Increased Focal Adhesion Kinase- and Urokinase-type Plasminogen Activator Receptor-associated Cell Signaling in Endothelial Cells Exposed to Asbestos

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Exposure of low-passage endothelial cells in culture to nonlethal amounts of asbestos, but not refractory ceramic fiber-1, increases cell motility and gene expression. These changes may be initiated by the fibers mimicking matrix proteins as ligands for receptors on the cell surface. In the present study, 1- to 3-hr exposures of endothelial cells to 5 mg/cm² of chrysotile asbestos caused marked cell elongation and motility. However, little morphological change was seen when chrysotile was added to cells pretreated with either mannansamine or to prevent assembly of glycoprophatidylinositol (GPI)-anchored receptors or with herbimycin A to inhibit tyrosine kinase activity. Affinity purification of GPI-anchored urokinase-type plasminogen activator receptor (uPAR) from chrysotile-exposed cells demonstrated that asbestos altered the profile of proteins and phosphoproteins complexed with this receptor. Tyrosine kinase activities in the complexes were also increased by asbestos. Immunoprecipitations with selective monoclonal antibodies demonstrated that both chrysotile and crocidolite asbestos increase kinase activities associated with p60 Src or p120 focal adhesion kinase (FAK). Further, chrysotile also changed the profile of proteins and phosphoproteins associated with FAK in intact cells. These data suggest that asbestos initiates endothelial cell phenotypic change through interactions with uPAR-containing complexes and that this change is mediated through tyrosine kinase cascades. — Environ Health Perspect 105(Suppl 5):1131–1137 (1997)

Key words: focal adhesion kinase, endothelial, urokinase-type plasminogen activator receptor, asbestos, Src kinase

Introduction

Inhalation of asbestos fibers is a major health risk causing pulmonary fibrosis (asbestosis), mesotheliomas, and lung carcinomas (1–3). Hallmarks of asbestosis include increased fibrinolysis (4), expansion of interstitial matrix components (5), and angiogenesis (6). These events are indicative of activated pulmonary cells, which require enhanced pericellular proteolytic activity and matrix interactions for cell motility and proliferation (7-9). Many studies have examined the pathogenesis of pneumoconiosis related to asbestos or other fibers, yet few have investigated cellular and molecular mechanisms for activation of vascular cells contacted by fibers (3,5).

Inhaled chrysotile asbestos fibers accumulate along capillaries and penetrate into the capillary lumen (3,10). Asbestos increases the permeability of the pulmonary endothelium, the proliferative rates of endothelial cells (6,10), and the synthesis of proteins associated with wound repair or fibrosis (11–13). Several weeks after a single 1-hr exposure to chrysotile asbestos, aberrant pulmonary endothelial and smooth muscle cell proliferation occurs in the small arterioles and venules of animals (10). Recent studies using cultured cells demonstrated that noncytotoxic concentrations of asbestos, but not refractory ceramic fiber-1 (RCF-1), induce an activated endothelial cell phenotype (14). This phenotype is elongated, has increased motility, and increased expression of adhesion molecules for phagocytes (14).

Vascular remodeling and activated endothelial cells are associated with increased proteolytic cascades, which are initiated by activation of urokinase-type plasminogen activator (uPA). 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 (8). Expression of uPA receptor (uPAR) is also increased in remodeling and can be induced by cytokines, hormones, and tumor promoters (8,9). The binding of uPA to uPAR is central to the role of endothelial cells in tissue remodeling and is essential for endothelial cell motility (8,15–17). The effects of uPA on cell morphology, motility, and proliferation require receptor occupancy, but often not uPA proteolytic activity (18–20).

uPAR is a glycoprophatidylinositol (GPI)-anchored surface receptor that localizes either to insoluble lipid–protein complexes (21,22) on the cell surface or to focal adhesions attached to matrix-bound vitronectin (23,24). Addition of the glycoconostol phospholipid to the carboxyl tail of uPAR allows targeting and insertion into the outer cell membrane. GPI-anchored receptors have no transmembrane domain but transduce signals through soluble kinases associated with the inner membrane leaflet through N-terminal lipid modifications (22,25–28). Both tyrosine (22,27) and serine/threonine kinases (28) complex with GPI-anchored receptors to mediate their responses. Further, uPAR complexes with integrin-containing signaling complexes (22) and can regulate integrin function (29).

