether asbestos-induced proteolysis correlates with increased steady state levels of uPA mRNA. The data in Figure 3 demonstrate that an 8-fold increase in message occurs between 4 and 8 hours of following addition of crocidolite. The slight increase in mRNA levels caused by chrysotile never reached significance. In a separate experiment, the effects of crocidolite on uPA mRNA levels were compared with those of chrysotile and RCF-1 fibers (Figure 4). Again, only crocidolite caused significant increases in mRNA levels.

Localized increases in steady state mRNA levels following asbestos exposure.

Quantitative increases in uPA mRNA following exposure to 5 $\mu g/cm^2$ crocidolite asbestos, but not after 10 $\mu g/cm^2$ chrysotile asbestos (Figures 3 and 4), appear inconsistent with significant increases in uPA proteolytic activity around fiber deposition sites (Figures 1 and 2). Therefore, *in situ* hybridization analysis was used to investigate the hypothesis that only fiber-contacted endothelial cells were responsible for demonstrated increases in uPA activity. The data in Figure 5 demonstrate a 24 hour exposure to chrysotile asbestos increases hybridization with antisense, but not sense, DNA complementary to uPA mRNA. Crocidolite distributes over the cultures more evenly than chrysotile. Therefore, cells were exposed to either 1 or 5 $\mu g/cm^2$ of crocidolite for 24 hours and then analyzed by *in situ* hybridization to address whether a lower fiber burden would cause localized increases in message. As shown in Figure 6, the effect of crocidolite on uPA mRNA levels is dose-dependent. Lower amounts of fiber increases mRNA levels in cells contacted by fibers. However, 24 hours of exposure to the 5 $\mu g/cm^2$ of crocidolite asbestos reveals a more global increase in silver grains indicating increased hybridization to uPA mRNA.

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 hours, fixed and then incubated with monoclonal antibody against human uPAR. As seen in Figure 7, control uPAR expression and expression 4 hours after adding chrysotile is low and limited to punctate extranuclear staining. However, 8 hours of chrysolite exposure results in a large increase in antigenicity and appears to correlate with changes in the shape of cells. Careful examination of the dichroic image indicates that all cells in the field are contacted by fiber. The chrysotile-induced increase in uPAR expression was sustained for more than 24 hours (data not shown).

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 (11,12). Wounded or activated endothelial cells increase surface expression of ICAM-1, uPAR, and release of pro-uPA (2,19,41). These changes are also pronounced at the migrating front of an activated endothelial cell culture (19). Asbestos may cause similar endothelial cell wounding and activation of a pro-inflammatory phenotype. Previous studies in this laboratory have demonstrated that asbestos alters cell morphology, increases ICAM-1 expression, and promotes localized increases in neutrophil adherence to fiber-contacted cells (39). The data in the present studies demonstrate that asbestos causes fiber-specific stimulation of uPA activity and increases the expression of both uPA and uPAR. Endothelial cell activation and elaboration of proteases and adhesion molecules could promote the vascular remodeling (7,25,32), development of vascularized granular tissue (30), altered fibrinolytic activity (8), and leukocyte extravasation (28,32) that occur following inhalation of asbestos.

uPA and uPAR are central to cell adhesion and motility in a variety of cell types (31,35,42), including endothelial cells (24). The increased expression of uPA and uPAR following 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 monolayer (39). The increased proteolytic activity shown in Figures 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 (24). Motility mediated by uPA/uPAR has also been shown to be required for angiogenic factor-induced endothelial cell migration (24). This suggests a role for endothelial cell uPA/uPAR in asbestos-induced angiogenesis in the peritoneum

of animals exposed to intraperitoneal injections of asbestos (7) and in the vascular remodeling seen after inhalation of chrysotile fibers (25).

