Vitronectin Enhances Internalization of Crocidolite Asbestos by Rabbit Pleural Mesothelial Cells via the Integrin $\alpha v\beta 5$

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Abstract

The mechanism by which pleural mesothelial cells, the likely progenitor cells of asbestos-induced mesothelioma, recognize and internalize crocidolite asbestos is unknown. Because incubation of asbestos fibers with serum increases their association with cells, we asked whether a protein coat on asbestos increased internalization of fibers via specific cellular receptors. Coating crocidolite with vitronectin, but not with fibronectin or other proteins, increased fiber internalization by rabbit pleural mesothelial cells, as measured by a new technique using fluorescence confocal microscopy. Receptors for vitronectin, $\alpha v\beta 3$ and $\alpha v\beta 5$, were identified on mesothelial cells. Inhibiting vitronectin receptors by plating cells on a vitronectin substrate or incubating cells with excess soluble vitronectin reduced internalization of vitronectin-coated crocidolite. Inhibition of $\alpha v\beta 5$, but not $\alpha v\beta 3$, with blocking antibodies similarly reduced internalization. In addition, $\alpha v\beta 5$, but not $\alpha v\beta 3$, showed immunocytochemical colocalization with fibers. Of biologic relevance, coating crocidolite with serum also increased internalization via $\alpha v\beta 5$, an effect dependent on the vitronectin in serum. We conclude that pleural mesothelial cells recognize and internalize vitronectin- and serum-coated asbestos via the integrin $\alpha v\beta 5$. Since integrins initiate some of the same signaling pathways as does asbestos, our findings may provide insights into the mechanisms of asbestos-induced biologic effects. (*J. Clin. Invest.* 1995. 96:1987–2001.) Key words: confocal microscopy • wollastonite • $\alpha v\beta 3$ • signaling • clustering

Introduction

Asbestos is an important cause of pleural disease, leading to inflammation, fibrosis, and the formation of a unique mesothelial tumor, mesothelioma. This tropism for the pleura may derive from a specific interaction of asbestos fibers with the mesothelial cells that line the pleural space. Asbestos is known to have many effects on mesothelial cells in culture including disruption of chromosomes (1), induction of proliferative genes such as c-*fos* and c-*jun* (2), induction of inflammatory mediators such as IL-8 (3) and fibronectin (4), and cytotoxicity (5). Indeed, asbestos is 10–100 times more toxic to mesothelial cells than to other cells such as epithelial cells or fibroblasts (1, 6). Although the mechanisms by which asbestos fibers produce these diverse effects remain largely unknown, internalization of the fiber by the mesothelial cell is likely to play an important role. It is known that asbestos fibers are rapidly internalized by mesothelial cells in vitro (7) and can be found in mesothelial cells in vivo (8, 9). Despite the studies demonstrating internalization of fibers, the mechanism by which the fibers are recognized and internalized by the mesothelial cell has not been addressed.

The term asbestos describes a family of silicate fibers, with each type defined by a different shape and surface chemistry. By virtue of their highly reactive surfaces, asbestos fibers can adsorb a variety of biologic materials which can, in some cases, modify the biologic activity of the asbestos fibers (10, 11). Certain proteins have been shown to adsorb in minutes and to remain bound despite extensive washing or competition with other proteins (12). The protein adsorption is specific to the type of fiber, presumably due to the specific charge or charge density of the fiber surface. For example, crocidolite and amosite, both negatively charged fibers, have a different pattern of serum protein adsorption than does chrysotile asbestos, a positively charged fiber (10, 12, 13). In one study, prior exposure of amosite asbestos to serum increased both the toxicity of asbestos for cells as well as the number of fibers associated with the cells (14), suggesting that a coating of proteins might increase either fiber adherence or internalization. Although it is recognized that proteins may alter the biologic activity of the fibers, it is not known whether a protein coating on asbestos fibers specifically alters the internalization of the fibers.

We therefore asked whether coating asbestos fibers with certain proteins enhanced asbestos internalization and, if so, whether this effect was through a specific interaction with cellular receptors. We studied crocidolite asbestos because of its strong epidemiological association with the mesothelial tumor, mesothelioma (15). To distinguish between intracellular and extracellular fibers, we developed a new method using fluorescence confocal microscopy. In this quantitative method, the cell membrane was labeled with a fluorescent probe before exposure to the asbestos; because internalized fibers retain a membrane coating (7, 16), the internal fibers fluoresced under confocal viewing. Using this internalization assay, we found that vitronectin, a major adhesive protein in serum and tissues, specifically enhanced the internalization of crocidolite asbestos fibers. We also found that internalization of vitronectin-coated crocidolite asbestos was mediated via the integrin $\alpha v\beta 5$, which immunochemically colocalized with the fiber. In addition to internalization, the interaction of the fiber and the integrin was associated with a cell shape change without cell toxicity, sug-

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gesting a role for $\alpha v\beta 5$ in asbestos-induced cell signaling. Serum coating was also found to increase internalization of crocidolite via $\alpha v\beta 5$, an enhancement shown to be due to the vitronectin in the serum. This novel functional interaction of the fiber and a cell surface receptor offers insights into possible mechanisms of asbestos-induced biological effects.

Methods

Fibers, antibodies, and extracellular matrix proteins. Crocidolite asbestos was obtained from the National Institute of Environmental Health and Safety (Research Triangle Park, NC). The fibers had been previously characterized and found to have a mean length of 19 μm (range 1–560 μm) and a mean width of 0.25 μm (17). By counts under phase-contrast microscopy, there were 200×10^6 fibers/g. Wollastonite, a relatively nonpathogenic calcium silicate fiber in both in vitro (18) and in vivo (19) studies, was used as a control fiber. The preparation of wollastonite used was Nyglos I (or Rimglos), milled to a geometry mimicking that of crocidolite asbestos (mean length 12 μm, range 2–80 μm, mean width 2 μm) (NYCO Minerals, Willsboro, NY). By phase-contrast microscopy, there were 120×10^6 fibers/g, indicating that the average wollastonite fiber was approximately twice the weight of the average crocidolite fiber. For these studies, to keep the number of added fibers constant, wollastonite was used at twice the surface concentration ($\mu g/cm^2$) as crocidolite.

A blocking mAb for the human $\alpha v\beta 5$ complex (P1F6) was a generous gift from E. Wayner (University of Minnesota, Minneapolis, MN) (20). A blocking mAb to human $\alpha v\beta 3$ (9G2.1.3), known to recognize and block rabbit $\alpha v\beta 3$, was provided by A. Chuntharapai and K. Jin Kim (Genentech Inc., So. San Francisco, CA) (21). A nonblocking mAb against the human αv subunit (H5P10; provided by A. Weinacker and A. Chen, Lung Biology Center, University of California San Francisco, San Francisco, CA) was generated by immunizing mice with secreted recombinant $\alpha v\beta 6$ (20). H5P10 demonstrated specificity for the αv subunit by immunoprecipitating secreted $\alpha v\beta 6$ and by specific staining of the surface of $\alpha v+\beta 6-$ hamster and human cell lines. A nonblocking mAb to the human and rabbit $\alpha v\beta 3$ complex (2B4) was provided by A. Chuntharapai (Genentech Inc.). The rat mAb directed against the $\beta 1$ subunit (AIIB2) was the gift of C. Damsky (University of California, San Francisco, CA) (22) and the rabbit polyclonal antiserum against the cytoplasmic domains of αv (332) was provided by Louis Reichardt (University of California, San Francisco, CA) (23). Rabbit anti-human vitronectin polyclonal antiserum was the generous gift of Deane Mosher (University of Wisconsin, Madison, WI). P1F6 and H5P10 were used in the forms of hybridoma supernatants or purified antibodies, as indicated. mAbs were purified by adsorption onto a column of rabbit anti-mouse IgG (Sigma Chemical Co., St Louis, MO) followed by elution using high salt buffers (ImmunoPure Gentle Ag/Ab Buffer System; Pierce Chemical Co., Rockford, IL), concentration by centrifugation (Centricon-10; Amicon Corp., Beverly, MA), and dialysis against PBS.

Extracellular matrix proteins used in these experiments included collagen I (Sigma Chemical Co.), fibronectin (Collaborative Research Inc., Bedford, MA), and laminin (Collaborative Research Inc.). Vitronectin was purified from human plasma by heparin affinity chromatography according to the method of Yatohgo et al. (24). BSA (fraction V; Sigma) was confirmed to have no detectable vitronectin by immunoblot. Delipidated bovine serum was obtained from Biomedical Technologies Inc. (Boston, MA).

Cells and cell culture. Mesothelial cells were harvested from the pleura of healthy New Zealand White rabbits as described previously (3). The cells were confirmed to be mesothelial by positive immunochemical staining for vimentin, keratin, hyaluronic acid mucin, and negative staining for factor VIII. For all experiments, cells were grown to subconfluence and used between passages three and six.

Asbestos preparation. For coating of fibers with proteins, crocidolite asbestos or wollastonite was incubated (100 μg of fiber in 200 μl PBS) with 60 μg of either vitronectin, collagen I, fibronectin, laminin, or albumin while vortexing for 1 h at room temperature. In some experiments, vitronectin was added at a range of concentrations. For serum coating of fibers, fibers were incubated with freshly prepared human serum from healthy donors overnight at 4°C on a rocker. For separation of fibers and better removal of unbound proteins, the suspension was sonicated (power 60 W for 5 s) (450 sonifier; Branson Ultrasonics Corp., Danbury, CT). Fibers were washed twice using cell culture medium (RPMI/DME), incubated with BSA (0.1%, vol/vol) during vortexing (10 min), washed twice again, resuspended in cell culture medium, sonicated, and then added to the cells at the desired surface concentration. In most experiments, crocidolite was used at 3.0 $\mu g/cm^2$, a moderate concentration at which internal and external fibers were easily quantifiable. Wollastonite was used at 6.0 $\mu g/cm^2$, a concentration that provided an equivalent number of fibers per area as crocidolite. By counting fibers at each step using phase-contrast microscopy, it was determined that fibers were not lost during the coating procedures.

To assure that proteins adsorbed to both fibers using the conditions above, the proteins on the fiber surface were detected by immunofluorescence. Fibers coated with vitronectin, fibronectin, human serum, or BSA were incubated overnight at room temperature with either rabbit anti-human vitronectin polyclonal antiserum (final concentration 1:30) (Chemicon International, Inc., Temecula, CA), rabbit anti-human fibronectin polyclonal antiserum (final concentration 1:15) (Chemicon), or no primary antibody. Glass beads were also coated with vitronectin as a positive control. The next morning fibers were washed, incubated (1 h at room temperature) with FITC-conjugated goat anti-rabbit IgG antibody (final concentration 1:50; Tago Inc., Burlingame, CA), and washed again. Fibers were then viewed under coverslips using a microscope (Carl Zeiss, Inc., Thornwood, NY) fitted for epifluorescence.

