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Vitronectin adsorption to chrysotile asbestos increases fiber phagocytosis and toxicity for mesothelial cells

J. WU,¹ W. LIU,¹ K. KOENIG,² S. IDELL,² AND V. C. BROADDUS¹

¹Lung Biology Center, San Francisco General Hospital, University of California, San Francisco, California 94143-0854; and ²The University of Texas Health Center at Tyler, Tyler, Texas 75708

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Vitronectin adsorption to chrysotile asbestos increases fiber phagocytosis and toxicity for mesothelial cells. *Am J Physiol Lung Cell Mol Physiol* 279: L916–L923, 2000.—Biological modification of asbestos fibers can alter their interaction with target cells. We have shown that vitronectin (VN), a major adhesive protein in serum, adsorbs to crocidolite asbestos and increases fiber phagocytosis by mesothelial cells via integrins. Because chrysotile asbestos differs significantly from crocidolite in charge and shape, we asked whether VN would also adsorb to chrysotile asbestos and increase its toxicity for mesothelial cells. We found that VN, either from purified solutions or from serum, adsorbed to chrysotile but at a lower amount per surface area than to crocidolite. Nevertheless, VN coating increased the phagocytosis of chrysotile as well as of crocidolite asbestos. VN coating of both chrysotile and crocidolite, but not of glass beads, increased intracellular oxidation and apoptosis of mesothelial cells. The additional apoptosis could be blocked by integrin-ligand blockade with arginine-glycine-aspartic acid peptides, confirming a role for integrins in the fiber-induced toxicity. We conclude that VN increases the phagocytosis of chrysotile as well as of crocidolite asbestos and that phagocytosis is important in fiber-induced toxicity for mesothelial cells.

crocidolite; apoptosis; integrin; arginine-glycine-aspartic acid peptides; dichlorofluorescein assay

ASBESTOS FIBERS ARE CONSIDERED to be a complete carcinogen for the formation of the mesothelium-derived tumor mesothelioma. The interaction of asbestos with mesothelial cells that leads to cancer is still unknown; however, it appears that fiber phagocytosis by the target cell may be an important step. Phagocytosis brings the long thin asbestos fiber in close contact with the nucleus, probably enhancing the toxic effect of reactive oxygen species (ROS) on the DNA and allowing the fiber to damage chromosomes during mitosis. In an animal study (34), the long thin shape of asbestos was found to be critical to its carcinogenicity, suggesting that mechanical effects within cells might be of crucial importance. In *in vitro* studies (18, 19, 35, 36) of asbestos, phagocytosis of fibers has been correlated with toxic effects in certain cell types. Determining the role of phagocytosis in asbestos-induced injury to me-

sothelial cells may hold clues to the important mechanisms of asbestos toxicity.

Biological modification of asbestos fibers may increase fiber phagocytosis. In a previous study, Boylan et al. (3) exposed crocidolite asbestos fibers to serum, pleural liquid, bronchoalveolar fluid, or purified vitronectin (VN) and showed that the fibers became coated with VN and were more readily phagocytosed by mesothelial cells. This phagocytosis was mediated via integrins capable of recognizing VN, the major adhesive protein in serum. Liu et al. (26) have recently shown that phagocytosis of crocidolite asbestos is important for mesothelial cell toxicity. Therefore, biological modification via VN adsorption may increase phagocytosis and possibly enhance cytotoxicity of the fibers *in vivo*.

Chrysotile shares many toxic effects with crocidolite, although the fibers have striking differences, including that of surface charge. Chrysotile is positively charged in physiological solutions, whereas crocidolite is negatively charged (25). The positive charge on chrysotile is known to account for one major difference with crocidolite; the hemolysis induced by chrysotile is a result of the interaction of the positive charge with sialic acid moieties on the surface of the erythrocyte (6). The differences in charge between the two fibers may also account for differences in protein adsorption and thus in the biological modification of each fiber in the body (11). VN, which contains a heparin-binding region, appears to bind preferentially to negatively charged materials (1) and might not be expected to adsorb as readily to chrysotile as to crocidolite. Although chrysotile fibers have been shown to be taken up by mesothelial and other cells as early as 15 min after exposure (22), the mechanism of uptake of this fiber has not been explored. The differences in surface charge between crocidolite and chrysotile may herald different mechanisms of entry of these fibers into cells.

Therefore, we asked whether chrysotile would adsorb VN and, if so, whether that adsorption would alter phagocytosis of chrysotile by mesothelial cells. To measure phagocytosis of chrysotile, a fiber too thin and variably shaped to be recognized by our other assays,

Address for reprint requests and other correspondence: V. C. Broaddus, Lung Biology Center, Box 0854, Univ. of California, San Francisco, CA 94143-0854 (E-mail: sfcourt@itsa.ucsf.edu).

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we developed a novel assay using radiolabeled VN and albumin. Finally, we asked whether adsorption of VN onto chrysotile would also alter its toxicity for mesothelial cells.