Increased expression of uPA and uPAR following exposure to fibrogenic fibers, such as asbestos, may be a global mechanism for fiber-induced pulmonary toxicity. The data in Figures 1 and 2 demonstrate, in a primary culture model, that chrysotile asbestos increases uPA proteolytic activity. This is consistent with previous observation made in transformed pulmonary epithelial cells (16). Further, we have also observed that asbestos increases uPA expression in low passage human pulmonary microvascular endothelial cells (data not shown). Pulmonary expression of uPA and uPAR correlates well with tumor metastasis and tissue remodeling (34). However, the mechanism for asbestos-induced increases in uPA or uPAR have not been identified. Further, 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 (28). 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 tumor proliferation in mesotheliomas (34). Finally, differentiation of macrophages and monocytes, their adhesion, and cytokine elaboration, are all modulated by uPA/uPAR (31,35). This suggests that induction of uPA/uPAR following inhalation of asbestos may play a central role in the etiology of fiber-induced diseases.

Localized induction of uPA proteolytic activity and mRNA following exposure of endothelial cells to low concentrations of chrysotile or crocidolite asbestos is consistent with the pattern of neutrophil adherence observed in similar experiments (39). The data in Figures 5 and 6 demonstrate little expression of uPA in cells not contacted by fibers. This data and localized neutrophil adherence (39) suggest that asbestos has a direct effect on primary endothelial cells and that asbestos-induced uPA and ICAM-1 expression (39) do not require elaboration of a diffusible cytokine or paracrine factor. Expression of uPAR in Figure 7 does appear to be global. However,

close examination of the dichroic image reveals that all cells in the field are contacted by fibers. These findings are consistent with changes observed in developing fibrotic lesions (11,41), where there is increased expression and coordination of proteolytic activity of uPA, increased expression of uPAR on cells in the region, increased expression of and adhesivity to ICAM-1, and an increased content of extracellular matrix components such as fibronectin (11,12). Therefore, these molecules and the coordination of their actions appear to be essential for the invasion and migration of endothelial cells in the direction of an activating stimulus. Phenotypic change in the endothelial cells, as well as in other target cells, may contribute to the complex adhesivity and tissue remodeling observed following asbestos fiber deposition within the pulmonary cellular milieu.

Direct activation of endothelial cells by asbestos could be explained by the fibers mimicking ligands for integrins, such as fibronectin or vitronectin (6,10). Boylan *et al.* have demonstrated that pleural mesothelial cells internalize vitronectin or serum-coated crocidolite asbestos fibers via an $\alpha v\beta 5$ integrin-dependent mechanism (6). The affinity of uPAR for vitronectin is higher than that of integrins (42). Additionally, Wei *et al.* demonstrated that vitronectin binding to uPAR in epithelial cell culture in the absence of uPA also activates morphologic change and increased adhesion (42). Binding of vitronectin to uPAR causes a clustering of the receptor and formation of signaling complexes with integrins (5). These complexes can then initiate tyrosine kinase-dependent signaling cascades for cytoskeletal rearrangement and cell migration (5). Activation of these signaling cascades could explain uPAR-dependent endothelial cell motility and deformability (24). In addition to changes in cell shape, activation of integrin signaling and tyrosine-kinase cascades would provide an axis for induction of gene expression (35). These observations and data from the current study demonstrating asbestos-induced shape change, increased proteolytic activity, and increased expression of both uPA and uPAR, suggest that asbestos may not only increase activity of the uPA/uPAR system, but also utilize interactions with uPAR to initiate cellular responses. However, further investigations are required to establish the role of uPA/uPAR in such a positive autocrine loop of initiating cellular activation that leads to

increased gene expression and pericellular protease activity.

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Figure 1. Localized activity of uPA. Post-confluent endothelial cells, grown on gelatin coated glass chamber slides, were exposed to 10 $\mu g/cm^2$ of chrysotile asbestos (A) or RCF-1 (C) or to 1 $\mu g/cm^2$ of crocidolite asbestos (B) for 24 hours. 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 hours to allow proteolytic clearance. Slides were evaluated by dark field microscopy for dark zones of proteolytic clearance. Data represent dark field photomicrographs at 20X magnification and are representative of at least five fields.

Figure 2. Specificity of asbestos-induced uPA proteolytic activity. The experiment in Figure 1 was repeated, with the exception that the amount of crocidolite added during the 24 hour 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 hr incubation with the overlay gels, the slides were evaluated by dark field microscopy. Data represent dark field photomicrographs at 20X magnification and are representative of at least five fields.