For further confirmation of protein coating, proteins were eluted from fibers and analyzed by immunoblotting for the presence of vitronectin as follows. Crocidolite and wollastonite fibers were incubated with vitronectin or BSA (30 μg/ml) as described above or incubated overnight with complex biologic liquids such as undiluted human serum, concentrated bronchoalveolar lavage liquid, or undiluted pleural liquid. Bronchoalveolar lavage (kindly provided by J. Balmes, University of California, San Francisco) was obtained from three otherwise healthy asthmatic volunteers, pooled, and concentrated by centrifugation (20-fold) (Centricon-10; Amicon Corp.). Pleural liquid was obtained from patients with documented malignant or tuberculous effusions, centrifuged, and the supernatant stored at −20°C until use. Proteins were then eluted from the fibers using detergent (10 mM $NaPO_4$, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, pH 7.4) (4°C, 20 min). These eluted proteins and vitronectin standards were diluted in reducing Laemmli sample buffer and boiled. Proteins in equal volumes of supernatant (35 μl) were then separated using Tris/glycine SDS-PAGE (7.5%) and transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon; Millipore Corp., Bedford, MA). To block nonspecific binding sites, the membrane was incubated with 10% BSA (wt/vol) for 2 h before incubation with rabbit anti-human vitronectin polyclonal antiserum (1:2,000) (Chemicon) overnight at 4°C. After three washes, the membrane was incubated with alkaline phosphatase conjugated to goat anti-rabbit IgG polyclonal antibody (1:3,000 in 4% BSA, wt/vol) (Pierce Chemical Co.), washed, and developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-1-phosphate (Promega Corp., Madison, WI). In some experiments, Coomassie blue staining of duplicate gels was used to confirm that similar amounts of protein were loaded in each lane.

Fluorescence confocal technique. To differentiate between intracellular and extracellular fibers, the cell membrane was labeled with a nontoxic fluoroprobe, a lipid soluble dye (1,1′-dioctadecyl-d,d,d′,d′-tetramethylindocarbocyanine, Dil)[1] (Molecular Probes, Inc., Eugene, OR), before each experiment. Once the membrane was labeled in this

1. *Abbreviation used in this paper:* Dil, 1,1′-dioctadecyl-d,d,d′,d′-tetramethylindocarbocyanine.

manner, fibers internalized by the cell fluoresced because internalized fibers were encased in a sleeve of fluorescent cell membrane; external, adherent fibers did not fluoresce. Dil was chosen for several reasons: it labels lipid membranes uniformly, it is nontoxic to cells, and it is resistant to photobleaching (25).

For validation of the technique, a series of experiments was performed using crocidolite and wollastonite fibers. First, fibers alone were studied. Uncoated fibers and fibers coated with albumin, collagen I, fibronectin, or vitronectin and then incubated with Dil (0.1–10 μg/ml) were found not to fluoresce. When fibers were coated with lipid vesicles (dipalmitoyl phosphotidylcholine) (100 μg/ml × 5 min) (Sigma) and then incubated with Dil (1 μg/ml), all fibers fluoresced showing that the fibers required a lipid coating to label with Dil. Second, fibers were studied after incubation for 2 h with Dil-labeled mesothelial cells. In 100 cells examined by fluorescence confocal microscopy, each fluorescent fiber was confirmed to be intracellular by virtue of its location in the same focal plane as intracellular organelles. Partially internalized fibers were detected by fluorescence along a portion of the fiber length (Fig. 1 *A*). For confirmation that all nonfluorescent fibers were extracellular, the fibers already incubated with Dil-labeled cells for 2 h as above were then incubated with Dil-labeled lipid vesicles. Then, all fibers fluoresced showing that the previously nonfluorescent fibers were extracellular and therefore accessible to the lipid. Third, fibers were incubated with Dil-labeled mesothelial cells under conditions in which internalization was inhibited, either by incubating the cells at 4°C or by exposing the cells to a cytoskeletal toxin (cytochalasin B, 5 μg/ml) (Sigma). When fibers were incubated with Dil-labeled cells at 4°C or 37°C, there was a similar number of fibers associated with the cells after washing; however, compared to cells incubated at 37°C, the cells at 4°C contained no fluorescent fibers. When fibers were incubated with Dil-labeled cells that had been treated with cytochalasin, there was a significant decrease in the number of fluorescent fibers compared to untreated cells (0.3±0.1 fiber/cell vs 1.2±0.4 fiber/cell [mean±SD]; cytochalasin vs control after exposure to 3.0 μg/cm^2 crocidolite for 2 h). Finally, a second technique for counting internalized fibers was developed and compared with the fluorescence confocal technique. Because trypsin/EDTA led to the removal of all adherent fibers from cells incubated with fibers at 4°C but not from cells incubated with fibers at 37°C, it was determined that this treatment led to the removal of extracellular, adherent fibers. After exposure to trypsin/EDTA (0.25%), cells were examined under dark-field conditions to count the number of fibers found associated with the cells. Because adherent fibers had been removed from the cell surface, these associated fibers were considered internalized fibers (Fig. 1 *B*). For comparison of the two techniques, different slides of Dil-labeled cells were incubated with fibers in parallel experiments so that intracellular fibers could be counted using fluorescence confocal microscopy on one group of cells or using dark-field microscopy of the matching group of cells detached using trypsin/EDTA.

Internalization experiment. Mesothelial cells were grown to 90% confluence in eight-chamber well slides (Nunc, Inc., Naperville, IL) on a matrix of collagen type I (10 μg/ml). The cells were loaded with Dil before the experiment so that the membrane could be labeled fully before the introduction of fibers and so that the fibers would not be exposed to free Dil. The Dil (diluted in DMSO at 0.5 mg/ml and stored at −20°C) was dissolved by incubation at 37°C and sonication. It was then diluted in delipidated serum to 20 μg/ml. Then, diluted to 1 μg/ml in culture media, the Dil solution was pipetted onto the cell monolayers. After 30 min at 37°C, the Dil solution was aspirated and the cells were washed twice with PBS containing 0.1% BSA. The various fibers coated with proteins as above and suspended in protein-free culture medium were then added to each well for a 2-h incubation at 37°C. After the incubation, the medium containing unbound fibers was aspirated, monolayers were washed twice with PBS/0.1% BSA, fixed with glutaraldehyde (2.5%, vol/vol) (Sigma), and washed twice with PBS/0.1% BSA. Using a split screen with fluorescent (rhodamine filter; excitation wavelength 540 μm, emission wavelength 580 μm) and dark-field views of the same field, internal (fluorescent) fibers, total fibers (all fibers seen on dark-field), and cell nuclei were counted in a blinded fashion. In fields in which the cells were not fully confluent, fibers were counted only if they were associated with a cell. 10 randomly selected fields were counted for each experimental condition (each well), leading to the examination of at least 100 cells. This number of fields was chosen because the cumulative average of fibers per cell reaches a constant value after approximately six to eight fields are counted (variance < 5%). The number of internal particles per cell and of external fibers per cell (total minus internal) was calculated.

Flow cytometry for identification of vitronectin receptor expression. Nearly confluent mesothelial cells (90% confluent) were detached using a brief exposure to dilute trypsin (0.25%), washed with PBS, and then incubated with normal goat serum (15 min at 4°C) (Sigma) to inhibit nonspecific binding of the secondary antibody. After two washes, cells were incubated with one of the antiintegrin antibodies (30 min on ice) and washed again. Cells were then incubated with the secondary antibody, phycoerythrin-conjugated goat anti-mouse IgG (Boehringer-Mannheim Biochemicals, Indianapolis, IN) (30 min on ice), washed twice more, fixed using paraformaldehyde (1% in PBS for 1 min; Sigma), and washed. The stained cells were analyzed for fluorescence using a flow cytometer (FACScan®; Becton Dickinson & Co., San Jose, CA).

Immunoprecipitation to confirm presence of known vitronectin receptors in rabbit pleural mesothelial cells. For surface labeling, rabbit pleural mesothelial cells (passage 4) were washed in PBS, detached by a brief exposure to dilute trypsin, and incubated (30 min at 4°C) with labeling buffer (50 mM Tris; 300 mM NaCl; 20 mM dextrose; 1 mM CaCl; pH 7.4), lactoperoxidase (200 mg), [^{125}I]sodium iodide (2 mCi) (Amersham Corp., Arlington Heights, IL), and glucose oxidase solution (0.214 U, Sigma). Washed cell pellets were lysed in cold immunoprecipitation buffer (100 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% NP-40) containing 1 mM PMSF (Gibco BRL, Gaithersburg, MD). For removal of nonspecific proteins, the supernatant from the lysate was precleared by incubation with protein A Sepharose beads (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) for 45 min at 4°C. The lysate was then split and the aliquots incubated (12 h at 4°C) with one of the following antibodies: 332 (anti-αv, cytoplasmic domain), 9G2 (anti-αvβ3), P1F6 (anti-αvβ5), or AIIB2 (anti-β1). After 12 h, rabbit anti-mouse IgG (Zymed Laboratories Inc., So. San Francisco, CA) was added to the samples containing 9G2 and P1F6 and rabbit anti-rat IgG (Zymed) was added to the sample containing AIIB2. For capture of the immune complexes, protein A Sepharose beads were added to each sample and incubated for 1 h at 4°C. The samples were then centrifuged and washed five times with immunoprecipitation buffer. Proteins were eluted from the beads by addition of nonreducing Laemmli sample buffer followed by boiling for 2 min. Proteins in the supernatant were separated using Tris/glycine SDS-PAGE (7.5%). The gel was fixed, stained with Coomassie blue, and subjected to autoradiography.

Cell adhesion assays to confirm the function of vitronectin receptors and blocking activity of antibodies. Wells of nontissue culture-treated polystyrene 96-well flat-bottom microtiter plates (Linboro/Titertek; Flow Laboratories, McLean, VA) were coated with various concentrations of vitronectin for 4 h at 37°C. Wells were then washed with PBS and blocked with BSA (1%, wt/vol). Mesothelial cells were detached using a nonenzymatic cell dissociation solution (Sigma), incubated with antibodies (50 μg/ml) for 15 min at 4°C, and added to wells at 25,000 cells/well. After centrifugation (top side of plate up) at 10 *g* for 5 min, the cells were allowed to attach at 37°C (5% CO_2) for 1 h. Nonadherent cells were removed by centrifugation (top side of plate down) at 48 *g* for 5 min. The cells that remained attached were fixed with 1% formaldehyde and stained using 0.5% crystal violet. The relative number of cells in each well was determined by measuring the absorbance at 595 nm in a microplate reader (Bio-Rad Laboratories, Richmond, CA). The data were expressed as the mean of the absorbance of three wells in triplicate plates minus the absorbance in uncoated wells.

