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Crocidolite Induces Prostaglandin I₂ Release Mediated by Vitronectin Receptor and Cyclooxygenase-2 in Lung Cells

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Abstract

Interstitial lung disease (ILD) produces disruption of alveolar walls with loss of functionality and scar tissue accumulation. Asbestosis is the ILD produced by the inhalation of asbestos fibers. This study attempts to elucidate the role of lung epithelial cells in the generation of asbestos-induced ILD. When exposed to crocidolite LA-4 cells had a decrease in viability and an increase in the release of lactate dehydrogenase (LDH) and 6-keto PGF_{1α}, a PGI₂ metabolite. PGI₂ release was mediated by cyclo-oxygenase-2 (COX-2) and vitronectin receptor (VNR). When LA-4 cells were treated with VNR inhibitors, either RGD (Arg-Gly-Asp) peptide or VNR blocking antibody, a statistically significant decrease in PGI₂ metabolite production was observed, but crocidolite-induced cytotoxicity was not prevented. These findings propose that crocidolite is coated by an RGD protein and binds VNR-inducing COX-2 expression and PGI₂ release. Moreover, when LA-4 cells were exposed to crocidolite in the presence of reduced serum culture media, PGI₂ production was prevented, and when bronchoalveolar lavage fluid (BALF) was added, PGI₂ production was rescued. Cytotoxicity did not occur, either in reduced serum culture media or when BALF was added. In conclusion, crocidolite requires the presence of an RGD protein coating the fibers to induce inflammation (PGI₂ production) and crocidolite alone cannot induce cytotoxicity in lung cells.

Keywords

Crocidolite; Pulmonary fibrosis; Cyclooxygenase 2; Prostaglandin I₂; Vitronectin receptor

Introduction

Asbestos fibers are a naturally occurring hydrated silicate mineral used for commercial applications. It has been reported that over the last century, more than 30 million tons of asbestos have been mined, processed, and applied in the United States [1]. Exposure to asbestos typically occurs during mining and milling of the fibers or during industrial application of asbestos in textiles, insulation, shipbuilding, brake lining mechanics, and other areas. Nonoccupational exposure is usually related to asbestos fibers inadvertently released into the environment and transported by asbestos-contaminated clothing or other materials [2].

Asbestos has two primary classes: serpentine and amphibole. Structurally, serpentine fibers are curly-stranded structures and amphibole fibers are straight, rigid, and needlelike. Three examples of amphibole fibers are crocidolite, tremolite, and amosite [3]. Regardless of their different physical properties, all types of asbestos fibers are considered fibrogenic to the lung, but some of them, including amphibole, are also considered carcinogenic.

Asbestosis is an interstitial lung disease (ILD) produced by the inhalation of asbestos fibers. ILD is a large and heterogeneous group of lower respiratory tract disorders. ILD is also known as “interstitial pulmonary fibrosis” or “diffuse parenchymal lung disease” [4,5]. The cause of most ILDs is unknown, but it has been suggested that a stimulus (e.g., asbestos, beryllium) produces repeated episodes of lung injury and the damaged alveolar epithelium induces accumulation and activation of immunoinflammatory cells, with subsequent migration and proliferation of fibroblasts and extracellular matrix deposition in the alveolar interstitium leading to fibrosis and loss of lung function [6,7].

Coating crocidolite asbestos with vitronectin enhances its cell internalization via the alpha V integrin VNR (vitronectin receptor) $\alpha_v\beta_5$ in mesothelial cells [8]. VNR and other integrins recognize the RGD (Arg-Gly-Asp) motif present in a variety of ligands such as vitronectin, fibronectin, osteopontin, bone sialoprotein, thrombospondin, fibrinogen, von Willebrand factor, tenascin, and agrin [9–12]. Additional studies have reported the involvement of VNR and RGD motif in crocidolite internalization and toxicity [13–15]. In endothelial cells, an increase in the release of PGI₂ has been reported after asbestos exposure [16,17]. It has been suggested that changes in the PGI₂ homeostasis could lead to endothelial damage, cell activation, and inflammation contributing to the development of ILD.