Endothelial cells responding to asbestos elongate and move around the fibers (14). Direct activation of endothelial cells by asbestos could be explained by the fibers mimicking ligands, such as fibronectin or...
vitronectin, which bind cell surface proteins (30,31). Boylan et al. (31) demonstrated that pleural mesothelial cells internalize vitronectin or serum-coated crocidolite asbestos fibers via an avb5 integrin-dependent mechanism. However, the affinity of vitronectin for uPAR is higher than its affinity for integrins (24). As discussed above, activation of uPAR promotes endothelial cell motility and deformability (17). Therefore, the role of uPAR in initiating cell signaling linked to morphological and phenotypical change was examined in second- to fourth-passage porcine aortic or human microvascular endothelial cells. The data obtained demonstrate that asbestos requires both GPI-anchored receptors and tyrosine phosphorylation to elicit morphological change. Chrysotile asbestos alters the profiles of proteins complexed with uPAR and activates tyrosine kinases in these complexes. Chrysotile and crocidolite asbestos, but not RCF-1, activate endothelial cell tyrosine kinases. These studies suggest that activation of uPAR-signaling complexes and tyrosine–kinase cascades provides an axis for induction of gene expression and phenotypic change in response to asbestos.

Materials and Methods

Materials. Culture media, balanced salts solutions, culture supplements, GRGDSP peptide, and trypsin were from Life Technologies (Gaithersburg, MD). Characterized fetal bovine serum (FBS) was from Hyclone Laboratories (Logan, UT). [35S]methionine (Express [35S]S, 1000 Ci/mmole) and [32P]orthophosphate (9000 Ci/mmole) were from Dupont/New England Nuclear (Boston, MA). Growth factors, protease inhibitors, and mannosamine were from Sigma (St. Louis, MO). All other reagents were of the highest purity available.

Cell Culture. Porcine endothelial cells were enzymatically isolated from fresh aortas as previously described (32). Third passage human pulmonary microvascular endothelial cells were obtained from Clonetics Corporation (San Diego, CA). Both cell types were cultured to confluence in 0.1% gelatin-coated 25 cm² flasks containing the defined endothelial cell medium (33): MCDB 131 media containing 10 ng/ml human epidermal growth factor, 1.0 µg/ml hydrocortisone, and 5% FBS. The cultures were maintained at 37°C under an atmosphere of 5% CO₂/95% air and post-confluent monolayers were used in all experiments. Greater than 95% of the cells tested positive for endothelial cell-specific markers such as antigenicity for anti-factor VIII antibody and for rapid uptake of Di-1 acetylated LDL (32). The culture medium was replaced 18 hr prior to the beginning of an experiment and fiber additions were made in the presence or absence of serum as indicated.

Fiber Samples. Reference samples of National Institute of Environmental Health Sciences chrysotile and crocidolite asbestos used in these studies were obtained from B. Mossman (University of Vermont, Burlington, VT). All fibers were greater than 10 µ. Working preparations of fibers were baked at 200°C for 12 hr to remove endotoxin or other biological contaminants. The fibers were then diluted in sterile Dulbecco’s modified Eagle’s medium (DMEM) and tested for endotoxin using the E-toxate assay (Sigma).

Metabolic Labeling of Intact Cells. Confluent cells were metabolically labeled with [32P]orthophosphate as previously described (34,35). Briefly, cells were rinsed in low phosphate MEM and then incubated in the same buffer with 0.1 mCi/ml of carrier-free [32P]orthophosphate for 1 hr at 37°C, under 5% CO₂/95% air. Asbestos was then added for up to 1 hr, and exposures were stopped by rinsing the cells twice with cold stop buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 5 mM EGTA, 100 mM NaF, 200 mM sucrose, 100 µM Na-orthovanadate, 5 mM pyrophosphate, 4 µg/ml leupeptin, 4 µg/ml soybean trypsin inhibitor, 1 mM benzamidine, 20 µM calpain inhibitor 1, 100 µM aprotinin, and 100 µM phenylmethylsulfonyl fluoride). The cells were then lyzed at room temperature in 10 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.2 M sucrose, 0.14 M NaCl, 1.0 mM orthovanadate, 2.5 mM MgCl₂, and protease inhibitors. Samples were then immediately added to uPA-coated plates as described below.