Role of vitronectin receptors in internalization. The role of the vitronectin receptors in internalization was studied by inhibiting the receptors in four ways: blocking a variety of integrins with soluble

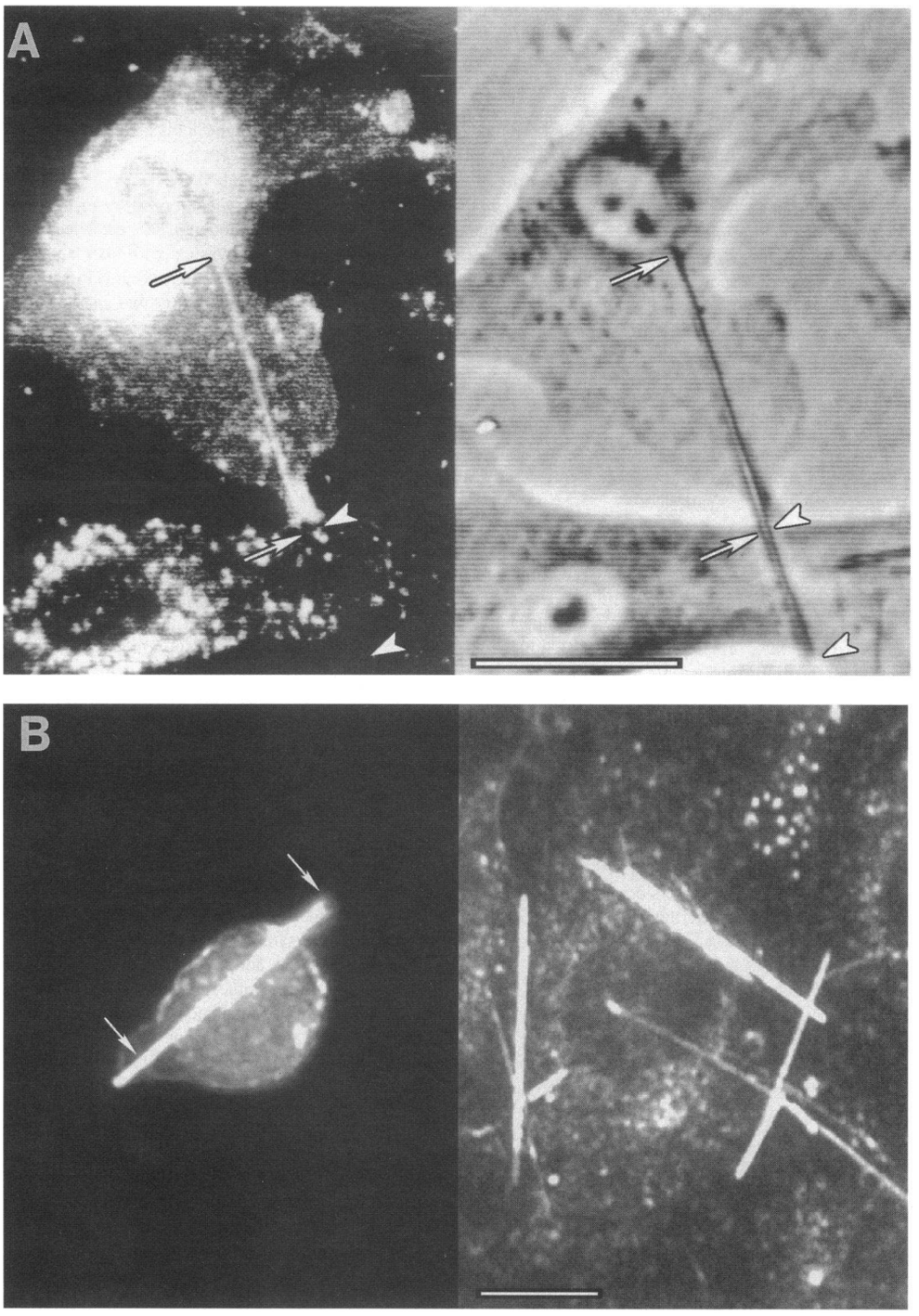

Figure 1. Intracellular fibers detected in rabbit pleural mesothelial cells using (*A*) fluorescence confocal and (*B*) dark-field microscopy. (*A*) For fluorescence confocal microscopy, mesothelial cells were labeled with the lipid-soluble fluoroprobe, DiI, before incubation with crocidolite fibers. On the phase-contrast image on the right, an asbestos fiber can be seen to extend over two cells. In the fluorescence confocal image on the left, the fiber can be seen to be fluorescent only along the portion of the fiber within the profile of the upper cell (*arrows*), indicating that the fiber is coated with membrane by this cell and is internalized. The rest of the fiber overlying the lower cell (*arrowheads*) is not fluorescent and thus extracellular. (*B*) For dark-field views, mesothelial cells were incubated with crocidolite, washed, and viewed before (*right*) and after (*left*) trypsin/EDTA treatment. Before trypsin/EDTA, multiple fibers appear associated with cells. Because trypsin/EDTA was found to remove adherent fibers (see text), fibers associated with cells after trypsin/EDTA were counted as intracellular (*arrows*). Bar, 25 μm.

RGD-containing peptides, redistributing vitronectin receptors from the apical to the basal surface by plating cells on bound ligand, competing for vitronectin receptors with excess soluble vitronectin, and neutralizing receptors with specific antibodies. For studies using RGD-peptides, mesothelial cells were grown on collagen I, to which cells adhere in an RGD-independent manner, and labeled with DiI. Cells were exposed to fibers in the presence of either GRGDSP or GRGESP peptides (0.5 mg/ml) (Gibco BRL) for 2 h. For studies on the effects of different substrates on fiber internalization, the different wells of eight-chamber well slides were coated with vitronectin or other proteins (10 μg/ml) for 6 h at 37°C and washed. Mesothelial cells were plated at high density to achieve near confluence by the next morning. At that time, the cells were labeled with DiI and exposed to vitronectin-coated crocidolite or control fibers for 2 h. For studies of the effects of soluble vitronectin, mesothelial cells grown on collagen I and labeled with DiI were exposed to fibers in the presence of soluble vitronectin (0–100 μg/ml) for 2 h. For studies using neutralizing antiintegrin antibodies, the blocking or nonblocking purified antiintegrin antibodies (10–100 μg/ml) were added to the suspension of coated fibers immediately before addition to DiI-labeled mesothelial cells grown on a collagen I substrate. At the end of each incubation, mesothelial cells were fixed and examined in a blinded fashion by fluorescence confocal microscopy to quantify the number of internal and external fibers per cell.

Immunodepletion of vitronectin in serum. For determination of the

role of vitronectin in serum-enhanced fiber uptake, serum was depleted of vitronectin using immunoaffinity chromatography. In brief, polyclonal rabbit anti-human vitronectin antibody (a gift of D. Mosher, University of Wisconsin, Madison, WI) was immobilized on cyanogen bromide-activated Sepharose (2 ml) according to the manufacturer's directions (Pharmacia LKB Biotechnology Inc.). After washing the column with PBS (six column vol), 2 ml of fresh human serum was applied to the column, recycled through the column 20 times, and saved for analysis and fiber coating. To elute bound proteins, the column was then washed with PBS (10 column vol) followed by four cycles of alternating 0.1 M glycine (pH 2.8) and 0.1 M triethylamine (pH 11). Eluates were characterized by immunoblot as above.

Distribution of integrins and colocalization with fibers. Vitronectin-coated crocidolite or wollastonite fibers prepared as above and suspended in culture medium were added at 3.0 $\mu g/cm^2$ to rabbit pleural mesothelial cells grown to near confluence on coverslips coated with collagen I or vitronectin (10 $\mu g/ml$). After 4 h, the cells were washed twice with PBS (containing Ca and Mg; Sigma), fixed for 5 min in cold 1:1 ethanol/acetone, air-dried at room temperature, and then rehydrated for 5 min in PBS (Ca- and Mg-free). For blocking the nonspecific binding of the secondary antibody, cells were incubated with sheep serum (1% vol/vol, 15 min; Sigma) before incubation overnight at 4°C with hybridoma supernatants, P1F6 (neat) or 9G2 (1:25 dilution), or with the sheep serum (1%) alone. The next day, the cells were incubated (1 h at room temperature) with biotinylated secondary antibody (sheep anti-mouse IgG, 1:50; Amersham) followed by streptavidin conjugated to fluorescein (1:50; Amersham). After the coverslips were mounted with Vectashield (Vector Laboratories, Inc., Burlingame, CA) to enhance and preserve fluorescence, the cells were viewed using epifluorescence microscopy.

Toxicity assays. For determining whether mesothelial cells were acutely injured at the concentrations of asbestos used, cells were studied for the release of lactate dehydrogenase and ^{51}chromium. After mesothelial cells had been incubated for 2 h with vitronectin-coated crocidolite (0–6 $\mu g/cm^2$), the conditioned medium was removed for lactate dehydrogenase measurement. The lactate dehydrogenase activity was measured using a kit (Sigma) and expressed as a percentage of the total lactate dehydrogenase activity released from an equal number of cells lysed in Triton X-100 (1%, vol/vol) (Sigma). The chromium release assay was performed with minor modifications from published methods to determine both cell lysis and detachment (26, 27). Cells grown in 24-well plates (Costar Corp., Cambridge, MA) were exposed to sodium ^{51}chromate (0.2 μCi/well; 1 μCi/ml) (Amersham) for 12 h. Cells were then washed to remove extracellular chromium and exposed to medium alone or vitronectin-coated fibers (0–12 $\mu g/cm^2$) for 2–8 h. At the end of each time period, the conditioned medium was removed, and the cells were washed three times with DME. An aliquot of the combined media and wash was saved for counting; the rest of the combined media and wash was pelleted so that the supernatant could also be counted. Cells in control wells were lysed in Triton X-100 (1%, vol/vol). The total counts were the sum of counts in the control cell lysates and the overlying media. The amount of chromium in each sample was measured using a gamma counter (Beckman Instruments, Palo Alto, CA). Chromium release was expressed as [(cpm test media-cpm control media) · 100/cpm total]. Cell detachment was expressed as [(cpm test media-cpm supernatant media) · 100/cpm total].

Statistical analysis. Data are expressed as mean±1 SEM, unless indicated. Statistical differences among groups were determined by one way analysis of variance with Tukey's test to discriminate where the difference lay (28). A P value < 0.05 was accepted as representing a significant difference.

Results

Coating of fibers. After incubation of fibers with either vitronectin or fibronectin, a continuous protein coat was detected by immunofluorescent labeling. Vitronectin-coated crocidolite, serum-coated crocidolite, and vitronectin-coated wollastonite, but not uncoated fibers, demonstrated immunofluorescent staining with antivitronectin antibodies. As a positive control for vitronectin binding, vitronectin-coated glass beads stained similarly to vitronectin-coated crocidolite fibers. Vitronectin-coated fibers did not stain with antifibronectin antibodies or secondary antibody alone (no primary). Similarly, fibronectin-coated fibers stained specifically with antifibronectin antibodies. When fibers were incubated with vitronectin (30 $\mu g/ml$), eluted vitronectin was detected from both crocidolite and wollastonite in approximately equal amounts (Fig. 2 *A*); after incubation with vitronectin at lower concentrations (3, 0.3 $\mu g/ml$), no vitronectin could be detected in the eluate from either fiber. When crocidolite was incubated with human biologic liquids including serum, bronchoalveolar lavage (concentrated 20-fold), and pleural liquid, vitronectin could also be eluted from the fiber (Fig. 2 *B*).

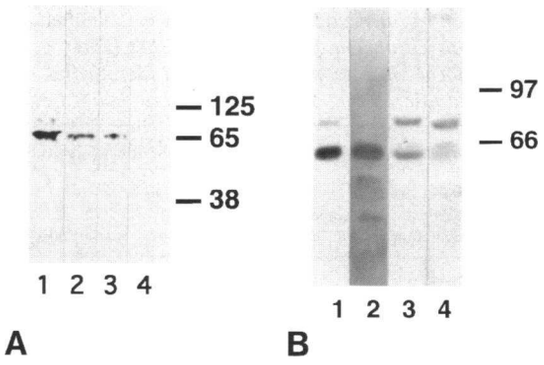

Figure 2. Immunoblots of proteins eluted from fibers incubated with purified proteins (*A*) or incubated with biologic liquids (*B*) and detected with an anti-vitronectin antibody. (*A*) An immunoblot of vitronectin standard (lane *1*) and proteins eluted from vitronectin-coated crocidolite (lane *2*), vitronectin-coated wollastonite (lane *3*), and BSA-coated crocidolite (lane *4*). (*B*) An immunoblot of proteins eluted from crocidolite fibers incubated with human serum (lane *1*), bronchoalveolar lavage liquid (lane *2*), and liquid from pleural effusions due to malignant (lane *3*) or tuberculous (lane *4*) pleural effusions. The bands seen correspond to either the 75-kD or the 65-kD form of vitronectin (36). Fibers were incubated with human vitronectin (30 $\mu g/ml$), BSA (30 $\mu g/ml$), serum (neat), bronchoalveolar lavage (concentrated 20-fold), or pleural liquids (neat), washed and sonicated. Adsorbed proteins were eluted and equal volumes analyzed by SDS-PAGE under reducing conditions with molecular weight standards. The immunoblots were stained with anti-human vitronectin polyclonal antiserum followed by an alkaline phosphatase-conjugated secondary antibody.