METHODS

Reagents and proteins. National Institute of Environmental Health and Safety (NIEHS) asbestos fibers were used for all experiments (7). Union Internationale Contre le Cancer (UICC) asbestos fibers were obtained from Dr. Marie-Claude Jaurand (Institut National de la Santé et de la Recherche Médicale, Paris, France) (37) and used together with the NIEHS asbestos fibers in experiments of protein adsorption and phagocytosis. Asbestos fibers were stored in 1-ml aliquots of 100 μg in PBS at -20°C and were used within 1 mo of preparation. NIEHS crocidolite had a mean length of 10 μm and a mean width of 0.3 μm ; NIEHS chrysotile had a mean length of 7 μm and a mean width of 0.2 μm (7). UICC crocidolite had a mean length of $2.1 \pm 3.6 \mu\text{m}$ and a mean diameter of $0.2 \pm 0.1 \mu\text{m}$, and UICC chrysotile had a mean length of $1.7 \pm 2.2 \mu\text{m}$ and a mean diameter of $0.05 \pm 0.04 \mu\text{m}$ (41). In some experiments, NIEHS chrysotile fibers were rigorously sonicated to shorten mean fiber length (100 W for 10 min; Branson Ultrasonics, Danbury, CT) as confirmed by examination with dark-field microscopy. Glass beads (mean diameter $1.6 \pm 0.3 \mu\text{m}$; Duke Scientific, Palo Alto, CA) were used as control particles.

Purified proteins included VN, purified as described (3) with the technique of Yatohgo et al. (40), and BSA (fraction V; Sigma, St. Louis, MO) that was confirmed to be VN free by immunoblot (3). Human serum was freshly prepared. Radiolabeled proteins included ^{125}I -BSA (ICN Pharmaceuticals) and ^{125}I -VN, iodinated by the IODO-GEN method (14). IODO-GEN (Pierce, Rockford, IL) was prepared so that 10 μg of IODO-GEN were dried in a $10 \times 75\text{-mm}$ glass tube. VN (50 μg) was added to the tubes with 5 μl of 20 mM HEPES buffer (pH 7.45, 150 mM NaCl and 0.5 mCi of Na^{125}I) to a final total volume of 100 μl . This was allowed to react on ice for 5 min. Then 25 μl of 1% KI in water were added to the tube to stop the reaction, and 25 μl of 1% ovalbumin in water were added. Free iodine was separated from the ^{125}I -labeled VN by gel filtration in a 12-ml column of Sephadex G-25 equilibrated with HEPES-buffered saline containing 1% BSA.

GRGDSP, the control GRGESP peptides, trypsin-EDTA, and mouse laminin were obtained from GIBCO BRL (Life Technologies, Gaithersburg, MD). Propidium iodide was obtained from Sigma.

SDS-PAGE and Western blot analysis of eluted proteins from serum-coated fibers. Asbestos fibers (750 μg) were incubated with undiluted human serum (100 μl ; ~ 6.0 mg of serum protein) for 1 h in clean Eppendorf tubes. After three washes with PBS, the asbestos fibers were sonicated (100 W for 8 s; Branson Ultrasonics) to disperse the fibers. After sonication, the fiber solutions were transferred to clean Eppendorf tubes and spun at 14,000 rpm for 10 min. Approximately 900 μl of supernatant were discarded, 5 $\times$ Laemmli sample buffer (20 μl) and β -mercaptoethanol (2 μl) were added, and samples were boiled at 100°C for 10 min to elute the proteins bound to the fibers. Eluted protein ($\sim 120 \mu\text{l}$) was analyzed by SDS-PAGE and Western blot analysis.

SDS-PAGE. Eluted proteins at equal volumes of eluate were analyzed by electrophoresis. Eluted proteins ($\sim 60 \mu\text{l}$) were loaded onto 10% SDS-polyacrylamide gels and run at 100 V in the stacking gel and 150–200 V in the separating gel for 3 h. Gels were stained with Coomassie blue.

Western blotting. Proteins from the SDS-polyacrylamide gel were transferred onto Immobilon-P transfer membrane (Millipore, Bedford, MA). Powdered milk [5% in Tris-buffered saline (TBS)] was used as the blocking solution throughout. The membrane was blocked for 1 h and washed with TBS four times. The membrane was incubated with the primary antibody mouse anti-human VN (Chemicon International, Temecula, CA), at 1:500 in blocking solution for 1 h. After a wash with TBS, the membrane was treated with the secondary antibody (goat anti-mouse Ig conjugated to horseradish peroxidase; Amersham Life Science, Piscataway, NJ) at 1:2,000 for 1 h and washed again with TBS. Proteins were detected with chemiluminescence (Amersham).

Coating of fibers. Fibers were coated with purified, radiolabeled BSA or VN at 10 $\mu\text{g}/\text{ml}$, similar to the concentrations of VN in lung lining fluid (29). The incubation time of 1 h was sufficient for maximal adsorption (11), as we confirmed. The following method is a general description of fiber coating with purified proteins. Eppendorf tubes were coated with 1 ml of 1% BSA for 1 h to minimize adsorption of radiolabeled protein to the tubes. In the BSA-coated tubes, 100 μg of asbestos fibers in 100 μl of PBS were incubated with 1 μg of radiolabeled protein (VN or BSA) for 1 h on a vortex at room temperature (RT). The fibers were then washed three times by adding 900 μl of PBS, centrifuging at 14,000 rpm for 10 min, aspirating 900 μl , and then adding 900 μl of fresh PBS. After removal of the supernatant from the third wash, the ^{125}I -protein-asbestos solution was transferred into a new set of Eppendorf tubes to minimize the presence of unbound proteins. Fresh $1\times$ PBS was added, bringing the final volume to 1 ml. All washes were saved for radioactivity determination to assess recovery of total radioactivity. Washes were spun at 14,000 rpm for 10 min and found to contain no asbestos fibers. The radioactivity of fibers plus wash was always at least 90% of the radioactivity of a comparable amount of radiolabeled protein spiked in a separate Eppendorf tube. The radioactivity of fibers and the wash was counted in a gamma counter to calculate the percentage of the total protein adsorbed by the fibers. Fibers were then used in the phagocytosis assays.