Figure 3. Time course for increased uPA steady state mRNA levels following asbestos exposure. Post-confluent endothelial cells were exposed to 10 $\mu g/cm^2$ chrysotile or 5 $\mu g/cm^2$ crocidolite asbestos for 1,4,8 or 24 hours. Following each exposure period, the monolayers were rinsed with HBSS and total cellular RNA was isolated. The RNA was then hybridized with [^{32}P]5' end-labeled 24 base cDNA complementary to porcine uPA. Non-hybridized complexes were then digested with S1 nuclease and the remaining hybridized duplexes were precipitated and quantified using a liquid scintillation counter. The fmol uPA

mRNA per mg of total cellular RNA was calculated. The data are the mean ± s.d fold increase in uPA mRNA relative to control. Significant differences ($p < 0.001$) are designated by *, n=4.

Figure 4. Fiber-Specific increase in uPA mRNA. Cells were then exposed to 10 $\mu g/cm^2$ chrysotile asbestos or RCF-1 or 5 $\mu g/cm^2$ crocidolite asbestos fibers for 24 hours. Total cellular RNA was then isolated and analyzed for steady-state levels of uPA mRNA by solution hybridization. Data are the mean fold increase in fmoles uPA mRNA per mg total RNA relative to control (n=4 for asbestos exposures and n=2 for RCF-1).

Figure 5. Localized increase of uPA mRNA following exposure to asbestos. Cells were exposed to 10 $\mu g/cm^2$ of chrysotile asbestos for 24 hours. Following exposure, cultures were rinsed with HBSS and fixed with 3.7% methanol free formaldehyde. RNA was then hybridized *in situ* to [^{33}P] 5'- end labeled sense and anti-sense cDNA probes complementary to porcine uPA mRNA. Following hybridization, the monolayers were rinsed with increasing stringency and the slides were then coated with photographic emulsion. Slides were incubated at 4°C for 1.5 weeks in the dark and then developed, counter-stained and coverslipped. The photomicrographs are representative of at least 5 fields containing fibers.

Figure 6. Dose-dependent increase of uPA mRNA in response to crocidolite asbestos. Cells were exposed to 1 $\mu g/cm^2$ and 5 $\mu g/cm^2$ of crocidolite asbestos for 24 hours. Following this exposure, cultures were rinsed, fixed with 3.7% methanol free formaldehyde, and RNA was hybridized to a [^{35}S] 5' end-labeled cDNA probe complementary to uPA mRNA. The slides were exposed to photographic emulsion for 3.5 weeks and then photographed. These data are representative of at least 5 fields containing fibers.

Figure 7. 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 $\mu g/cm^2$). Chrysotile exposures were for

either 4 (B) or 8 (C) hours. At the end of the 8 hour 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 antimouse 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.