Fluorescence confocal technique. In a parallel experiment, mesothelial cells exposed to vitronectin-coated crocidolite or wollastonite for 2 h were analyzed for intracellular fibers by two techniques: by fluorescence confocal microscopy (Fig. 1 *A*) or by dark-field microscopy after trypsin/EDTA (Fig. 1 *B*). Because trypsin/EDTA treatment removed all fibers from cells incubated with fibers at 4°C, this technique was determined to remove adherent fibers from mesothelial cells and to allow the counting of associated fibers as intracellular. When compared in a blinded fashion over a range of fiber dosages, the two techniques for counting internal fibers were equivalent (Table I). In separate experiments, both Dil-loaded and unloaded cells demonstrated the same number of intracellular fibers after tryp-

Table I. Comparison of Internalized Fiber Counts in Mesothelial Cells Incubated with Vitronectin-coated Fibers Using Either Fluorescence Confocal Microscopy of Dil-labeled Mesothelial Cells or Dark-Field Microscopy of Trypsinized Mesothelial Cells

Fiber	Fiber concentration	Temperature of incubation	Internalized fibers per cell: Fluorescence confocal microscopy	Internalized fibers per cell: Trypsin/EDTA
	$\mu g/cm^2$			
Crocidolite	3.0	4°C	0	0.02±0.02
	3.0	37°C	0.7±0.7*	0.6±0.4*
	4.5	37°C	1.0±0.4*	0.9±0.5*
	6.0	37°C	1.5±0.8*‡	1.3±0.6*‡
Wollastonite	6.0	4°C	0	0.03±0.02
	6.0	37°C	0.5±0.2*	0.6±0.2*

Data are means±SD of experiments performed in triplicate. Mesothelial cells were incubated with vitronectin-coated crocidolite or wollastonite for 2 h in parallel plates, one at 4°C and the other at 37°C. For each fiber and temperature condition, cells were either labeled with Dil for later confocal viewing or incubated with an equal volume of culture medium without Dil for later detachment with trypsin/EDTA. Fiber counts were performed in a blinded fashion on 200 cells in each category. In cells labeled with Dil and examined with fluorescence confocal microscopy, fibers were counted that were fluorescent along all or part of their length; in cells detached with trypsin/EDTA and examined under dark-field microscopy, fibers were counted that were associated with cells. * Different from fiber internalization at 4°C; ‡ different from fiber internalization at lower concentrations of crocidolite.

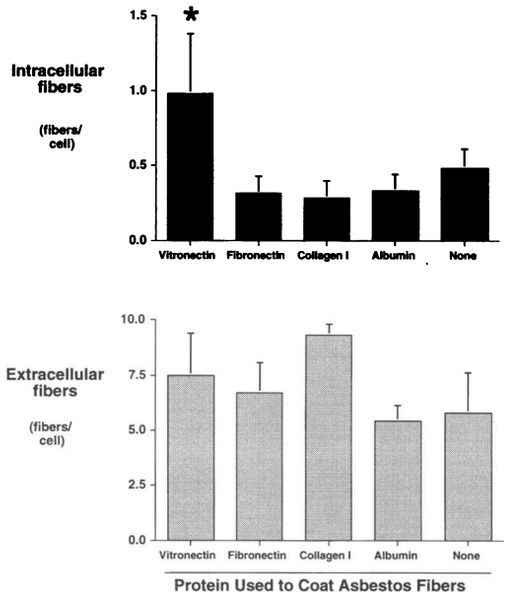

Figure 3. The effect of different proteins adsorbed onto crocidolite asbestos fibers on fiber internalization (*top*) and adherence (*bottom*) by rabbit pleural mesothelial cells. Crocidolite fibers were incubated with vitronectin and thoroughly washed leaving a protein coat. Coated fibers were incubated with Dil-labeled mesothelial cells for 2 h in a protein-free medium at 4.5 $\mu g/cm^2$. Internal (fluorescent) and external (non-fluorescent) fibers were counted in a blinded fashion using fluorescence confocal microscopy. When crocidolite fibers were coated with vitronectin, fiber internalization was significantly greater than when fibers were coated with other proteins or with no protein (*A*). The increase in internalization was not associated with an increase in the number of external, adherent fibers (*B*). Data are means±SEM; * different from other proteins; $P = 0.03$, $n = 3$ experiments; 125 cells counted in each experiment.

sin/EDTA treatment, indicating that Dil loading did not influence fiber internalization.

Effect of vitronectin-coating on internalization. Coating with vitronectin significantly increased the internalization of crocidolite asbestos by mesothelial cells compared to coating with other proteins or no protein (Fig. 3). The effect of vitronectin was specific for internalization of the fibers because it did not alter the number of fibers adherent to the cells. Coating with a range of vitronectin concentrations demonstrated a significant increase in internalization at 30 μg/ml, the concentration used in these studies (Fig. 4). There was no further increase with increasing concentrations of vitronectin. Vitronectin coating did not increase the internalization of the control fiber, wollastonite.

Expression and function of known vitronectin receptors. By flow cytometry, rabbit pleural mesothelial cells expressed two known integrin receptors for vitronectin, $\alpha v\beta 5$ and $\alpha v\beta 3$ (Fig. 5 *A*). The nonblocking antibodies to αv (H5P10) and $\alpha v\beta 3$ (2B4) also recognized receptors on rabbit mesothelial cells (data not shown).

By immunoprecipitation of surface labeled proteins, rabbit pleural mesothelial cells were confirmed to express $\alpha v\beta 3$ and $\alpha v\beta 5$ (Fig. 5 *B*). With precipitation using an antibody to the cytoplasmic domain of αv (lane *1*), a band was identified corresponding to the position of β subunits, $\beta 3$ and $\beta 5$. With precipitation using antibodies to $\alpha v\beta 3$ (lane *2*) or $\alpha v\beta 5$ (lane *3*), the presence of both β subunits was confirmed. No band corresponding to the molecular weight of the $\beta 1$ subunit was precipitated with antibody to αv, demonstrating the absence from these cells of $\alpha v\beta 1$, a putative receptor for vitronectin. Cellular ex-

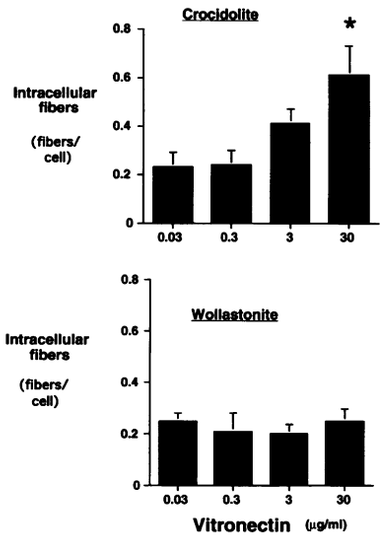

Figure 4. Dose-response of vitronectin concentrations used to coat fibers on internalization of crocidolite (*top*) or wollastonite (*bottom*) by rabbit pleural mesothelial cells. Fibers were incubated with various concentrations of vitronectin and thoroughly washed leaving a protein coat. Coated fibers were incubated with Dil-labeled mesothelial cells for 2 h in a protein-free medium at 3.0 $\mu g/cm^2$. Internal (fluorescent) fibers were counted in a blinded fashion using fluorescence confocal microscopy. When crocidolite fibers were incubated with vitronectin at 30 μg/ml, fiber uptake was increased significantly, but no enhancement of wollastonite fiber uptake was seen at any concentration. Data are means±SEM; * different from other protein concentrations; $P < 0.02$, $n = 3$ experiments; 150 cells counted in each experiment.

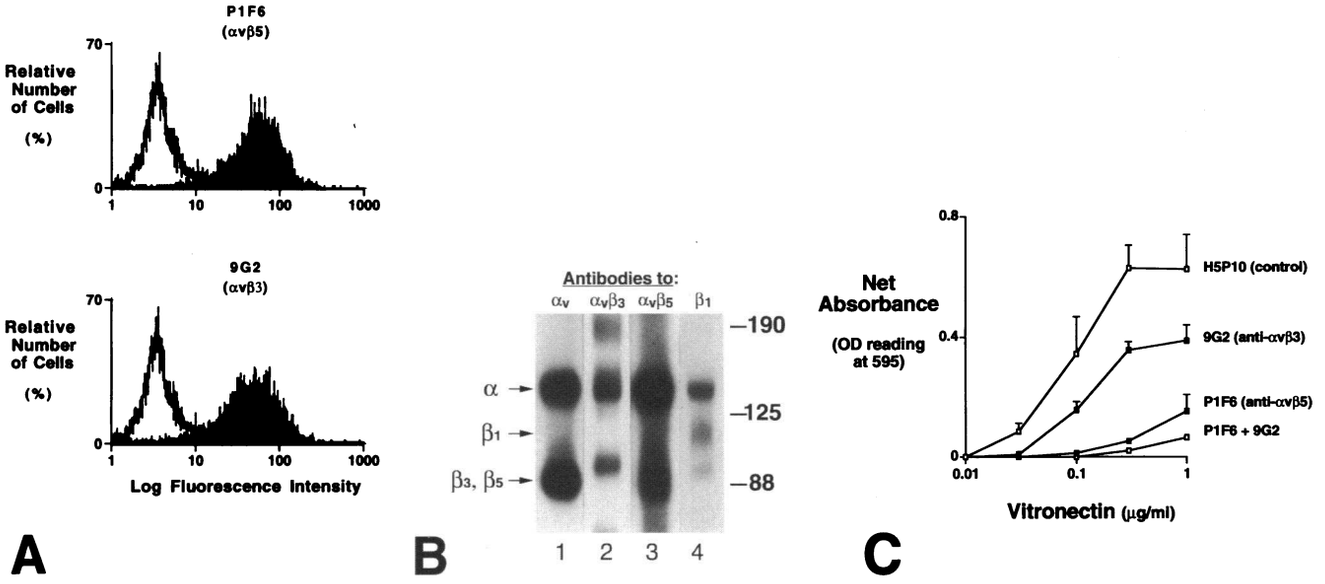

Figure 5. Identification of vitronectin receptors, $\alpha v\beta 5$ and $\alpha v\beta 3$, in rabbit pleural mesothelial cells. (*A*) Flow cytometry of mesothelial cells stained with antibodies to $\alpha v\beta 5$ (P1F6) and $\alpha v\beta 3$ (9G2). After incubation with either no antibody (*white curves*) or with monoclonal anti-integrin antibody (*black curves*), cells were incubated with goat anti–mouse IgG conjugated with phycoerythrin and analyzed for fluorescence by flow cytometry. (*B*) Immunoprecipitation of surface-labeled proteins from unstimulated rabbit pleural mesothelial cells demonstrating the presence of the vitronectin receptors $\alpha v\beta 5$ and $\alpha v\beta 3$. Lysates from ^{125}I–surface-labeled mesothelial cells were immunoprecipitated using antibodies against αv (332, lane *1*), $\alpha v\beta 3$ (9G2, lane *2*), $\alpha v\beta 5$ (P1F6, lane *3*), and $\beta 1$ (AIIB2, lane *4*). The proteins in all lanes were analyzed under nonreducing conditions followed by autoradiography. Anticipated positions of integrin subunits are shown to the left. The positions of molecular size markers (kD) are shown to the right. (*C*) Cell adhesion assay demonstrating the participation of $\alpha v\beta 5$ and $\alpha v\beta 3$ in the attachment of rabbit pleural mesothelial cells to vitronectin. Rabbit pleural mesothelial cells were incubated with blocking antibodies to $\alpha v\beta 5$ and/or $\alpha v\beta 3$ or a nonblocking control antibody and then allowed to attach to individual wells of 96-well microtiter plates that had been coated with increasing concentrations of vitronectin.

pression of $\beta 1$ was confirmed in association with other α subunits (lane *4*).