Cells and culture. Rabbit mesothelial cells were harvested as described (3) and maintained in standard medium: RPMI 1640 medium and DMEM (1:1), 10% fetal bovine serum (GIBCO BRL), 2 mM L-glutamine (GIBCO BRL), 100 U/ml of penicillin, and 100 $\mu\text{g}/\text{ml}$ of streptomycin. Cells between passages 3 and 7 were used in all studies. The experimental medium was the same as that described above but was serum free to avoid additional protein adsorption.

Fiber phagocytosis protocol. The cells were incubated with the radiolabeled VN-coated or BSA-coated fibers at two different temperatures for 4 h and then washed free of nonadherent fibers. At 4°C , with phagocytosis blocked, the association of fibers with the cell monolayer indicated adherence of fibers. At 37°C , with normal phagocytosis, fiber association with the cell monolayer indicated both adherence and phagocytosis.

Eight-well chamber tissue culture slides (Nunc International, Naperville, IL) were coated with mouse laminin (200 μl , 10 $\mu\text{g}/\text{ml}$) for 1 h at 37°C , washed with PBS, and plated with 25,000 cells/well the night before the experiment, with a goal of 90% confluence.

On the day of the experiment, cells were washed once with PBS and incubated with 250 μl of serum-free medium. The slides were placed at 37°C or 4°C for 15 min and tritured, radiolabeled VN- or BSA-coated fibers equilibrated at either 37°C or 4°C were added at $7.5 \mu\text{g}/\text{cm}^2$. Cells and fibers were incubated at either 37°C or 4°C for 4 h. The cells were gently

washed to remove nonadherent fibers. Cell counts showed that at 4°C, no cells were lost with washing, whereas at 37°C, ~10% of the cells were lost with washing. Fortunately, the cell loss was equal whether cells were exposed to VN-coated or BSA-coated fibers, allowing comparison between them at 37°C. Adsorbed protein remained tightly adherent to the fibers during the time of the assay; when VN- or BSA-coated fibers were incubated without cells for 4 h at either 37°C or 4°C, <1% of adsorbed protein could be found in the supernatant.

All wash fluids were saved for counting of nonadherent fibers. Adherent cells were then detached with trypsin (0.25% wt/vol) and EDTA (0.5 mM) and saved for counting of adherent plus internalized fibers. Radioactivity was detected with the Beckman gamma 5500 counting system (Beckman Instruments, Fullerton, CA).

The percentage of fibers associated with cells was calculated as [radioactivity of adherent + internalized fibers (e.g., cells after washing) × 100]/total radioactivity (e.g., cells + wash).

The recovery of radioactivity was determined by comparing the total recovered experimental counts with the total counts of standard vials to which identical amounts of protein-coated fibers had been added. The total recovery was ≥90%.

Dichlorofluorescein assay of intracellular oxidation. To determine whether VN coating of chrysotile fibers increased intracellular oxidation, cells exposed to protein-coated fibers or glass beads were incubated with an oxidation-sensitive fluorescent probe, 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR). Intracellular deacylation of DCFH-DA results in the formation of the nonfluorescent 2',7'-dichlorofluorescein (DCFH); with oxidative stress, DCFH is oxidized to the fluorescent dichlorofluorescein (DCF) by a variety of ROS and reactive nitrogen species (10) and is sensitive to a general level of oxidative stress (2). DCFH-DA, divided into aliquots in DMSO from a stock solution of 5 M and stored in a desiccator in the dark at -20°C, was diluted in PBS immediately before the experiment. After their exposure to BSA- or VN-coated fibers or glass beads (5 µg/cm²) for 4 h, the mesothelial cells were detached with trypsin-EDTA, which was then neutralized, combined with the floating cells, and incubated with DCFH-DA (5 µM) for 1 h before and continuously during flow cytometric analysis. Propidium iodide (15 µg/ml) was added before flow cytometric analysis to allow exclusion of cells that were permeable and thus would not retain the fluorescent probe. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA), and acquisition and data analysis were performed with the use of CELLQuest software (Becton Dickinson). At least 5,000 cells were analyzed for adequate statistical collection. Intracellular oxidative shift was measured as the percentage of cells with fluorescence greater than that of 95% of the control, unexposed cells. Finally, maximal cell fluorescence was measured after excess H₂O₂ was added to confirm equivalent loading of DCF. Maximal fluorescence exceeded 85% in all cases.

Annexin V assay for apoptosis. Apoptosis was measured by the binding of green fluorescent protein (GFP)-annexin V to the phosphatidylserine residues on the outer leaflet of the apoptotic cellular membrane with a GFP-annexin V fusion protein constructed as described by Ernest et al. (13). After exposure to BSA- or VN-coated fibers or glass beads (5 µg/cm²) for 18 h, mesothelial cells were collected and centrifuged (1,000 rpm for 10 min). In some experiments, arginine-glycine-aspartic acid (RGD)- or arginine-glycine-glutamic acid (RGE)-containing peptides (0.5 mg/ml) were added to the

cells 1 h before the fibers. The cell pellet was resuspended in serum-free RPMI 1640 medium-DMEM buffer and stained with GFP-annexin V fusion protein (3 µg/ml in HEPES buffer) for 10 min on ice. Propidium iodide (15 µg/ml) was added just before analysis by flow cytometry. Early apoptotic cells, i.e., those with positive staining for annexin V but negative staining for propidium iodide, were measured. Cells were analyzed with the FACScan flow cytometer, with acquisition and data analysis as described above. Five thousand events per sample were acquired to ensure adequate mean data.