Cells in 35-mm wells were metabolically labeled with [35S]methionine. They were then rinsed with serum-free, methionine/cysteine-deficient DMEM. One milliliter of the same media containing 0.1 mCi/ml [35S]methionine was then added. After 2 hr, an additional milliliter of complete DMEM containing 10% serum was added. Eighteen hours later, cells were exposed to asbestos as described above. Samples were collected and prepared as described for those labeled with [32P]orthophosphate.

Solid-Phase Affinity Plates for uPAR. The methods described by Busso et al. (28) for uPAR signaling in human epithelial cells and by Bohuslav et al. (22) for uPAR signaling in monocytes were used with slight modification. Microtiter wells were coated for 2 hr with 50 µl of 50 µg/ml of recombinant pro-uPAR (a gift from J. Henkin, Abbott Laboratories, Abbott Park, IL) in phosphate-buffered saline (PBS). The wells were rinsed three times with 100 µl of PBS containing 1% bovine serum albumin (BSA) and then incubated with 200 µl of the same buffer for an additional 2 hr. The wells were then rinsed with cell lysis buffer and 100 µl of cell lysate was added to each well. To control for non-specific absorption, aliquots of lysates were also added to wells coated with PBS plus 1% BSA, but no pro-uPAR. The lysates were incubated overnight at 4°C with gentle agitation. They were then rinsed 3 times with 200 µl of lysis buffer and then twice with kinase buffer. The samples were then either used for in vitro kinase assays or collected for electrophoresis by addition of 50 µl of electrophoresis buffer, transfer to a fresh storage tube, and then boiling for 5 min.

Immunoprecipitations. Immunoprecipitations were performed as previously described (35). Briefly, following asbestos exposure, cells were lysed in cold RIPA buffer (35) and centrifuged to remove debris. Supernatants were incubated overnight at 4°C with specific antibody and protein A Sepharose beads (Pharmacia, Piscataway, NJ) coated with rabbit antitoxin IgG (200 µg of antibody/ml of beads). The complexes were rinsed 3 times with RIPA buffer and twice with kinase buffer. Kinase assays were performed immediately to preserve activity.

Kinase Assays. Tyrosine kinase activity of proteins purified with microtiter affinity plates or by immunoprecipitation were performed essentially as described (35). Kinase buffer (45 µl; 20 mM Hepes, pH 7.5; 5 mM MnCl₂, 5 mM MgCl₂, and 0.1 mM orthovanadate) was added to pellets of immunobeads or to microtiter wells. The kinase reactions were initiated by adding 5 µl of 32PγATP (5 µCi, 10 µM) and were incubated at 22°C for 20 min. Incubations with immunobeads were stopped by placing on ice, centrifuging at 13,000 × g and 4°C for 30 sec, and replacing the kinase buffer with 50 µl of electrophoresis buffer. All samples were boiled for 5 min and separated by polyacrylamide gel electrophoresis (PAGE). Gels were stained with Coomassie Blue to assure equal protein recoveries and autoradiographs were prepared for comparison of incorporated 32P04. These reaction conditions favor autophosphorylation and substrate phosphorylations by tyrosine kinases.
Control

Mannosamine

Herbimycin A

Gly-Arg-Gly-Asp-Ser-Pro

Figure 1. Morphological change in response to asbestos. Postconfluent endothelial cells were incubated with medium alone or medium containing inhibitors for varying periods of time before addition of 5 μg/cm² of chrysotile asbestos to half of the cultures for 3 hr. The cultures were then photographed without fixing. Mannosamine (10 mM) was added for 6 hr before asbestos exposure. Herbimycin A (1 μM) was added for 18 hr before asbestos exposure. GRGDSP peptide (100 μg/ml) was added for 1 hr before asbestos exposure. An F is placed below some of the large fibers or fiber bundles in the field. The cells in the photographs are representative of those in at least five microscopic fields.