By cell adhesion studies, rabbit pleural mesothelial cells were shown to use both $\alpha v\beta 3$ and $\alpha v\beta 5$ to bind vitronectin (Fig. 5 *C*). The anti–human integrin antibodies used in these experiments (9G2, P1F6) were thus confirmed to have blocking activity for the rabbit integrins.

Role of vitronectin receptors in internalization. Mesothelial cells exposed to soluble GRGDSP peptides (0.5 mg/ml) internalized ~ 70% fewer vitronectin-coated crocidolite fibers (at 3.0 $\mu g/cm^2$ for 2 h) than did cells exposed to control GRGESP peptides (0.39±0.07 fibers/cell vs. 0.13±0.04 fibers/cell; GRGDSP vs GRGESP, $P < 0.05$). There was no difference in the number of external, adherent fibers.

Mesothelial cells plated on a vitronectin substrate internalized significantly fewer vitronectin-coated crocidolite fibers than did cells plated on other protein substrates (Figs. 6 *A* and 7). A vitronectin substrate did not affect the number of vitronectin-coated crocidolite fibers bound to the cell surface (Fig. 6 *B*) or the number of internalized control fibers (collagen I–coated crocidolite or vitronectin-coated wollastonite) (Fig. 6, *C* and *D*).

Similarly, mesothelial cells exposed to excess soluble vitronectin internalized significantly fewer vitronectin-coated crocidolite fibers than did cells exposed to no soluble vitronectin (Fig. 8 *A*) or to a soluble control protein, albumin (50 μg/ml) (0.35±0.2 fibers/cell). The addition of soluble vitronectin altered neither the number of bound vitronectin-coated crocidolite fibers (Fig. 8 *B*) nor the number of internalized control fibers (Fig. 8, *C* and *D*).

A blocking antibody to $\alpha v\beta 5$ (P1F6) inhibited internalization of vitronectin-coated crocidolite fibers, but neutralizing antibody to $\alpha v\beta 3$ (9G2) and nonblocking antibodies to αv (H5P10) or $\alpha v\beta 3$ (2B4) had no effect (Fig. 9 *A*). Results were not different over the range of antibody concentrations between 10 and 100 μg/ml; data are shown for 50 μg/ml. The neutralizing antibody altered neither the number of vitronectin-coated crocidolite fibers bound to the surface of the mesothelial cells (Fig. 9 *B*) nor the number of control fibers internalized by the cells (Fig. 9, *C* and *D*). There was no additional decrease in internalization when the two blocking antibodies (P1F6 and 9G2) were used in combination.

Effect of serum coating. The vitronectin in human serum was found to be completely depleted by immunoaffinity chromatography (Fig. 10 *A*). When fibers were incubated with either serum or immunodepleted serum, vitronectin could be eluted only from the fibers incubated with serum (data not shown). The binding of vitronectin to the column was confirmed by detection of vitronectin in the eluate (Fig. 10 *A*). This eluted vitronectin was shown to increase internalization of coated fibers (data not shown).

Serum coating of crocidolite fibers increased fiber internalization to a similar degree as vitronectin coating (Fig. 10 *B*). When fibers were coated with serum depleted of vitronectin, internalization was reduced to baseline levels. When vitronectin was added back to the immunodepleted serum at 300 μg/ml, but not 30 μg/ml, the increased internalization of coated fibers was restored. There was no difference in fiber adherence (2.8±0.2, external fibers/cell for all conditions; mean±SEM).

The internalization of serum-coated fibers was inhibited by

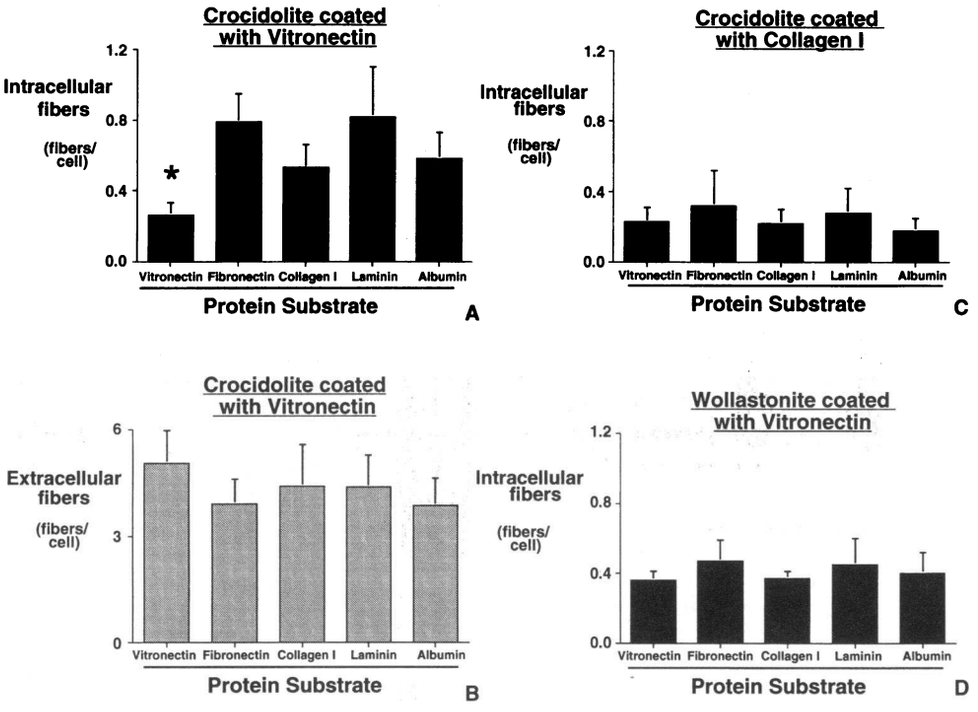

Figure 6. Effect of different extracellular matrix substrates on internalization (*A*, *C*, and *D*) and extracellular binding (*B*) of fibers. Mesothelial cells grown overnight on various protein substrates were labeled with Dil and then incubated with coated fibers for 2 h. Internal (fluorescent) and external (nonfluorescent) fibers were counted in a blinded fashion using fluorescence confocal microscopy. When grown on a vitronectin substrate, mesothelial cells internalized significantly fewer vitronectin-coated crocidolite fibers than when grown on other protein substrates (*A*). Growth on a vitronectin substrate did not alter the external binding of vitronectin-coated crocidolite, indicating that the decrease in internalization was not due to a decrease in binding (*B*). Growth on vitronectin substrates also did not alter the internalization of either collagen-coated crocidolite fibers (*C*) or vitronectin-coated wollastonite fibers (*D*). Data are means±SEM; * different from other protein substrates; $P = 0.035$, $n = 6$ experiments; 120 cells counted in each experiment. Crocidolite used at 3.0 $\mu g/cm^2$, wollastonite used at 6.0 $\mu g/cm^2$.

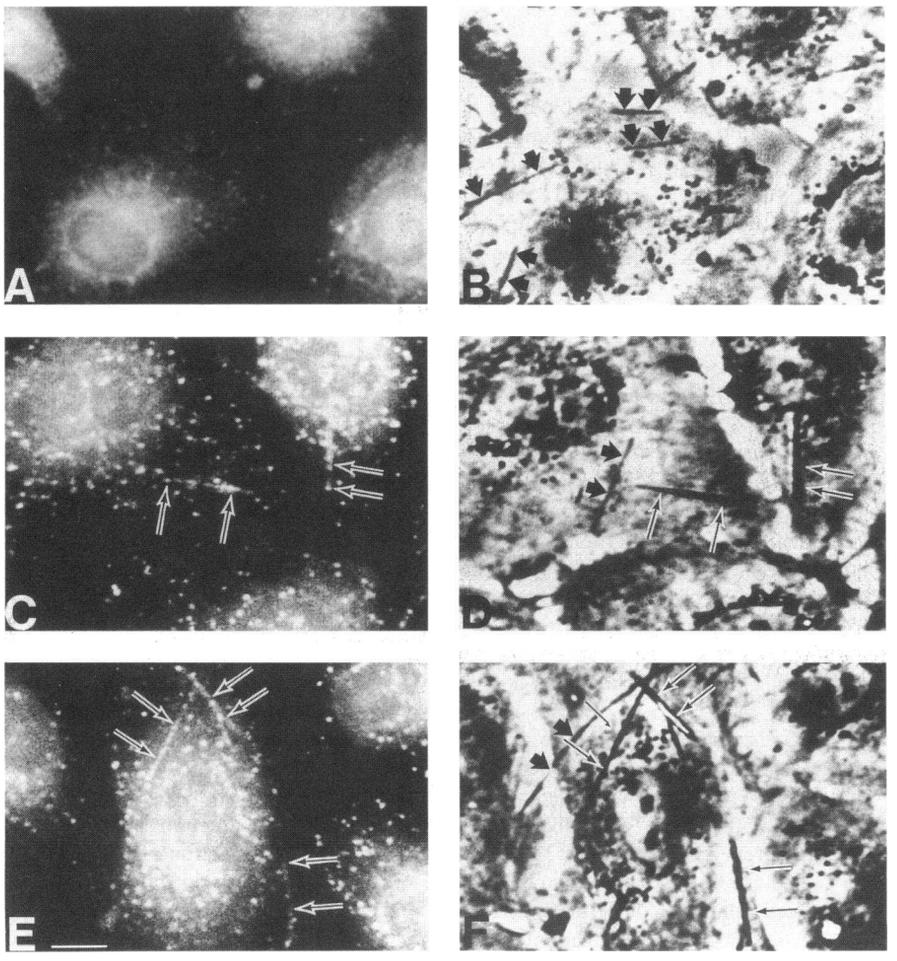

Figure 7. Fluorescence confocal microscopic views (*left*) with corresponding phase-contrast views (*right*) of Dil-labeled mesothelial cells grown overnight on vitronectin (*A* and *B*), fibronectin (*C* and *D*), or laminin (*E* and *F*) substrates and then incubated with vitronectin-coated crocidolite for 2 h. On the phase-contrast views, similar numbers of asbestos fibers are seen (*double arrows*). In the cells grown on a vitronectin substrate (*A*), none of the asbestos fibers are fluorescent whereas in the cells grown on fibronectin (*C*) or laminin (*E*) substrates, many fluorescent fibers are seen (*double arrows*). Fibers seen on phase contrast that correspond to the fluorescent fibers (internal) are marked by long double arrows, and fibers that do not correspond to fluorescent fibers (external) are marked by short, solid double arrows. Bar, 10 μm.