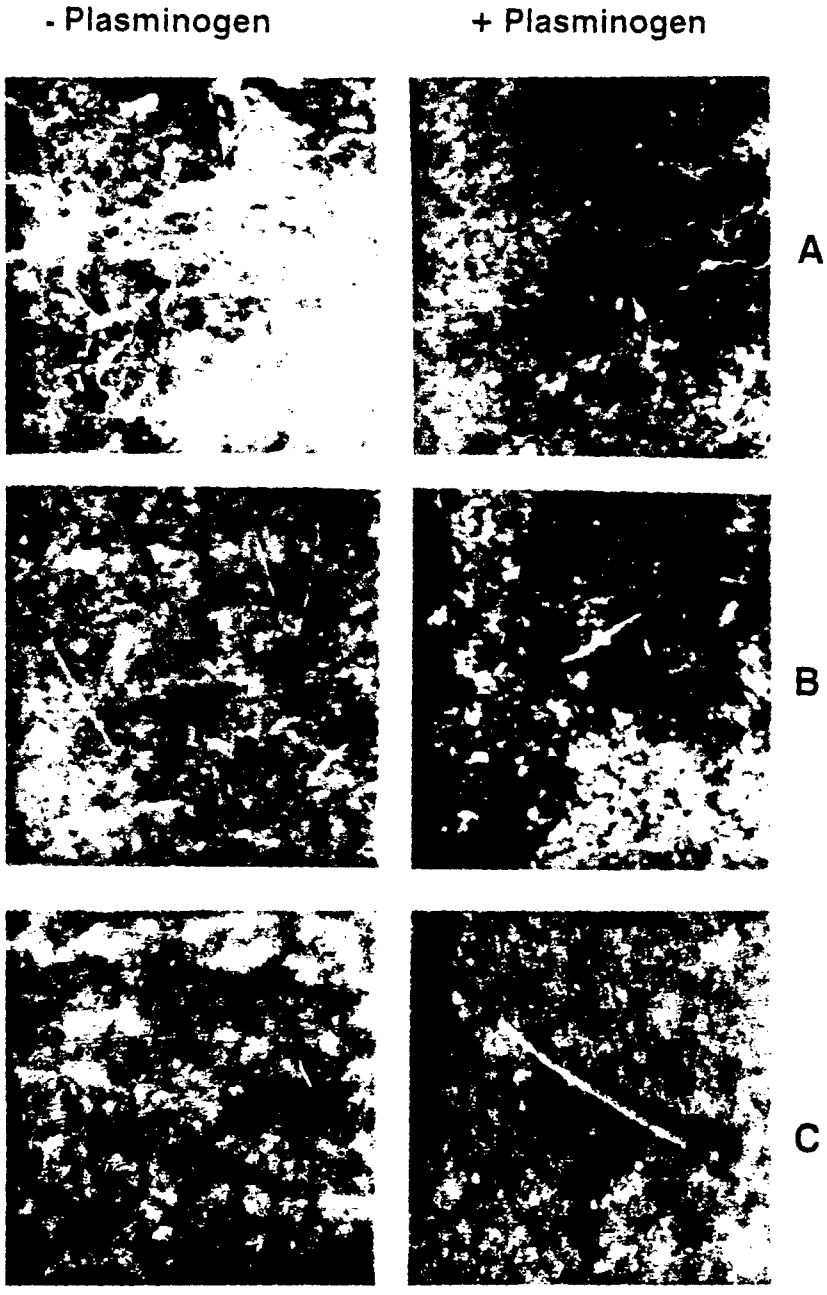


FIGURE 1

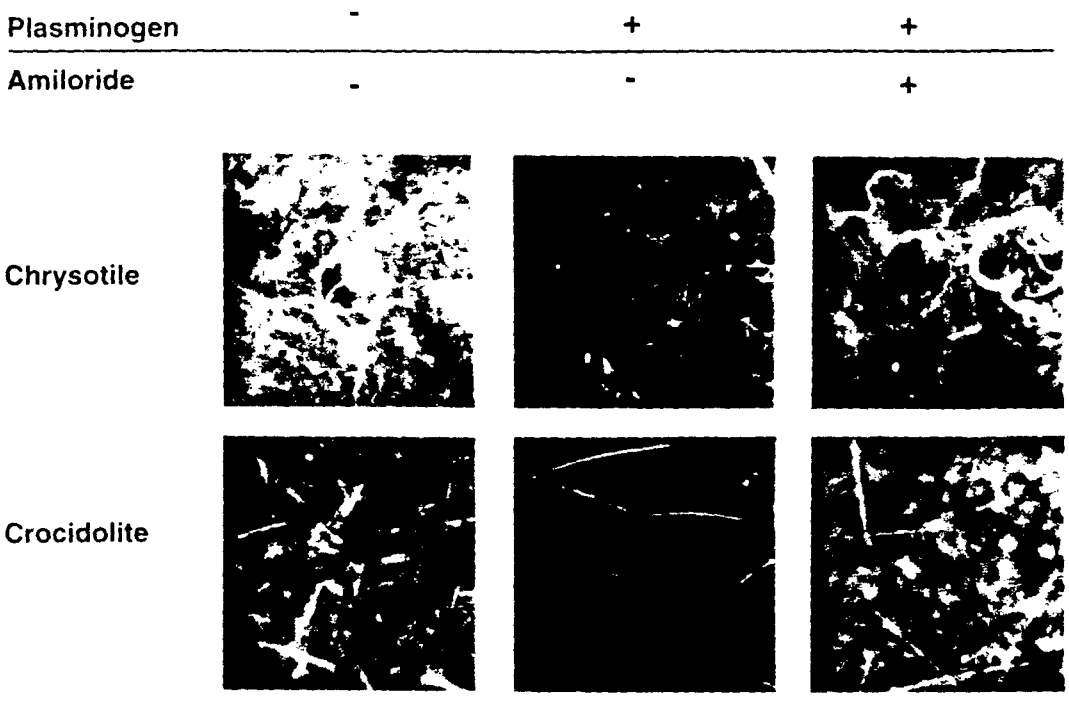
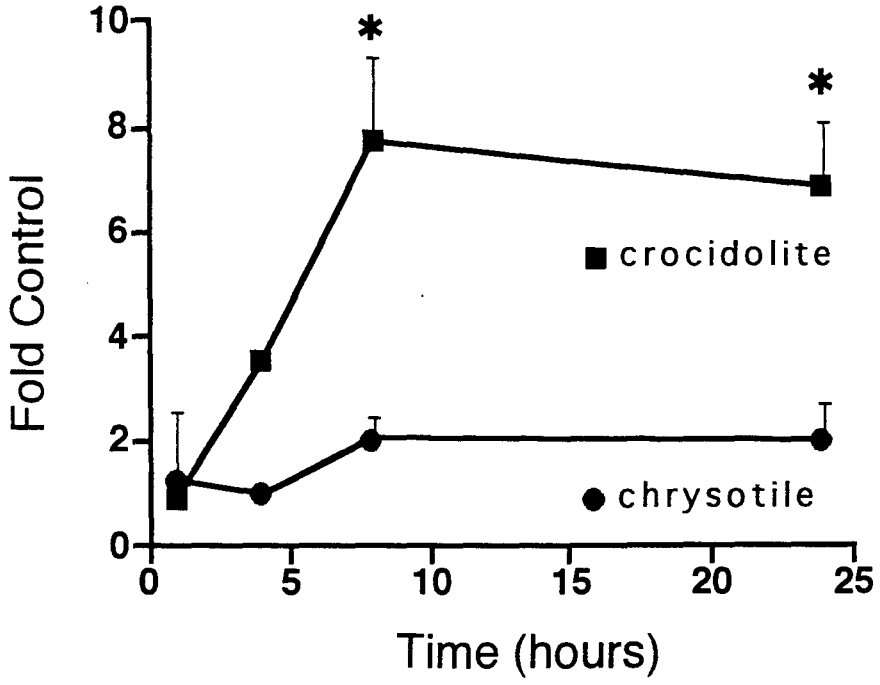


FIGURE 2

Treadwell et al
Asbestos-induced expressionof uPA and uPAR