Results and Discussion

Inhibition of GPI-Assembly or Tyrosine Kinase Activity Prevents Asbestos-induced Changes in Morphology

Nonlethal levels of asbestos, but not RCF-1 fibers, cause marked changes in the morphology and phenotype of endothelial cells (14). As shown in Figure 1B, the normal endothelial cell monolayer is disrupted by a 3-hr exposure to chrysotile asbestos as the cells move over and around the fibers. The elongation of the cells can be observed as early as 2 hr after addition of chrysotile and is sustained beyond 72 hr (the longest time point examined). No overt toxicity is observed during these prolonged exposures (14).

Re-alignment of the cells along the fibers implies that matrix contacts have been altered. Asbestos engages integrins on mesothelial cells (31) and changes in integrin signaling could mediate the motility of the endothelial cells in Figure 1. However, addition of a saturating amount of synthetic RGD-containing peptide (100 μg/ml), which is known to block integrin binding (30,31), did not prevent asbestos-induced endothelial cell elongation (Figure 1G,H). uPA and uPAR are also central to cell adhesion and motility in various cell types (18,20,24), including endothelial cells (17). In addition to binding uPA and facilitating its activation, uPAR regulates β₁-integrin function (29) and serves as a high-affinity binding site for vitronectin (24). Therefore, the role of uPAR in asbestos-induced changes in endothelial cell shape was examined in control and mannosamine-treated cells. Mannosamine prevents assembly of the GPI anchor and thereby insertion of uPAR and other GPI-anchored proteins into the plasma membrane (24,36). The data in Figure 1C,D indicate that a 6-hr mannosamine treatment causes a slight increase in vacuoles, but has little effect on endothelial cell shape. However, endothelial cells treated with mannosamine prior to addition of asbestos were resistant to asbestos-induced shape change. The fact that chrysotile fibers are still attached to these cells indicates that GPI-anchored proteins mediate morphological change in response to asbestos, but additional proteins or lipids facilitate fiber binding. This is similar to previous results demonstrating that vitronectin is required for integrin-dependent internalization of crocidolite, but not for adherence of crocidolite to cells (31).
Both integrins and GPI-anchored proteins signal through the Src family of tyrosine kinases (22,37,38). Therefore, herbimycin A, a relatively selective inhibitor of Src-related kinases (39), was used to determine whether these kinases are required for morphologic change in response to chrysotile fibers. Eighteen- to twenty-four-hour treatment with herbimycin A causes some loosening of contacts between endothelial cells (Figure 1 E,F). However, herbimycin A-treated cells did not elongate in response to asbestos. Again, the chrysotile fibers still attached to these cells. This suggests that the mechanisms for fiber binding are proximal to or independent from signals involved in morphologic change.

**Asbestos Activates uPAR Signaling Complexes**

uPAR associates with signaling complexes in specialized regions of the cell membrane (22,28). Therefore, cells were metabolically labeled with [35S]methionine to examine whether chrysotile asbestos affects the composition of proteins complexed with uPAR. After exposing the cells to chrysotile fibers, lysates were prepared and aliquots were affinity purified in microtiter wells coated with pro-uPA. Nonadherent proteins were rinsed away; adherent proteins were lysed from the wells and then separated by PAGE. Data presented in Figure 2A demonstrate that asbestos causes proteins to complex with uPAR. The mass of several proteins in the complex, especially those with apparent molecular weights of 116, 60, 42.7, and 38 kDa, increase relative to the mass of a 66-kDa protein. Synthesis of new proteins would be limited at these early time points, which suggests that asbestos mobilizes proteins in the cells.