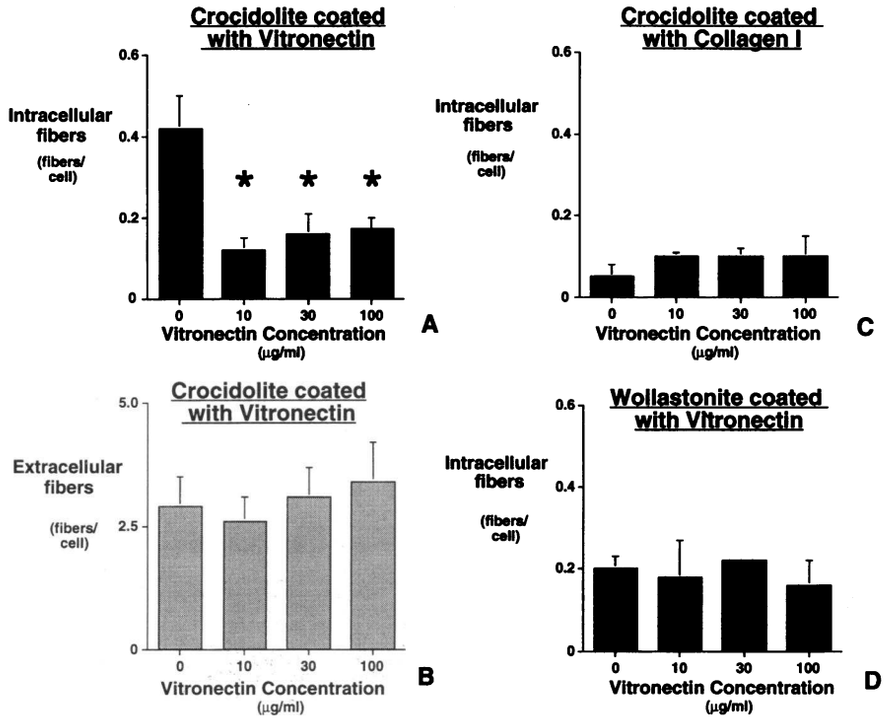

Figure 8. Effect of excess soluble vitronectin on internalization (*A*, *C*, and *D*) and external binding (*B*) of fibers. Mesothelial cells grown on collagen I were labeled with Dil and then incubated with coated fibers together with soluble vitronectin. Soluble vitronectin significantly decreased uptake of vitronectin-coated crocidolite at all concentrations of vitronectin studied (*A*) but did not alter the number of external, adherent fibers (*B*). Soluble vitronectin did not affect uptake of collagen-coated crocidolite (*C*) or vitronectin-coated wollastonite fibers (*D*). Data are means±SEM; * different from no soluble vitronectin; $P = 0.03$, $n = 4$ experiments; 200 cells counted in each experiment. Crocidolite used at 3.0 $\mu g/cm^2$, wollastonite used at 6.0 $\mu g/cm^2$.

blocking integrins with RGD-containing peptides (0.25±0.03 vs 0.05±0.01 internal fibers/cell GRGDSP vs GRGESP, respectively, means±SE; $P = 0.0001$) whereas there was no effect on BSA-coated fibers (0.06±0.03 vs 0.08±0.02 internal fibers/cell, GRGDSP vs GRGESP, respectively). The increased internalization of coated fibers due to serum as well as to depleted serum repleted with 300 μg/ml vitronectin was significantly inhibited by blocking $\alpha v\beta 5$ (Fig. 11).

Distribution of integrins. The two vitronectin receptors, $\alpha v\beta 5$ and $\alpha v\beta 3$, were distributed in different patterns in mesothelial cells (Fig. 12). In cells examined at 4 h, the integrin, $\alpha v\beta 5$, was diffusely distributed while the integrin, $\alpha v\beta 3$, was localized in focal contacts. Interestingly, $\alpha v\beta 5$ colocalized with many, but not all, vitronectin-coated crocidolite fibers. This colocalization was apparent with vitronectin-coated crocidolite fibers, but with neither collagen I–coated crocidolite fibers nor

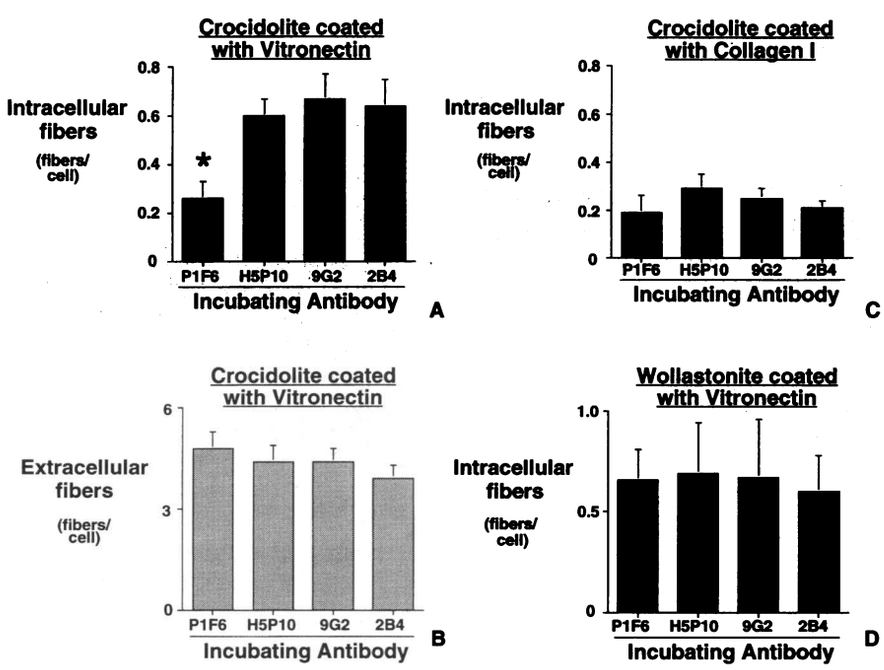

Figure 9. Effect of neutralizing antibody to $\alpha v\beta 5$ (P1F6) on internalization (*A*, *C*, and *D*) and binding (*B*) of vitronectin-coated crocidolite fibers and control fibers. Vitronectin or collagen I–coated fibers were incubated with Dil-labeled mesothelial cells for 2 h in the presence of the following antibodies (50 μg/ml): a blocking antibody to $\alpha v\beta 5$ (P1F6), nonblocking antibody to the αv subunit (H5P10), blocking antibody to $\alpha v\beta 3$ (9G2), or nonblocking antibody to $\alpha v\beta 3$ (2B4). Counts of internal (fluorescent) and external (nonfluorescent) fibers were conducted in a blinded fashion using fluorescence confocal microscopy. The blocking antibody to $\alpha v\beta 5$ (P1F6) significantly decreased internalization of vitronectin-coated crocidolite fibers (*A*) without altering the adherence of the fibers (*B*). The anti-integrin antibodies did not inhibit internalization of collagen-coated crocidolite fibers (*C*) or vitronectin-coated wollastonite fibers (*D*). Data are means±SEM; * different from other antibodies; $P = 0.04$, $n = 7$ experiments; 180 cells counted in each experiment. Crocidolite used at 3.0 $\mu g/cm^2$, wollastonite used at 6.0 $\mu g/cm^2$.

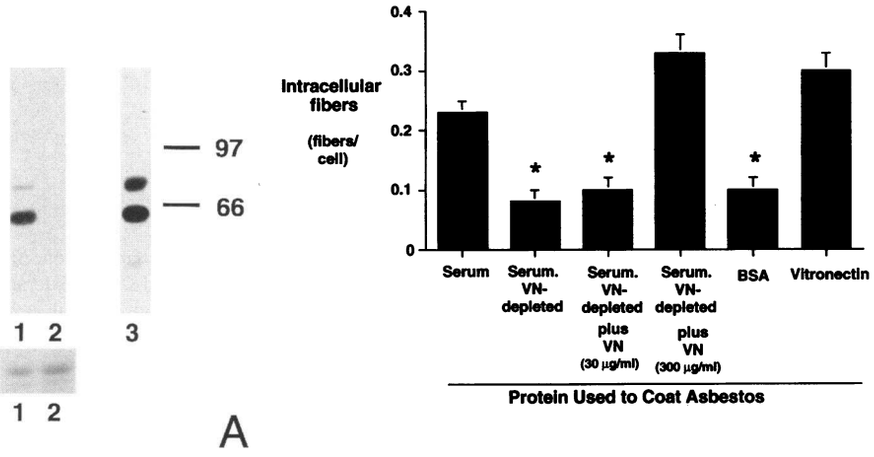

Figure 10. Effect of serum on internalization of crocidolite asbestos and the role of vitronectin in serum in this internalization as demonstrated by immunodepletion experiments. (*A*) Immunoblot of vitronectin from serum (lane *1*), serum depleted of vitronectin using immunoaffinity chromatography (lane *2*), and the eluate from the affinity column (lane *3*). Equal volumes were analyzed by SDS-PAGE under reducing conditions with molecular weight standards. The immunoblots were stained with anti–human vitronectin polyclonal antiserum followed by an alkaline phosphatase–conjugated secondary antibody. Lower panel shows Coomassie blue staining confirming that similar amounts of protein were loaded in lanes *1* and *2*. (*B*) The effect of coating with serum, serum depleted of vitronectin, and vitronectin-depleted serum repleted with vitronectin on crocidolite internalization by rabbit pleural mesothelial cells. Crocidolite fibers were incubated with serum (neat), serum immunodepleted of vitronectin (neat), vitronectin-depleted serum with added vitronectin (30 or 300 μg/ml), BSA (30 μg/ml), or vitronectin alone (30 μg/ml). For these studies coated fibers were washed and then incubated with Dil-labeled mesothelial cells for 2 h in a protein-free medium at 3.0 $\mu g/cm^2$. Internal (fluorescent) and external (nonfluorescent) fibers were counted in a blinded fashion using fluorescence confocal microscopy. Data are means±SEM; * different from serum; $P < 0.01$, $n = 3$ experiments; 200 cells counted in each experiment.

vitronectin-coated wollastonite fibers (not shown). The distribution of $\alpha v\beta 5$ and $\alpha v\beta 3$ and the colocalization of $\alpha v\beta 5$ was not detectably different in cells plated on vitronectin or collagen, although the staining of $\alpha v\beta 3$ in focal contacts was brighter in cells plated on vitronectin.

Toxicity after exposure to crocidolite. At the concentrations at and above those used in these studies, crocidolite asbestos did not cause toxicity as judged by assays of lactate dehydrogenase in the medium or chromium release from chromium-loaded cells (Table II). Cell detachment was negligible in all groups. At incubations of 4, 6, and 8 h, there was also no significant increase in chromium release from asbestos-exposed cells compared to control cells.

By 6 h, mesothelial cells exposed to vitronectin-coated crocidolite (3.0 $\mu g/cm^2$) demonstrated a change in shape from cuboidal and flattened to spindle-like and retracted. The blocking antibody to $\alpha v\beta 5$, but not other antibodies, inhibited the shape change (Fig. 13).

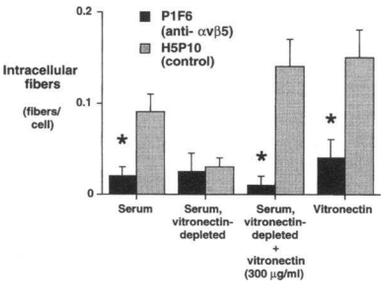

Figure 11. Effect of antibody to $\alpha v\beta 5$ on internalization of crocidolite fibers coated with serum, serum immunodepleted of vitronectin, depleted serum reconstituted with vitronectin (300 μg/ml), and vitronectin (30 μg/ml). Coated fibers were incubated with Dil-labeled mesothelial cells for 2 h in the presence of blocking antibody to $\alpha v\beta 5$ (P1F6) or nonblocking antibody to the αv subunit (H5P10). Counts of internal fibers were conducted in a blinded fashion using fluorescence confocal microscopy. Data are means±SEM; * different from control antibody; $P < 0.003$, $n = 3$ experiments; 200 cells counted in each experiment.