Statistics. Data were analyzed for significance with Super-ANOVA (Abacus Concepts, Berkeley, CA) with ANOVA with Tukey's test. Data are means ± SD unless otherwise noted. A difference was regarded as significant if $P < 0.05$.

RESULTS

SDS-PAGE and Western blot analysis of eluted proteins from fibers. Elution of serum proteins adsorbed to asbestos fibers showed that, in general, a wide range of serum proteins adsorbed to both fibers. Consistently, a larger amount of protein adsorbed to chrysotile fibers than to crocidolite fibers ($n = 4$ experiments; Fig. 1). The similarity in elution pattern for both crocidolite and chrysotile fibers suggested that the larger surface area of chrysotile was an important factor in its greater protein binding ability. Of the eluted proteins from both fibers, the most intense band(s) comigrated with purified VN and/or albumin (Fig. 1, lanes 2 and 3).

By Western blotting of the eluted serum proteins, VN was confirmed to adsorb to both fibers from serum (Fig. 2).

Adsorption of purified proteins to fibers. To quantitate adsorption of proteins to fibers, radiolabeled purified VN or BSA (1 µg) was incubated with fibers (100 µg), and the percentage of adsorption was quantitated. Purified VN adsorbed to both fibers. For crocidolite, significantly more VN than BSA bound to the fibers ($P < 0.001$; Fig. 3). No preferential adsorption of VN compared with BSA was found for chrysotile. The sim-

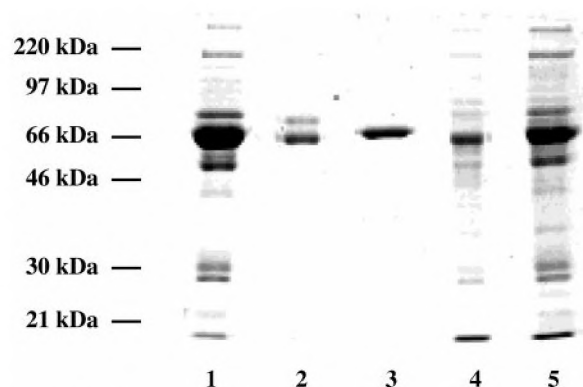


Fig. 1. SDS-PAGE analysis of serum proteins eluted from crocidolite and chrysotile fibers from the National Institute of Environmental Health Sciences (NIEHS). Lanes 1–3, human serum (25 µg), 5 µg of purified vitronectin (VN), and 5 µg of purified BSA, respectively. Lanes 4 and 5, proteins eluted from crocidolite and chrysotile fibers (750 µg), respectively, incubated for 1 h with human serum. In general, darker bands can be observed in lane 5 compared with those in lane 4. Nos. at left, molecular mass.

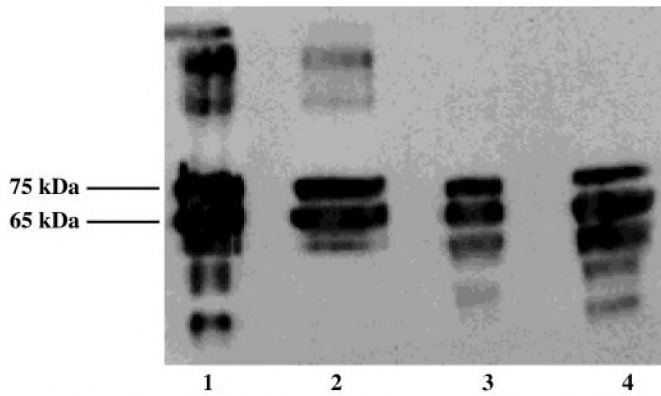


Fig. 2. Western blot of VN in serum proteins eluted from crocidolite and chrysotile fibers (NIEHS). *Lanes 1 and 2*, 5 and 1 µg of purified VN, respectively. *Lanes 3 and 4*, VN eluted from crocidolite and chrysotile fibers (750 µg), respectively, incubated with human serum. The 75- and 65-kDa bands are the major bands of VN. Bands < 65 kDa may represent fragments; bands > 75 kDa likely represent multimers.

ilar binding of VN and BSA to chrysotile suggested that the adsorption was nonspecific and that differences in total binding were because of the greater surface area of chrysotile. Indeed, when published values of the surface area of each fiber were used to calculate the specific adsorption, it was shown that chrysotile adsorbed less VN per surface area than crocidolite (Table 1). The differences in VN binding to the fibers led us to examine whether VN binding altered fiber phagocytosis and toxicity for mesothelial cells.