After asbestos inhalation, fibers deposited in the lungs typically remain in close contact with lung epithelial cells. Since this fiber-cell interaction could potentially initiate or inhibit cellular functions, the purpose of this study was to characterize the cellular response of lung epithelial cells directly exposed to asbestos fibers.

Methods

Cell Line

LA-4 cells (lung adenoma clone 4) derived from A/He strain mouse, characterized as alveolar type II cell like [18], were cultured in Ham's F12 K medium (Mediatech, Inc., Herndon, VA) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B (Mediatech), and 15% fetal bovine serum (FBS) (Hyclone, Logan, UT). Cell cultures were incubated at a temperature of 37°C in an atmosphere of 95% air and 5% CO₂ (ThermoForma, Mariette, OH).

Asbestos Exposure

All asbestos exposure experiments were performed using the amphibole crocidolite, which was chosen due to its high toxicity. Adherent cells were trypsinized with 2–3 ml of trypsin/EDTA (Mediatech), counted using a Z1 Coulter Particle Counter (Beckman Coulter, Hialeah, FL), and plated 24 h prior to exposure at 50,000 cells in a volume of 200 µl of medium per well in a 96-well plate. Cell culture medium was changed at exposure time. Crocidolite suspension (10 mg/ml) was freshly made by mixing crocidolite obtained from Research Triangle Institute (Research Triangle Park, NC) with pure water. The suspension was sonicated using a Sonicator Ultrasonic processor XL (Misonix, Farmingdale, NY) for 1 min, mixed with fresh cell culture medium, and added to the 96-well plate. Cells were exposed to crocidolite for 24 h before performing any assessment unless indicated.

Cell Cytotoxicity Assays

Viability Assay—After exposure, culture medium was removed leaving 100 μ l of medium in each well and 20 μ l of CellTiter96 AQueous One Solution Reagent (Promega, Madison, WI) was added into each well and incubated for 1–4 h at 37°C in a humidified 5% CO₂ atmosphere. Optical density (OD) was measured using a microplate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 490 nm and for reference at 650 nm. For normalizing purposes each control group (nonexposed) was considered to have 100% viability.

Cytotoxicity Assay—Lactate dehydrogenase (LDH) release was measured using the Cytotoxicity Detection Assay (LDH) (Roche Applied Science, Indianapolis, IN). Cells were plated 24 h before exposure as previously described. At exposure time, cell culture medium was replaced with 1% FBS in order to decrease the LDH background absorbance, as recommended by the manufacturer. Cells exposed to Triton X-100 (Sigma-Aldrich Inc., St Louis, MO) at 1% were used as positive control. After exposure, 100 μ l of cell culture medium was transferred to a 96-well plate, mixed with 100 μ l of reaction mixture as described by manufacturer, and incubated for 30 min at room temperature protected from light. OD was measured at wavelengths of 492 and 650 nm. Cytotoxicity was calculated as a percentage between minimum or 0% (nonexposed) and maximum or 100% (Triton X-100) control values.

6-Keto Prostaglandin F_{1 α} Detection Assay

PGI₂ is nonenzymatically hydrated to become 6-keto PGF_{1 α} , which is a more stable metabolite. In order to detect PGI₂, experiments were conducted to measure its metabolite 6-keto PGF_{1 α} . Cells were plated at 40,000–50,000 cells/well in a 96-well plate. After 24 h of exposure, as previously described 6-keto PGF_{1 α} was detected using an ACE competitive enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. For each condition, 50 μ l of cell culture medium was transferred to a precoated 96-well plate, mixed with 6-keto PGF_{1 α} tracer and antiserum, and incubated for 18 h at 4°C. Plates were developed using Ellman's reagent (Cayman Chemical, Ann Arbor, MI) and read at a wavelength of 405 nm.

Cyclooxygenase Inhibitors

The COX-1/COX-2 inhibitor indomethacin and the COX-2 inhibitor indomethacin heptyl ester were used at doses 10 and 100 times their respective IC₅₀ [19]. Inhibitors were added at the same time that fresh cell culture medium containing crocidolite was added to the cells as previously described.