Protein interactions or mobilization is facilitated by changes in their phosphorylation state. Cells were labeled with [32P]orthophosphate to examine the effect of asbestos on the phosphorylation state of proteins complexed with uPAR. Figure 2 shows that chrysotile increases the phosphorylation of many proteins in the complex, most notably a 36-kDa protein and proteins with molecular weights greater than 97 kDa. The effect is transient but illustrates that there are many proteins of low abundance that become hyperphosphorylated. Also, asbestos changes the stoichiometry of the phosphorylated proteins in the complex. The most pronounced examples of this are the 38- and 60-kDa proteins. The increase in the phosphorylation state of these proteins precedes significant increases in their mass in the complex (35S bands compared to 32P bands in Figure 2A). The increased phosphorylation may serve as a recognition signal for the proteins. However, other signals must be required for dissolution of the complexes, since the increased mass of the proteins in the complex is sustained longer than the increases in phosphorylation.

**Asbestos Increases Kinase Activities in uPAR Complex**

The data in Figure 2A indicate that the content of phosphoproteins in uPAR complexes is increased by asbestos. Therefore, in vitro kinase assays in the microtiter wells were used to study whether asbestos activates kinases complexed with membrane-bound uPAR. Lysates were prepared from intact cells exposed to asbestos for up to 60 min. The lysates were incubated in microtiter wells coated with pro-uPA or with the BSA-blocking solution alone. The wells were rinsed and kinase reactions were conducted in situ. The autoradiograph of the resulting phosphoproteins is shown in Figure 2B and illustrates that asbestos causes time-dependent increases in the phosphorylation of several bands. The density of bands at 212, 98, and 60 kDa reached maximal intensity by 30 min. The strong intensity of a 48-kDa band did not peak until 45 min, indicating that asbestos differentially activates multiple kinases or kinase cascades in the intact cells. The specificity of the assay is demonstrated by the lack of kinase activity in the BSA wells. The differences between the phosphorylation profiles in Figure 2A,B illustrates the limitations of the two analyses. uPAR and integrin signaling are highly dependent on the context of the intact cell and its matrix attachments. The in vitro kinase assays demonstrate that substrate phosphorylation by kinases is activated by asbestos, but do not provide much information regarding the substrates that become phosphorylated in the intact cell. Also, phosphatase activity is inhibited in the in vitro assay, which allows more phosphorylated substrates to accumulate. However, the data in Figures 1 and 2 demonstrate that adding asbestos to intact endothelial cells initiates uPAR-associated signaling cascades that precede cell elongation.

**Asbestos Increases Tyrosine Kinase Activities Associated with Src and FAK**

The data in Figure 1E,F indicate that Src-related kinases mediate asbestos-induced shape changes. Further, Src associates with FAK complexed with integrins (38). Therefore, in vitro kinase assays using endothelial cell immunoprecipitates were used to determine whether asbestos activates Src or FAK. RIPA lysates were prepared from control cells or cells exposed to equally nontoxic concentrations of chrysotile or crocidolite asbestos for 15 min. The samples were split into two aliquots and
immunoprecipitated with monoclonal antibody recognizing either to Src (clone 327, Oncogene Science, Uniondale, NY) or to FAK (clone 2A7, obtained from T. Parsons, University of Virginia, Charlottesville, VA). The immunobead/antigen complexes were assayed for kinase activity and the autoradiographs of the resulting phosphoproteins are shown in Figure 3A. Both types of asbestos induced formation of a doublet of 57- to 60-kDa phosphoproteins in the Src immunoprecipitates and of a similar band immunoprecipitating with FAK. There is no increased phosphorylation of a 120-kDa protein, which is the expected molecular weight of FAK. This indicates that auto-phosphorylation of FAK is not increased by asbestos. However, asbestos does appear to either increase substrate phosphorylation by FAK or to increase the activity of a kinase co-precipitating with FAK. Autophosphorylation of an Src-related kinase brought into association with FAK in response to asbestos would explain the appearance of the 57- to 60-kDa band. However, further experiments will be required to identify this phosphoprotein.

**Asbestos Alters the Profile of Proteins Co-precipitating with FAK**

FAK is found in large intracellular signaling complexes at sites of integrin contacts with the extracellular matrix (38). Therefore, cells were metabolically labeled with [35S]methionine and then exposed to chrysotile to determine whether asbestos alters the composition of these complexes. The autoradiograph in Figure 3B presents the profile of [35S] labeled proteins immunoprecipitated from control and asbestos-exposed cells with monoclonal antibody to FAK. Exposure to chrysotile results in a profound change in the protein profile by 15 min. There is loss of proteins in many regions of the autoradiograph. However, there is a significant gain in a 60-kDa band. This suggests that asbestos induces a dissolution of focal contacts and changes the proteins associated with FAK.