Fiber adherence and phagocytosis. When fibers were incubated with mesothelial cells for 4 h at 4°C to inhibit phagocytosis, BSA- and VN-coated fibers had a similar adherence to the cells (Fig. 4A). At 37°C, how-

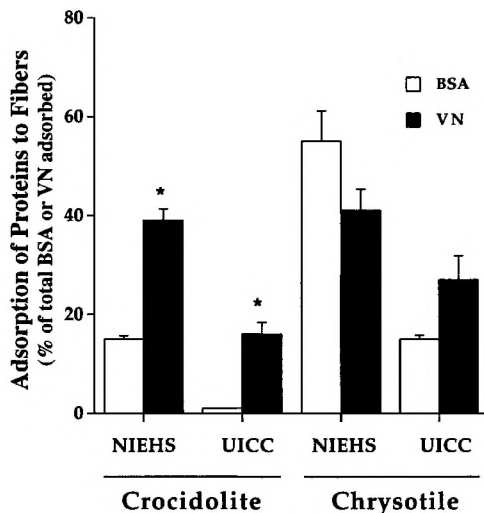


Fig. 3. Percent of adsorption of radiolabeled BSA and VN to crocidolite and chrysotile fibers. Fibers were incubated with purified, radiolabeled BSA or VN (1 µg) for 1 h and washed thoroughly. UICC, Union Internationale Contre le Cancer. Values are means $\pm$ SD; $n = 3$ experiments. VN adsorption was greater than that of BSA to crocidolite but not to chrysotile. *Significantly different from fibers with BSA adsorption, $P < 0.01$.

Table 1. Surface area and relative adsorption of vitronectin per surface area of crocidolite and chrysotile asbestos

	Vitronectin Adsorbed, µg protein/100 µg fiber	Surface Area, m ² /g	Specific Vitronectin Adsorption, µg protein/m ² fiber
Crocidolite (NIEHS)	0.39 $\pm$ 0.05	9.8	398 $\pm$ 51
Crocidolite (UICC)	0.16 $\pm$ 0.04	8.3	193 $\pm$ 53
Chrysotile (NIEHS)	0.41 $\pm$ 0.09	54.2	76 $\pm$ 16*
Chrysotile (UICC)	0.27 $\pm$ 0.08	21.3	128 $\pm$ 40*

Values are means $\pm$ SD of 3 experiments. Vitronectin adsorption was determined by amount of radiolabeled vitronectin (1 µg) adsorbed by 100 µg of fibers over 1 h. Surface area data are means of surface area determined by nitrogen adsorption for Union Internationale Contre le Cancer (UICC; 37) and National Institute of Environmental Health Sciences (NIEHS; 7). *Significant difference between specific vitronectin adsorption by chrysotile and crocidolite ($P < 0.05$).

ever, VN-coated fibers displayed a significantly greater association with the cells than BSA-coated fibers for both chrysotile and crocidolite (Fig. 4B). Because fiber cell association is due to adherence plus phagocytosis, increases in fiber cell association at 37°C that were not seen at 4°C were interpreted to be a result of fiber phagocytosis. By this assay, then, VN was shown to enhance phagocytosis of both chrysotile and crocidolite

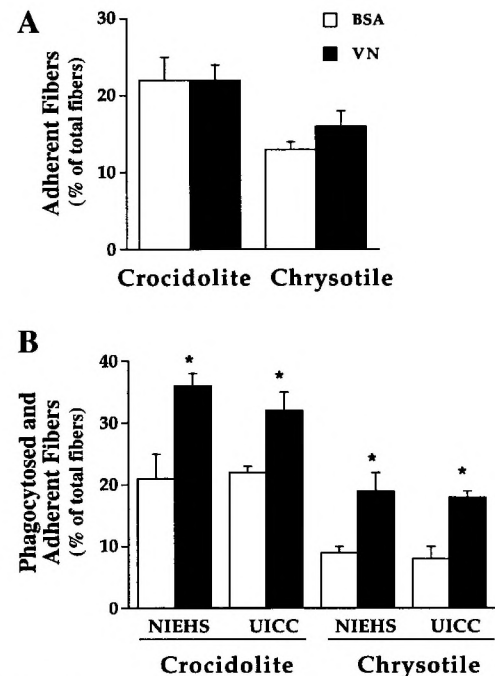


Fig. 4. Effect of protein adsorption on the percentage of fibers associated with rabbit pleural mesothelial cells. Radiolabeled BSA- or VN-coated fibers (7.5 µg/cm²) were incubated with rabbit mesothelial cells for 4 h either at 4°C to prevent phagocytosis (A) or at 37°C to allow phagocytosis (B). After cells were washed, the radioactivity associated with the cells indicated fibers that were either adherent only (A) or adherent plus phagocytosed (B). Values are means $\pm$ SD; $n = 10$ experiments. *Significantly different from fibers with BSA adsorption, $P < 0.01$.

fibers, as has been previously shown (3) only for crocidolite.

Intracellular oxidation. Crocidolite and chrysotile fibers (NIEHS) both induced an increase in intracellular oxidation as measured by a shift in fluorescence of the intracellular probe DCF (Fig. 5). Compared with BSA coating, VN coating of fibers led to greater intracellular oxidation for crocidolite but not for the standard chrysotile. VN coating of chrysotile did increase oxidation when the chrysotile was sonicated to reduce mean length, suggesting that the shape or length of the standard chrysotile fiber interfered with cell interactions in this assay. Glass beads were also phagocytosed by cells (VN coated, $80 \pm 6\%$ cells with >4 intracellular beads; BSA coated, $71 \pm 11\%$ with >4 intracellular beads; $P > 0.05$). However, VN coating of glass beads did not affect intracellular oxidation, suggesting that the process of phagocytosis alone did not contribute to intracellular oxidation.