Discussion

In this study, crocidolite asbestos has been shown to interact with mesothelial cells via a specific cell surface receptor leading to enhanced internalization of the fiber. We initially found that internalization of crocidolite asbestos was enhanced significantly by the adsorption of vitronectin on its surface. The effect of vitronectin was shown to be specific because internalization increased independently of any change in the external binding of crocidolite and because internalization was greater only for vitronectin-coated crocidolite, not for collagen-coated crocidolite or for a different fiber, wollastonite, even when also coated with vitronectin. The enhanced internalization was mediated by a receptor for vitronectin, specifically the integrin $\alpha v\beta 5$. The association of this integrin and fiber was supported by the finding that $\alpha v\beta 5$ colocalized immunocytochemically with the vitronectin-coated asbestos fibers. In addition to internalization, the interaction of fiber and receptor led to a cell shape change not associated with evidence of toxicity.

The strategy for determining that the vitronectin-induced internalization was due to a specific receptor had four stages. First, RGD-containing peptides were used to block integrins recognizing vitronectin via an RGD tripeptide sequence. RGD-containing peptides blocked the effect of the vitronectin coating on internalization, suggesting that an integrin or integrins mediated the effect of vitronectin. Second, vitronectin was used as a substrate with the aim of sequestering vitronectin receptors at the cell base making them less available for ligand binding at the apex. Compared to other extracellular matrix substrates, the vitronectin substrate was effective in inhibiting the vitronectin-induced internalization of crocidolite. In previous studies, the use of bound ligand to reduce the number of available apical receptors successfully demonstrated the role of Fc receptors in macrophage phagocytosis (29), of fibronectin receptors in the uptake of fibronectin-bound beads (30), and of the integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ in internalization of adenovirus (31, 32). It has recently been shown that cells grown on vitronectin have

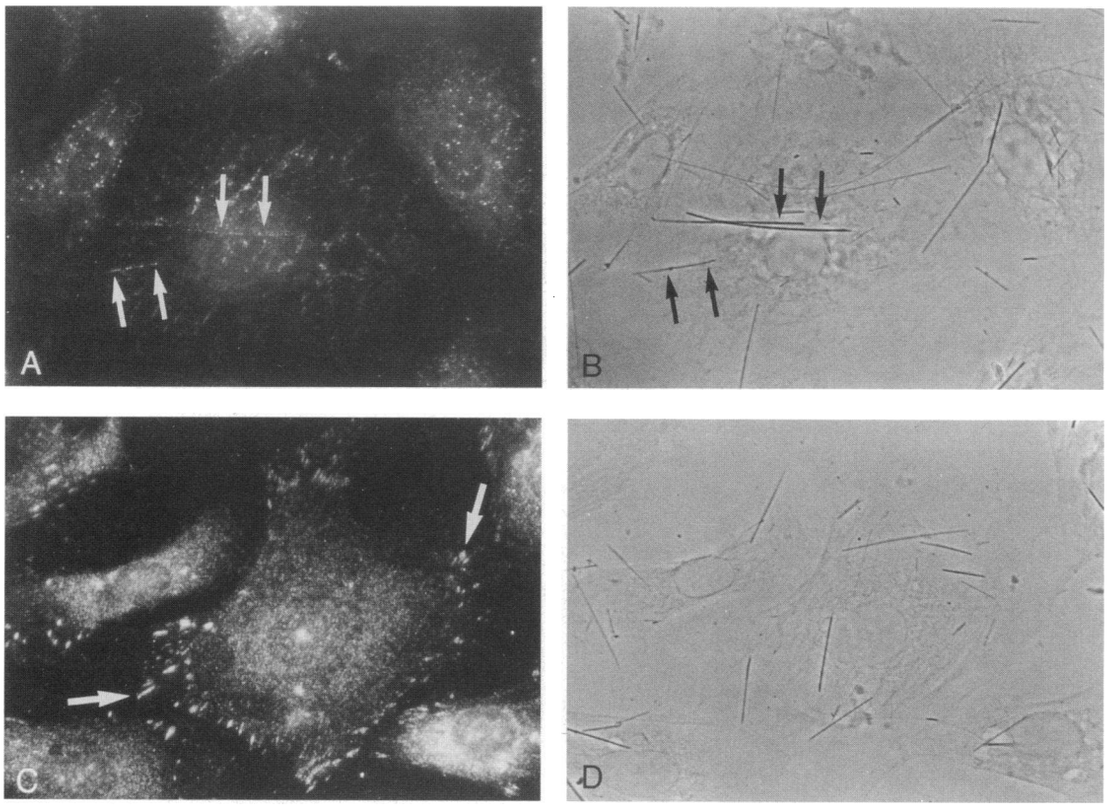

Figure 12. Immunofluorescent localization of vitronectin receptors in rabbit pleural mesothelial cells exposed to vitronectin-coated crocidolite asbestos. Rabbit pleural mesothelial cells grown overnight on a vitronectin substrate were exposed to vitronectin-coated crocidolite asbestos for 4 h, fixed, permeabilized, and stained with hybridoma supernatants immunoreactive to $\alpha v\beta 5$ (P1F6) or $\alpha v\beta 3$ (9G2). Fluorescent views (*left*) show the distribution of $\alpha v\beta 5$ (*A*) and $\alpha v\beta 3$ (*C*), and corresponding phase-contrast views (*right*) show the location of asbestos fibers. The $\alpha v\beta 5$ complex colocalizes with some of the fibers overlying the cells (*A* and *B, double arrows*) whereas the $\alpha v\beta 3$ complex does not colocalize with fibers and is seen in areas of focal contacts (*C, arrows*). (×700.)

an ~ 50% decrease in binding of radiolabeled antibodies to the αv subunit compared to cells grown on other substrates (32). Third, soluble vitronectin was added to compete for those integrins functioning as vitronectin receptors. Vitronectin, and not albumin, was able to block the vitronectin-enhanced internalization. Finally, after confirmation that mesothelial cells expressed two vitronectin receptors $\alpha v\beta 5$ and $\alpha v\beta 3$, specific antibodies were used to block their function. P1F6 has previously been used to block the uptake of conformationally altered vitronectin by $\alpha v\beta 5$, at least in human cells (33). 9G2 has been used in both human and rabbit cells to block $\alpha v\beta 3$ (21). Here, we showed that both antibodies recognized their receptors by flow cytometry, immunoprecipitated the receptors, blocked cell adherence to vitronectin, and stained the receptors in a pattern described for these receptors in other cells (34). Blockade of $\alpha v\beta 5$, not $\alpha v\beta 3$, was able to inhibit the enhanced internalization of vitronectin-coated crocidolite.

Table II. Measurements of Mesothelial Cell Toxicity and Detachment after Exposure to Vitronectin-coated Crocidolite for 2 h

Fiber	Concentration	Lactate dehydrogenase	Chromium release	Cell detachment
	μg/cm²	*percent total*	*percent total*	*percent total*
Crocidolite	0.0	6.8±0.1	9.7±5.0	0.4±0.1
	3.0	7.8±1.6	9.1±0.8	0.9±0.1
	6.0	9.1±0.1	9.0±2.9	0.6±0.8
	12.0	—	8.1±2.5	0.3±0.2

Data are means±SD. Lactate dehydrogenase and chromium release are expressed as percentage of the total contained in an equivalent number of cells. Cell detachment represents the quantity of chromium associated with cells free in the media expressed as a percentage of the total chromium contained in media and adherent cells.

The biological relevance of these findings is strongly supported by experiments in which the vitronectin present in biological liquids was shown to coat fibers and increase their internalization via $\alpha v\beta 5$. Vitronectin, a multifunctional adhesive protein with diverse roles in cell adhesion, opsonization, and inflammation (35, 36), is present in large concentrations in serum (200–400 μg/ml). In addition, vitronectin diffuses into the extravascular tissues where it may bind and become relatively concentrated compared with other serum proteins (37). Vitronectin has been demonstrated to be present, for example, in bronchoalveolar lavage at basal levels and at increased concentrations after inflammation or injury. In our studies, serum, as well as bronchoalveolar lavage and pleural liquid, was shown to coat crocidolite asbestos with vitronectin. Serum coating also enhanced the internalization of crocidolite asbestos, an effect completely removed by depleting serum of vitronectin and re-

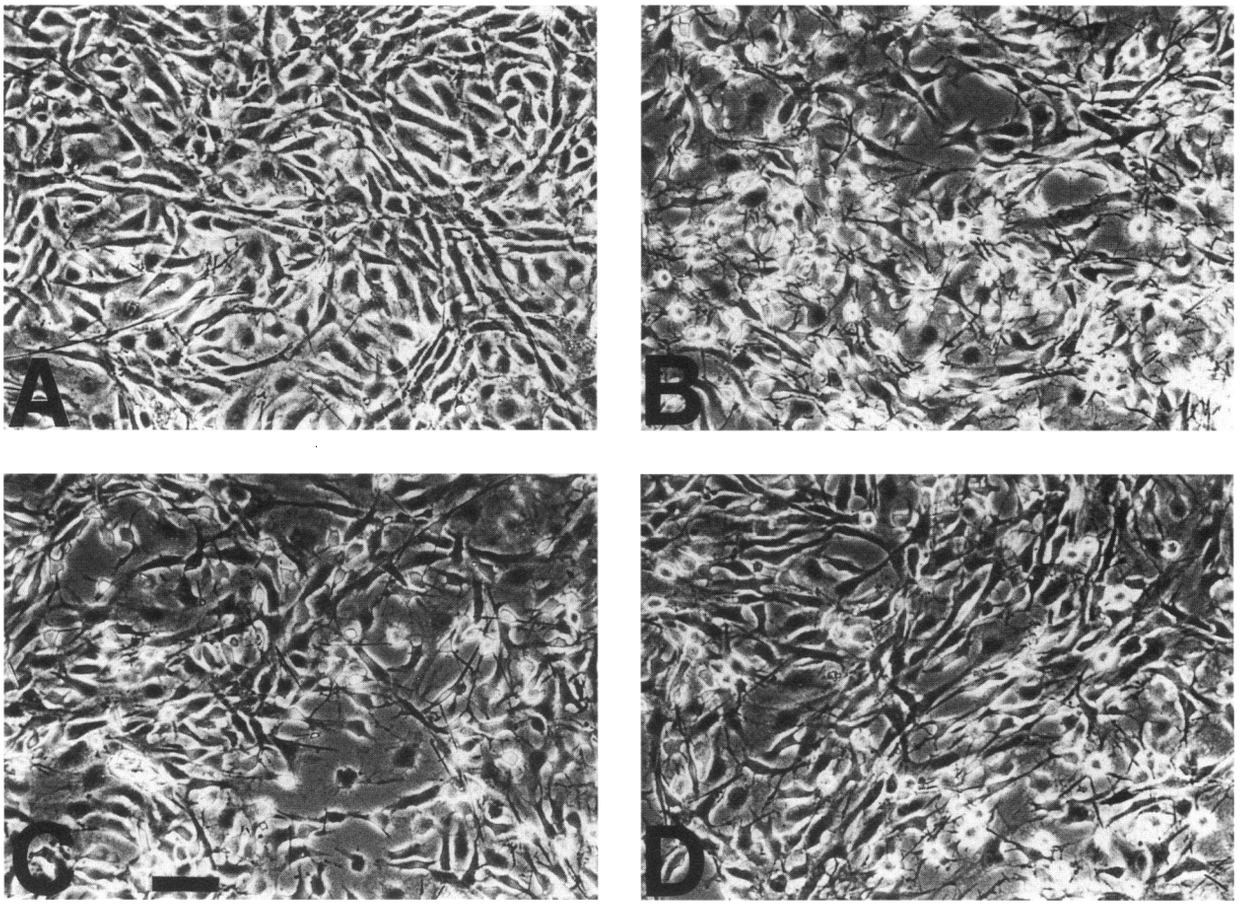

Figure 13. Phase-contrast microscopy of mesothelial cells exposed to vitronectin-coated crocidolite fibers in the presence of anti-integrin antibodies. Rabbit pleural mesothelial cells grown on a collagen I substrate were exposed to vitronectin-coated asbestos (3.0 $\mu g/cm^2$) for 6 h in the presence of blocking antibody to $\alpha v\beta 5$ (P1F6) (*A*), blocking antibody to $\alpha v\beta 3$ (9G2) (*C*), or nonblocking antibodies to αv (H5P10) or $\alpha v\beta 3$ (2B4) (*B* and *D*). Bar, 50 μm.