Cyclooxygenase-2 mRNA Quantification by Real-time PCR

Two to four hours after crocidolite exposure (10 μ g/cm²), LA-4 cells were lysed using Trizol reagent (Invitrogen, Carlsbad, CA) and total RNA was isolated following the manufacturer's instructions. First-strand reverse transcription was performed using RETROscript™ (Ambion Inc., Austin, TX) with oligo-dT primers, and amplification was performed using the Platinum® Quantitative PCR Super-Mix-UDG (Invitrogen) following the manufacturer's recommendations. Real-time probes and primers from murine COX-2 and GAPDH (endogenous control) were obtained from TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA). Amplification was run and signals detected using an ABI Prism 7700 Sequence Detection System (Applied Biosystems).

Vitronectin Receptor Expression

Three hundred thousand LA-4 cells were labeled with fluorescent murine-specific anti- α_v antibody (anti-CD51, RMV-7, Armenian hamster IgG, phycoerythrin-labeled [BD PharMingen, San Jose, CA]). After 20 min of incubation at 4°C, fluorescence was detected on

a FACs Aria Flow cytometer using FACs Diva software (Becton Dickson, Franklin Lakes, NJ). In order to exclude dead cells, Hoechst 33258 dye (Invitrogen) was added to a final concentration of 1 $\mu\text{g/ml}$. Cells that took up the dye were considered dead cells and excluded from the analysis.

Vitronectin Receptor Inhibition

RGDS Peptide—The tetrapeptide Arg-Gly-Asp-Ser or RGDS (Sigma-Aldrich, St Louis, MO) was used as a competitive inhibitor of VNR binding, and the Arg-Gly-Glu-Ser or RGES (Sigma-Aldrich) was used for nonspecific binding control. Peptides were added at a final concentration of 10 and 100 $\mu\text{g/ml}$ simultaneously with crocidolite exposure as previously described.

VNR Blocking Antibody (RMV-7)—The blocking antibody RMV-7 was used to specifically inhibit VNRs. RMV-7 antibody was added at a final concentration of 2 $\mu\text{g/ml}$ at 1 h before crocidolite exposure as previously described.

Bronchoalveolar Lavage Fluid (BALF) Isolation

BALB/c mice were obtained from our animal facility. Mice 6–8 weeks old were used for BALF isolation. All animal procedures were approved by the University of Montana Institutional Animal Care and Use Committee (IACUC). Five female mice were euthanized by a lethal injection of pentothal. Lungs were surgically removed with the heart and lavaged one time only to prevent dilution of the BALF. The lavage was done using 0.9 ml of cold sterile phosphate-buffered saline containing 100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 0.25 $\mu\text{g/ml}$ amphotericin B (Mediatech). All samples were mixed and centrifuged at 1500 rpm for 10 min. The BALF was separated and the cell pellet discarded.

Asbestos Exposure in the Presence of BALF

LA-4 cells were trypsinized and plated as previously described. At exposure time the medium was changed to Optimem[®] I reduced serum medium (Mediatech). Then BALF was added to attain a final concentration of 25%, followed by crocidolite at a final concentration of 10 and 15 $\mu\text{g/cm}^2$. Cells were incubated for 24 h before performing any measurement.

Statistical Analysis

Experiments were repeated at least three times. Results are presented as the mean and SEM unless otherwise indicated. When required, data were normalized with their respective controls in order to exclude differences in background conditions. Data were analyzed using the *t* test for independent variables, nonparametric statistics methods such as the Mann-Whitney U test when comparing two groups, and the Kruskal-Wallis test when comparing three groups or more. Statistical analyses were performed using GB-Stat version 6.5.6. Alpha error was set at $P < 0.05$.