Figure 3 Time course for increased uPA steady-state mRNA levels.



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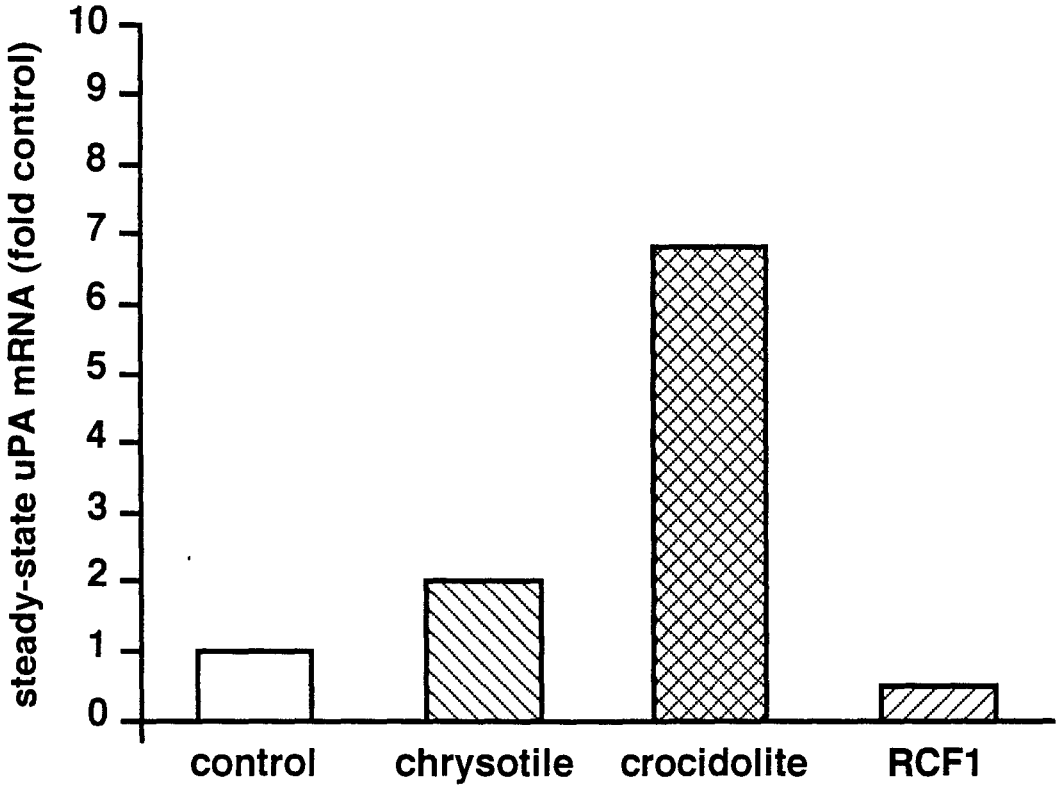
Asbestos-induced expression of uPA and uPAR
Figure 4 Fiber-specific increase in uPA mRNA