stored by repleting with vitronectin. Finally, the internalization due to serum coating was shown to be mediated by the integrin, $\alpha v\beta 5$. These data strongly suggest that the mechanisms of uptake outlined in this study would apply to the clinical situation. It is interesting that repletion of depleted serum with 300 μg/ml vitronectin, but not 30 μg/ml, restored the enhanced internalization originally seen in serum. Since purified vitronectin, when used alone, induced maximal uptake at 30 μg/ml, the need for a higher concentration of vitronectin in serum indicates competition for binding by the other proteins in serum. These data are consistent with studies showing that vitronectin in plasma preferentially coats surfaces; nonetheless, to achieve a similar coating, 10 × more vitronectin is needed when used together with other proteins as when used alone (38). Bronchoalveolar lavage liquid, a sampling of alveolar and airway lining fluid, also coated crocidolite with vitronectin, suggesting that when fibers contact the lung at distal airway and alveolar duct bifurcations (39, 40) they become coated with vitronectin. After deposition in the airspaces, asbestos can move into the lung interstitium and to the pleural space (41). Because protein adsorption can be a long lasting, nearly irreversible step (42), it is possible that fibers reaching the pleural mesothelium would remain coated with vitronectin.

The $\alpha v\beta 5$ receptor appeared to account for all the vitronectin-enhanced portion of internalization, as seen after coating with either purified vitronectin or serum. Although $\alpha v\beta 3$ accounted for some of the mesothelial cell binding to vitronectin, there was no inhibition of fiber internalization by the anti-$\alpha v\beta 3$ antibody (9G2) even at high doses. In addition, there was no additional inhibition when the antibodies were used in combination. In all experiments, the percentage of the internalization that could be blocked was the same, whether the blockade was by RGD-containing peptides, soluble vitronectin, vitronectin substrates, or the blocking antibody to $\alpha v\beta 5$. The fact that $\alpha v\beta 5$ accounted for all the vitronectin-enhanced internalization suggests that no other vitronectin receptors, such as $\alpha v\beta 1$ (43), the newly characterized $\alpha v\beta 8$ (44), or other unknown receptors participate in internalization. By cell adhesion data, $\alpha v\beta 5$ and $\alpha v\beta 3$ mediated all cell binding to vitronectin, making the existence of other vitronectin receptors unlikely. Not all internalization was blocked by inhibition of vitronectin receptors; the basal internalization rate (0.1–0.3 fibers/cell) was consistently ~ 30–40% of the total maximal internalization and was unaffected by maneuvers to alter integrin function or number. This basal rate may represent a nonspecific particulate uptake by the mesothelial cells. The increase in internalization of asbestos due to vitronectin-coating above the basal rate could be entirely accounted for by $\alpha v\beta 5$.

Through its interaction with the vitronectin coat, the $\alpha v\beta 5$ receptor specifically mediated the internalization of crocidolite fibers. Internalization was clearly not related to external binding and thus to the availability of fibers on the cell surface. External binding, in fact, was unaffected by any of the maneuvers that affected internalization. We can conclude that the binding of fibers, even those coated with extracellular matrix proteins, is not principally due to integrins in mesothelial cells, at least those integrins recognizing RGD-tripeptide epitopes. Because bound fibers were removed by treatment with trypsin/EDTA, it is likely that nonintegrin proteins are responsible for adherence of the fibers to the cell. The specificity of $\alpha v\beta 5$ for crocidolite was also shown by its lack of involvement in the internalization of wollastonite. Although wollastonite was also coated with vitronectin, vitronectin receptors, in general, and $\alpha v\beta 5$, in particular, were apparently not involved in its internalization. Compared to crocidolite, wollastonite may become coated with vitronectin at a different density or with a different orientation so that different regions of vitronectin are accessible to receptors, as has been proposed for fibrinogen binding to crocidolite and chrysotile asbestos (45). Or, even with identical vitronectin coating, wollastonite may be of the wrong shape to engage the receptors in the manner necessary to induce the signal for internalization. Indeed, colocalization of the $\alpha v\beta 5$ receptor could not be detected with the vitronectin-coated wollastonite suggesting either an inability of the vitronectin receptors to detect the vitronectin on the wollastonite surface or a more diffuse interaction that could not be visualized. The pathologic effect of crocidolite is attributed in large part to its long and narrow shape (46). Perhaps, when compared to wider fibers, the narrower fibers can better cluster the $\alpha v\beta 5$ receptors and thereby induce integrin-mediated signals such as internalization. Whether internalization of fibers is critical for their pathogenicity is not yet known and can only be answered with techniques allowing identification of internal fibers.

For discriminating readily between the internal fibers and external adherent fibers, a new technique was needed. Our fluorescence confocal technique enabled us to count large numbers of cells with an objective end point in a blinded fashion. The use of the membrane coat to identify internalized fibers was particularly necessary in mesothelial cells, which are extremely flat and did not permit the use of confocal microscopy to localize fibers easily by optical sectioning through the cell. Other techniques have been applied to confirm an intracellular location of fibers, including a combination of scanning and transmission electron microscopy (47–49), scanning electron microscopy with backscatter (50), transmission electron microscopy on macrophages induced to spread by exposure to phorbol ester (51), high voltage electron microscopy with stereoscopic imaging (52), and high resolution, video-enhanced light microscopy (53). Each technique limited viewing to a small number of cells or, often, to sections of cells. Our technique has some limitations for detecting small fibers due to the fluorescent labeling of endocytotic vesicles and of the endoplasmic reticulum. Small fibers or particles, such as riebekite, cannot be distinguished from endocytotic vesicles, limiting this technique to fibers longer than 2–3 μm. Also, signals from small fibers in the juxtanuclear region can be obscured by the labeling of the endoplasmic reticulum. Fortunately, long fibers are easily distinguished from vesicles and are not obscured by endoplasmic reticular staining because they extend beyond the juxtanuclear region. It is these longer fibers that are the ones most associated with pathological effects of asbestos, both in vitro and in vivo (46). This technique therefore offers a means of identifying and quantifying internalization of the fibers most toxic to cells.

The vitronectin receptors we detected on the mesothelial cells, $\alpha v\beta 5$ and $\alpha v\beta 3$, have different roles in internalization, in ligand recognition and in cell distribution. Interestingly, $\alpha v\beta 5$ has been shown to mediate the internalization, but not the binding, of conformationally altered vitronectin by cells (33). The integrin $\alpha v\beta 3$ did not have a role in vitronectin uptake in that study, although it was not reported whether the cells expressed $\alpha v\beta 3$. Because the vitronectin on the surface of crocidolite is likely to be in the conformationally altered form, the role of $\alpha v\beta 5$ in uptake of conformationally altered vitronectin may be important for the uptake of the coated fiber. Both $\alpha v\beta 5$ and $\alpha v\beta 3$ mediate the internalization, but again not the binding, of adenovirus via an interaction with RGD sequences on the viral protein coat (31). However, the engagement of $\alpha v\beta 5$, not $\alpha v\beta 3$, leads to an increase of permeability of the cell membrane promoting entry of viral genome into the cytoplasm (32). Although both receptors recognize ligands via the RGD site, $\alpha v\beta 5$ adhesion is basically restricted to vitronectin, whereas $\alpha v\beta 3$ has a wider range of ligand specificity including fibronectin, fibrinogen, vWf, and collagen (54). In our cells, as has been described in others (34), the integrins demonstrated a significant difference in distribution. $\alpha v\beta 5$ was distributed in a diffuse pattern, whereas $\alpha v\beta 3$ localized to focal contacts (see Fig. 12). A new finding of this study, however, was that $\alpha v\beta 5$ colocalized with many of the vitronectin-coated asbestos fibers. The clustering of receptors resulting from such colocalization may induce the signals for internalization or perhaps for other cell responses.

Internalization of asbestos may be important for its biologic effects. Major theories for the biological activity of asbestos include the generation by asbestos of reactive oxygen species, the disruption of mitotic machinery leading to aneuploidy and other chromosomal aberrations, and the transfer via the asbestos surface into the cell of toxins or exogenous DNA (15). In general, it is clear that long and thin fibers (> 8 μm, < 0.2 μm) induce more cell damage and ultimately more experimental mesothelioma than do shorter fibers (46). This well-recognized importance of fiber shape remains unexplained. However, by whatever mechanism asbestos causes injury, internalization of the fibers may be necessary for the injury or may enhance the injury. For example, if oxygen radical formation is important, the intracellular location of the fiber may magnify the effect. Clearly, if fibers interact with cells by mechanically interfering with chromosomal segregation during mitosis, the intracellular location of fibers is critical. Finally, if materials such as DNA, RNA, or toxins adsorbed on asbestos are important, then the internalization of the fiber, and perhaps a membrane permeabilization such as that mediated by $\alpha v\beta 5$ (32), may be important for entry of toxins into the cell. Asbestos has, in fact, been shown to mediate the transfection of cells by exogenous DNA, with an efficiency comparable to experimental methods (55). Questions about the specific role of internalization in mediating the biological effects of asbestos have not yet been addressed.

The interaction of integrins with asbestos may lead to the induction of other signals in addition to that for fiber internalization. Integrins are recognized as important signaling molecules (56) and can activate signals after being clustered, even when cell shape changes such as spreading are prevented (57, 58). Indeed, integrins are also known to induce some of the same signals as does asbestos. In particular, integrin binding initiates

expression of early growth response genes such as jun (59), a response also described in mesothelial cells exposed to asbestos (2). The interaction of vitronectin-coated asbestos with $\alpha v\beta 5$ in our experiments led to internalization within 2 h and later to a change in cell shape not associated with toxicity. Cell shape change, a characteristic consequence of the interaction of integrins with extracellular matrix, suggests a possible role for integrin signals in the response to asbestos. If integrins are important for mediating the biologic consequences of asbestos, then the importance of fiber shape might be explained by longer fibers potentially clustering receptors and inducing signals better than shorter fibers. Integrins can also transduce signals even after they and their ligands are internalized, indicating a possible mechanism by which asbestos could induce persistent signals (58).

In conclusion, pleural mesothelial cells have been found to recognize and internalize vitronectin-coated and serum-coated crocidolite using the specific surface receptor $\alpha v\beta 5$. The interaction of asbestos with a cell surface receptor is a novel finding and one that suggests unique means by which asbestos may interact with cells.

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