Results

Crocidolite Asbestos is Cytotoxic to LA-4 Lung Epithelial Cells

To determine if crocidolite could induce toxicity in LA-4 cells, a viability assay was performed using increasing doses of crocidolite. Three doses of crocidolite were used: 5, 10, and 20 $\mu\text{g/cm}^2$, and Triton X-100 1% was used for positive control. LA-4 cells showed a dose-dependent decrease in viability (Fig. 1a). To confirm these results, a cytotoxicity assay was performed using the crocidolite dose of 20 $\mu\text{g/cm}^2$. The results showed an increase in LDH release in the exposed group, confirming the results of the viability assay (Fig. 1b).

Crocidolite Asbestos Promotes COX-2-dependent PGI₂ Release from LA-4 Lung Epithelial Cells

Asbestos is known to induce lung inflammation that progresses to fibrosis. PGI₂ release is an indicator of cellular activation and inflammation. Endothelial cells release PGI₂ when exposed to asbestos prompting speculation that epithelial cells may respond in a similar way. To address this, it was necessary to verify whether LA-4 cells release PGI₂ after crocidolite exposure. PGI₂ production was determined by measuring its metabolite 6-keto PGF_{1 α} in LA-4 cells after crocidolite exposure at a final concentration of 10 $\mu\text{g}/\text{cm}^2$ for 24 h. A statistically significant increase of PGI₂ metabolite was observed in the exposed group (Fig. 1c). Since PGI₂ synthesis requires cyclooxygenase activity, COX inhibitors were used to determine if PGI₂ synthesis was either COX-1- or COX-2-mediated. Indomethacin (COX-1 and COX-2 inhibitor) and indomethacin heptyl ester (COX-2 inhibitor) decreased the production of PGI₂ metabolite after crocidolite exposure (Fig. 1d), suggesting that COX-2 was required for the increase in PGI₂ production.

Based on these findings using COX inhibitors, COX-2 mRNA was quantified using real-time PCR to determine whether asbestos induced its expression. COX-2 mRNA levels increased more than five times 2 h after exposure, returning to its basal concentration 4 h after exposure (Fig. 2a), suggesting a transitory increase.

Asbestos Toxicity is not Dependent on Cyclooxygenase Activity

To determine whether COX-1 and/or COX-2 contributed to asbestos-induced cytotoxicity, COX inhibitors at increasing doses were used before asbestos exposure. After 24 h of exposure, COX inhibitors did not protect LA-4 cells from asbestos-induced cytotoxicity (Fig. 2b, c). These findings suggest that LA-4 cells have the ability to respond in two different ways after crocidolite exposure: A cytotoxic response, which is COX-independent, and a PGI₂ response, which is COX-2-dependent.

The Vitronectin Receptor Plays an Important Role in Asbestos-induced PGI₂ Production

As previously described, asbestos coated with vitronectin binds mesothelial cells through the vitronectin receptor (VNR). Therefore, we determined if asbestos-induced cytotoxicity and inflammation required lung epithelial cell VNR ligation. Antibodies recognizing the α_v subunit detected VNR expression on the surface of LA-4 cells. This finding verified that LA-4 cells constitutively express the α_v subunit, which is the common subunit in all VNR (Fig. 2d). To determine if the PGI₂ release was VNR-mediated, inhibitors of VNRs were used. In the groups exposed to crocidolite (20 $\mu\text{g}/\text{cm}^2$) and treated with either RGDS peptide or VNR blocking antibody, a statistically significant decrease in PGI₂ metabolite production was observed, confirming VNR involvement in the PGI₂ release (Fig. 3a, b).

Bronchoalveolar Lavage Fluid Proteins are Required for Asbestos-induced PGI₂ Production

In all previous experiments involving VNR it was assumed that the crocidolite fibers were coated with the extracellular matrix (ECM) proteins present in the FBS of the cell culture medium. To verify this assumption and to establish that the required ECM proteins are also present in the BALF, LA-4 cells were exposed to crocidolite in the presence of reduced serum medium and BALF. LA-4 cells in reduced serum medium did not release PGI₂ when exposed to crocidolite; however, when BALF was added to the reduced serum medium, LA-4 cells released PGI₂ when exposed to crocidolite (Fig. 4a). This finding suggests that proteins present in BALF and also in FBS are required in order to induce the release of PGI₂. When viability was evaluated in the presence of reduced serum, LA-4 cells were protected from crocidolite-induced toxicity and the addition of BALF did not increase the toxicity (Fig. 4b), suggesting

that factors present in the FBS are required in order to induce cell toxicity of LA-4 cells when exposed to crocidolite.