Apoptosis. VN-coated fibers induced more apoptosis than BSA-coated fibers for both crocidolite and chrysotile (NIEHS) in mesothelial cells (Fig. 6). BSA-coated asbestos induced the same amount of apoptosis as uncoated asbestos (chrysotile: $20 \pm 7\%$ BSA-coated, $18 \pm 8\%$ uncoated, $n = 4$; crocidolite: $26 \pm 5\%$ BSA-coated, $24 \pm 7\%$ uncoated, $n = 4$ experiments). VN itself was shown to have no effect because VN-coated glass beads did not induce apoptosis. RGD peptides, but not control RGE peptides, reduced the apoptosis caused by VN-coated chrysotile fibers (Fig. 7). RGD

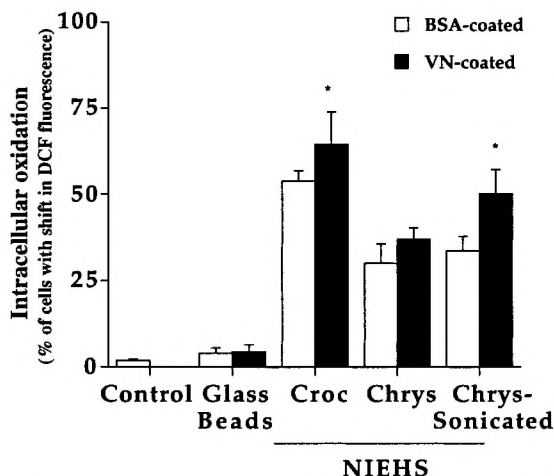


Fig. 5. Intracellular oxidation in primary mesothelial cells induced by BSA- or VN-coated asbestos fibers. After a 4-h exposure to protein-coated fibers or glass beads ($5 \mu\text{g}/\text{cm}^2$), cells were collected and incubated with 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate ($5 \mu\text{M}$) for 1 h before and continuously during flow cytometric analysis. Intracellular oxidation was measured as the percentage of cells with a fluorescence $> 95\%$ of the control, unexposed cells in each experiment. DCF, dichlorofluorescein. Values are means $\pm$ SD; $n = 3$ experiments. Compared with that for BSA-coated fibers, greater intracellular oxidation was observed for cells exposed to VN-coated crocidolite (Croc) and chrysotile (Chrys) fibers sonicated to reduce mean length. A significant difference was not observed for standard chrysotile fibers. No increase in intracellular oxidation was detected by VN-coated glass beads despite an observed increase in phagocytosis. * Significantly different from fibers with BSA adsorption, $P < 0.05$.

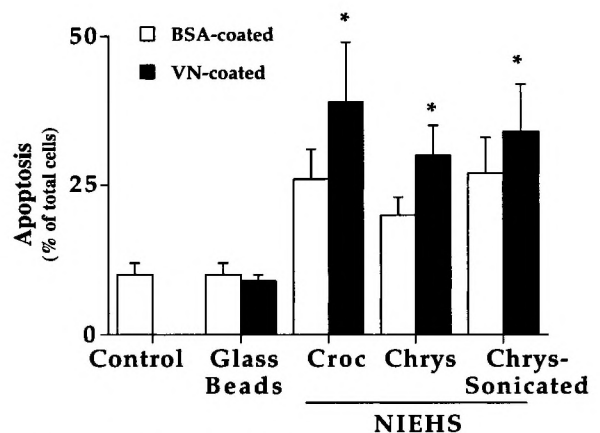


Fig. 6. The effect of BSA- or VN-coated asbestos fibers on apoptosis of rabbit mesothelial cells. Mesothelial cells were exposed to BSA- or VN-coated fibers or glass beads ($5 \mu\text{g}/\text{cm}^2$) for 18 h, detached, combined with floating cells, and stained with green fluorescent protein-annexin V ($3 \mu\text{g}/\text{ml}$) and propidium iodide ($15 \mu\text{g}/\text{ml}$). Values are means $\pm$ SD; $n = 4$ experiments. Apoptotic cells were early apoptotic, i.e., those staining positively for annexin V and negatively for propidium iodide. In all instances, cells incubated with VN-coated asbestos fibers exhibited more apoptosis than cells incubated with BSA-coated asbestos fibers. Apoptosis resulting from uncoated asbestos was no different from that resulting from uncoated asbestos (see RESULTS). * Significantly different from fibers with BSA adsorption, $P < 0.01$.

peptides had no effect on BSA-coated chrysotile fibers, indicating that the effect of RGD was VN dependent.

DISCUSSION

In this study, we have shown that despite differences in the two fiber types, VN adsorbs to both chrysotile

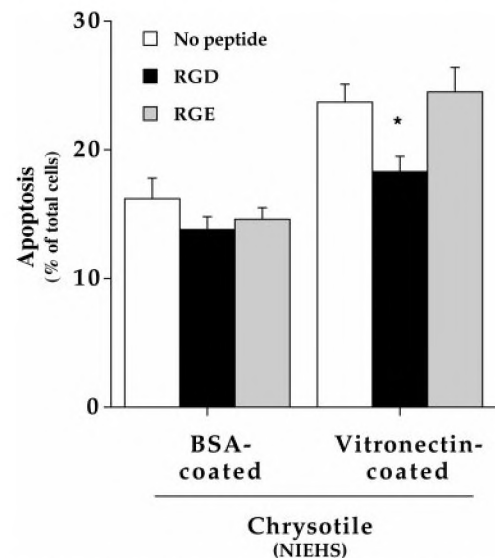


Fig. 7. The effect of arginine-glycine-aspartic acid (RGD)-containing peptides on the ability of BSA- and VN-coated chrysotile fibers to induce apoptosis in rabbit mesothelial cells. Cells were treated with RGD or control arginine-glycine-glutamic acid (RGE) peptides ($0.5 \text{ mg}/\text{ml}$) 1 h before the addition of fibers. Values are means $\pm$ SD; $n = 3$ experiments. RGD but not RGE peptides decreased apoptosis for primary mesothelial cells treated with VN-coated chrysotile fibers. RGD peptide had no effect on apoptosis of cells incubated with BSA-coated chrysotile fibers. * Significantly different from no peptide, $P < 0.05$.

and crocidolite asbestos. Although VN shows a lower specific adsorption to chrysotile than to crocidolite, the VN coating increases phagocytosis of both fiber types. VN coating also increases the cellular toxicity of chrysotile as well as crocidolite fibers, presumably by increasing fiber phagocytosis.