Chrysotile
Sense
10X

Chrysotile
Sense
40X

Chrysotile
Anti-Sense
10X

Chrysotile
Anti-Sense
40X

FIGURE 5

5 μg/cm^2 Crocidolite
Sense
40X

5 μg/cm^2 Crocidolite
Anti-Sense
40X

1 μg/cm^2 Crocidolite
Sense
40X

1 μg/cm^2 Crocidolite
Anti-Sense
40X

Figure 6

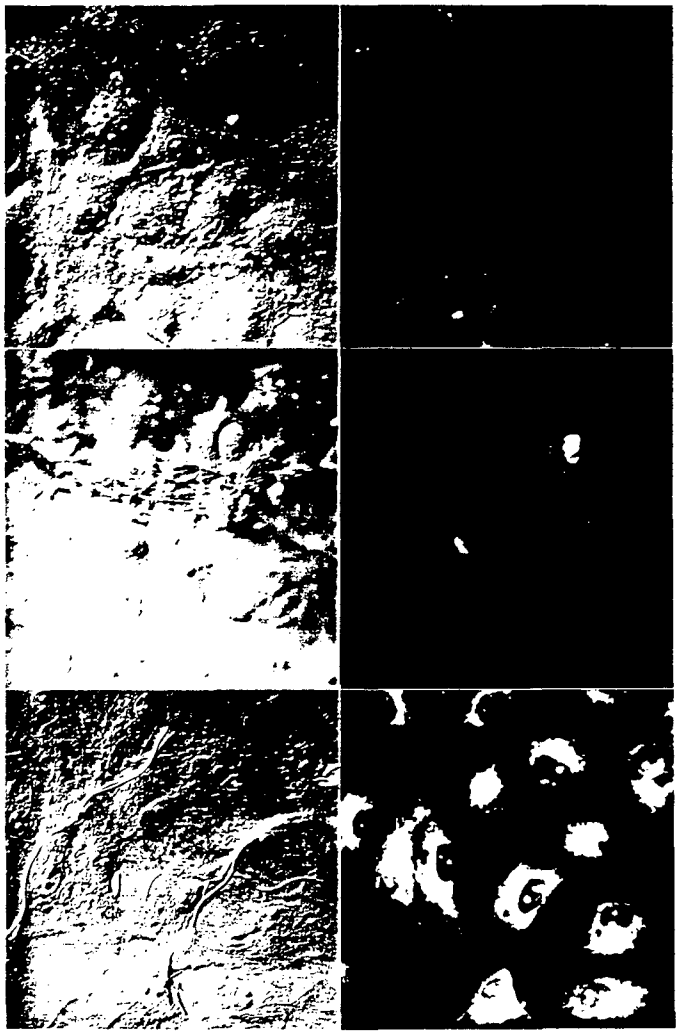


FIGURE 7