Increased phosphorylation of focal adhesion proteins disrupts cytoskeletal elements and facilitates cell elongation (40,41). Therefore, cells labeled with [32P]orthophosphate were exposed to chrysotile to determine whether asbestos increases phosphorylation of FAK-associated proteins in the intact cell. Immunoprecipitates of control and chrysotile-exposed cells demonstrate increased phosphorylation of many proteins after 30 min of exposure to the fibers, including a pronounced band at 60 kDa (Figure 3C). The increases in the 60-kDa band relative to the 116- and 50-kDa bands indicate that asbestos increases their phosphorylation state but not the distribution of ATP pools or cellular phosphate incorporation.

Endothelial cells from different organs and species are heterogeneous in their responses. Therefore, chrysotile-induced phosphorylations were examined in [32P]orthophosphate-labeled human pulmonary microvascular endothelial cells. The experimental paradigm was identical to the studies in Figure 3C, except that the lysates were split into two aliquots. The first aliquot was immunoprecipitated with mAb 2A7 and the second with a monoclonal antibody to phosphotyrosine (clone pY69, Santa Cruz

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Tyrosine phosphorylation and changes in signaling proteins complexed with FAK. (A) Lysate were prepared from control cells or cells exposed to either 10 μg/cm² of chrysotile or 5 μg/cm² of crocidolite asbestos for 15 min. The lysates were immunoprecipitated with monoclonal antibodies recognizing either Src (mAb 327) or FAK (mAb 2A7). Kinase assays were performed using these precipitates and the resulting phosphoproteins were separated by PAGE and detected by autoradiography. (B) [35S]Methionine-labeled cells were exposed to chrysotile (5 μg/cm²), lysed in the lysis buffer for uPAR extraction, and then separated by PAGE. The composite image is of the Coomassie Brilliant Blue-stained molecular weight markers and the autoradiograph of the dried gel. (C) Porcine aortic cells were labeled for 1 hr with [32P]orthophosphate before adding buffer or chrysotile (5 μg/cm²). Lysates were prepared in RIP buffer and immunoprecipitated with mAb 2A7. The proteins were separated by PAGE, stained with Coomassie Brilliant Blue, and then detected by autoradiography of dried gels. 3D: Human microvascular endothelial cells were labeled with [32P]orthophosphate as in Figure 3C and exposed to chrysotile (5 μg/cm²). RIP lysates were prepared and immunoprecipitated with either mAb 327 or pY69, which recognize phosphotyrosine. The precipitated proteins were separated by PAGE and detected by autoradiography. All experiments were performed in triplicate.
Biotechnology, Santa Cruz, CA). The proteins in the immunoprecipitates were separated on 8% polyacrylamide gels and phosphoproteins are shown in the autoradiography in Figure 3D. As observed in the porcine aortic cells, treatment of the human microvascular cells with 5 μg/cm² of chrysotile asbestos resulted in time-dependent increases in phosphorylation of 60- and 212-kDa proteins co-precipitating with FAK (Figure 3D) relative to a 116-kDa band. Increases were also observed in the pY69 immunoprecipitates, which suggests that the proteins become hyperphosphorylated on tyrosine residues.

**Conclusion**

These data support the hypothesis that sublethal amounts of asbestos initiate motility changes through activation of uPAR- and integrin-associated signaling cascades. Elongation of the cells in response to chrysotile asbestos is greatly reduced by preventing insertion of uPAR into the plasma membrane or by inhibiting tyrosine kinase signaling cascades. Asbestos increases the association of proteins with uPAR signaling complexes. However, at the same time, asbestos causes dissolution of proteins associated with FAK. Asbestos activates p60 Src and perhaps other Src-related kinases, which may mediate the changes in uPAR and FAK protein complexes. More investigations will be required for the identification of the proteins that are altered in response to asbestos and the relationships of these alterations to facilitating endothelial cell activation and motility in response to asbestos.

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