We studied VN specifically because it is the major adhesive protein of serum and of other biological liquids and was found in an earlier study (3) to adsorb to crocidolite and enhance its phagocytosis by mesothelial cells. VN, which contains a heparin-binding region, avidly adsorbs to glass and other negatively charged surfaces (1, 17). The binding of native VN leads to a change in its conformation and to exposure of other portions of the molecule, including an RGD site that can interact with several different integrins (30). Because of its avid adsorption to negatively charged surfaces, VN was expected to adsorb to crocidolite, a negatively charged fiber, but was not necessarily expected to adsorb to chrysotile, a positively charged fiber.

Despite the positive charge of chrysotile, we found that VN did adsorb to chrysotile both from purified VN solutions and from serum. The total adsorption of VN to chrysotile appeared equal or greater than its adsorption to crocidolite, but because of the greater surface area of chrysotile, the specific adsorption of VN (adsorption per surface area) was actually less for chrysotile than for crocidolite. In addition, VN showed no preferential adsorption to chrysotile as was the case with crocidolite. Preferential adsorption of VN to copolymers and other negatively charged materials, in which VN competes successfully with other more abundant proteins in plasma to become the major plasma protein adsorbed, has been described (1). Thus compared with crocidolite, the lower specific adsorption and the lack of preferential binding of VN to chrysotile suggested a different and perhaps reduced biological effect of VN. In addition, a reduced effect of VN for chrysotile could be expected if most of the VN adsorbed within the rolled scroll structure of the chrysotile, making it unavailable to interact with cells, or, because proteins may bind in different orientations (21, 38), if the VN was oriented without its RGD site exposed. Thus we pursued studies examining the effect of VN on chrysotile fiber phagocytosis and fiber toxicity.

Fiber phagocytosis is difficult to detect, to discriminate from fiber adherence, and to quantitate, especially in thin mesothelial cells ($<1\ \mu\text{m}$ over the cytoplasmic area) (39). Transmission electron microscopy can be used to confirm intracellular locations of fibers, but it is unwieldy to use for quantitation. For crocidolite fibers, Boylan et al. (3) previously developed two assays for quantitation of internal fibers: a confocal microscopy technique that used a membrane-specific fluoroprobe, 1,1'-dioctadecyl-1,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), to show whether a fiber was internal (coated with a fluorescent membrane) or external (not coated) and a simpler trypsin technique for removing external fibers, allowing the cell-associated internal fibers to be counted by dark-field microscopy. Neither of these techniques could be used for chrysotile, a

thinner, curly, variably shaped fiber that is difficult to see by fluorescent confocal or dark-field microscopy. For this fiber, we tried a new approach that involved comparing the association of the VN- or BSA-coated fibers with mesothelial cells kept at 4°C to prevent phagocytosis and at 37°C to allow phagocytosis. At 4°C , a temperature at which cellular uptake of fibers is blocked, there was no difference in adherence of the VN- and BSA-coated crocidolite or chrysotile. This was expected because Boylan et al. (3) have previously shown that adherence either at 4°C or at 37°C is the same for VN- or BSA-coated crocidolite fibers. Then, at 37°C , a greater association of VN-coated fibers than of BSA-coated fibers with cells was interpreted as being a result of a greater phagocytosis of the VN-coated fibers. Because this approach confirmed the previous findings, which used other assays with crocidolite, we concluded that it was valid for chrysotile. In this way, we could conclude that VN coating increased phagocytosis of chrysotile as well as of crocidolite fibers.

The major limitation of this assay of fiber phagocytosis was that it could be affected by cell loss as a result of washing. After the washing steps, we found that some cells were lost in the 37°C wells ($\sim 10\%$), whereas none were lost in the 4°C wells. There were no differences in cell loss between wells exposed to VN- and BSA-coated asbestos, permitting the comparison between those conditions. In fact, we are more confident of our findings because a greater cell loss from wells exposed to the VN-coated asbestos would have decreased radioactivity from those wells making it more difficult to detect increased phagocytosis of the VN-coated fibers. A related problem was the inability to test RGD peptides or cytochalasin because these interventions increased cell losses in the washing steps. Nonetheless, this assay was able to show an increase in phagocytosis of crocidolite asbestos as a result of VN coating, an effect that mirrored previous findings (3). Using this same assay, we conclude that, for chrysotile asbestos, the VN coating had a similar effect.

Although it is known that both fibers can be phagocytosed by mesothelial cells (3, 22), the role of phagocytosis in cellular toxicity by asbestos has been unclear. Asbestos fibers can cause damage to the cell surface; chrysotile in particular can induce hemolysis by interacting with the cell membrane (6). Asbestos fibers may also cause damage intracellularly by interacting with chromosomes or producing ROS in proximity to the DNA. The role of phagocytosis in fiber toxicity has been especially difficult to establish for mesothelial cells. Certain studies (19, 35, 36) that used other cell types suggested an important role for fiber uptake in DNA or chromosomal damage or transformation frequency. In a recent study, Liu et al. (26) have shown that phagocytosis of crocidolite asbestos is necessary for intracellular oxidation and apoptosis. Here, we confirm that study and show that the increase in phagocytosis of chrysotile is also associated with increased intracellular oxidation and increased apoptosis of mesothelial cells. The process of phagocytosis itself or the interaction with VN receptors did not contribute to the

biological effects because phagocytosis of glass beads (either BSA or VN coated) had no effect. The role of phagocytosis was particularly evident in the apoptosis studies in which VN coating led to more apoptosis by crocidolite and chrysotile, both sonicated and unsonicated. The increased apoptosis could be blocked by RGD-containing peptides, known to block VN-dependent phagocytosis without altering fiber adherence (3). There were similar findings in the oxidation assay; in this assay, however, the VN coating increased intracellular oxidation of crocidolite and the shorter sonicated chrysotile but not of the longer unsonicated chrysotile fibers. The difference in results for the longer chrysotile asbestos may arise because in the apoptosis studies, cells and fibers remained undisturbed during the entire assay, whereas in the oxidation study, cells were harvested before incubation with the fluoroprobe, a step that may have dislodged some of the longer fibers. Longer fibers may also take more time to phagocytose, allowing their toxicity to be demonstrated in the 18-h apoptosis assay but not in the 4-h oxidation assay.

Although asbestos has been suspected of generating intracellular ROS, the evidence for intracellular oxidation has been mostly indirect, such as the increase in cellular antioxidant enzymes, protection by antioxidant enzymes, or detection of oxidized bases (8, 12, 15, 20). Attempts to measure ROS directly have failed to confirm increases due to asbestos (16, 24, 28), although asbestos-induced increases in reactive nitrogen species have been detected (9). Here, in using the DCF fluoroprobe, which can detect both reactive oxygen and nitrogen species (10), we have been able to confirm that both fibers can increase intracellular oxidation.

The VN coating on both fibers increased asbestos-induced apoptosis of mesothelial cells. The increased apoptosis was likely a result of the increased intracellular oxidation because Broaddus et al. (5) have previously shown that asbestos-induced apoptosis of mesothelial cells is mediated by ROS. Ultimately, we attribute the effect of VN on apoptosis to an increased phagocytosis of fibers because the cells were otherwise exposed to the same numbers of VN- and BSA-coated fibers during the assay. The fibers that settled on the cells at $5 \mu\text{g}/\text{cm}^2$ would have been distributed as external, both adherent and nonadherent, and as internal. BSA-coated fibers were phagocytosed at a basal rate similar to that of uncoated fibers. VN coating thus served to shift more of the asbestos fibers from the outside to the inside of the cell, where the fibers were able to induce greater damage. RGD peptides, by blocking the phagocytosis and not the adherence of fibers, therefore acted to block the shift of fibers from the outside to the inside of cells. Indeed, the effect of the RGD peptides was specific for VN-induced effects and did not alter BSA-coated fiber-induced apoptosis. Thus the role of integrin-dependent phagocytosis and its role in enhancing fiber-induced apoptosis are shown for both crocidolite and chrysotile.

Protein adsorption to asbestos fibers has been explored in many previous studies (11, 21, 38), including some that have examined the biological consequences

of serum and selective protein adsorption to asbestos on epithelial cells or macrophages (23, 31, 32). Our study differed from these in that it examined VN, a biologically important opsonin for phagocytosis, and examined the biological consequence of VN adsorption for two different fibers in mesothelial cells. The relative adsorption of serum proteins did not differ greatly between the two fibers (Fig. 1), unlike the observation of Desai and Richards (11), who attributed differences in serum protein binding to differences in fiber composition and charge. Instead, our findings were similar to those of Valerio et al. (38) in that protein adsorption was not heavily influenced by surface charge. Instead, other forces may play a more important role in protein adsorption, including hydrophobic interactions and protein charge density (38).

The biological environment is complex and may have multiple effects on the fibers and the cells. Biological materials other than serum proteins, such as surfactant proteins and lipids, antibodies, and DNA that may alter the fibers' interaction with cells, may adsorb to asbestos fibers. Protein adsorption, at least, is associated with partial denaturation of the protein by a conformational change that renders the adsorption nearly irreversible (33). Thus proteins can be expected to remain on the surface of fibers as they move and accumulate in the pleural space (27). In addition, the biological environment may alter the cellular response to asbestos. In the in vitro environment, increases in asbestos phagocytosis may lead to increased apoptosis, whereas in the in vivo environment with its growth factors, extracellular matrix, and cell-cell interactions, apoptosis may be inhibited. If so, in the biological setting, some of the damaged cells may avoid apoptosis and survive with their damaged DNA, thus increasing the likelihood of eventual malignant change (4). We speculate that the biological modification of asbestos that leads to increased phagocytosis could enhance carcinogenicity of asbestos in the in vivo setting where multiple factors tend to oppose apoptosis.

In conclusion, although the crocidolite and chrysotile asbestos fibers are different in composition, charge, and shape, they each become coated with VN when exposed to purified protein or to serum. VN coating leads to increased phagocytosis of chrysotile as well as of crocidolite fibers. Increased phagocytosis then leads to increased intracellular oxidation and increased apoptosis of mesothelial cells. Thus for both types of asbestos fibers, biological modification by protein adsorption can enhance toxicity in vitro and possibly in vivo.

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