Asbestos Toxicity is not Dependent on VNR

As previously established, the cytotoxic response is COX-independent. To determine whether a protein containing a RGD motif or whether VNR was required for asbestos-induced cytotoxicity in LA-4 cells, cell toxicity assays were performed using RGDS peptide and VNR blocking antibody. Neither RGDS peptide nor VNR blocking antibody prevented crocidolite-induced cytotoxicity (10 $\mu\text{g}/\text{cm}^2$) of LA-4 cells (Fig. 5a, b).

Discussion

Asbestos is known to induce lung inflammation that progresses to fibrosis. It has been postulated that ILD develops in an inflammatory environment with abnormal repair mechanisms. Crocidolite-induced direct damage to lung cells such as macrophages and epithelial cells (e.g., alveolar type I and type II cells) may generate the conditions required for ILD development. Alveolar type II cells exemplify the importance of epithelial cells in ILD pathogenesis since they secrete pulmonary surfactant (to prevent alveolar collapse), and as a consequence of their stem cell function, they replace the damaged type I cells [20]. Our study used the immortalized murine cell line LA-4, which had previously been reported to be of alveolar type II cell origin [18], to study the role of lung epithelial cells in asbestos-induced response. Our results show that LA-4 cells were susceptible to crocidolite-induced toxicity.

PGI₂ release, a product of arachidonic acid metabolism, is an indicator of asbestos-induced cell activation and inflammation [16,17]. Interestingly PGI₂ is highly unstable since it is spontaneously nonenzymatically hydrated to 6-keto PGF_{1 α} , a more stable metabolite. In this study 6-keto PGF_{1 α} production was considered an indicator of PGI₂ synthesis. PGI₂ has diverse effects in endothelial cells, epithelial cells, macrophages, and fibroblasts. Curiously, all these different cells are known to play a role in ILD development. This study has shown that the LA-4 epithelial cells produce PGI₂ after asbestos exposure. This finding raises the possibility that epithelial production of PGI₂ following asbestos exposure may modulate the function of several cell types involved in the development of ILD.

PGI₂ synthesis requires the action of the COX enzymes COX-1 (constitutively expressed) and COX-2 (inducible). Several studies have suggested that PGI₂ biosynthesis is highly but not solely COX-2-dependent [21,22]. Lovgren et al. [23] reported that COX-2-dependent PGI₂ production played an important role in the development of lung fibrosis in the bleomycin-induced pulmonary fibrosis mouse model. Murakami et al. [24] reported that repeated administration of a long-acting PGI₂ agonist attenuated the development of bleomycin-induced pulmonary fibrosis and also improved survival in these mice. The present study showed that PGI₂ production, induced by crocidolite exposure, was prevented in the presence of COX-2 inhibitors, suggesting that PGI₂ production in LA-4 cells was COX-2-dependent. Moreover, a 2-h crocidolite exposure increased the expression of COX-2 mRNA fivefold in LA-4 cells. This increase was transitory but enough to maintain PGI₂ synthesis for a minimum of 24 h. Based on cited reports and our findings, we propose that lung epithelial cells exposed to crocidolite express COX-2 which promotes PGI₂ production.

The VNRs are a group of integrins that share a common α_v subunit. Murphy et al. [25] reported that engagement of the VNR $\alpha_v\beta_3$ induces COX-1 and COX-2 expression in endothelial cells. There are five different types of VNRs (based on their β subunit): $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$. VNRs $\alpha_v\beta_6$ and $\alpha_v\beta_8$ activate transforming growth factor (TGF β) in lung and skin by binding TGF β latency-associated peptide (LAP), and β_6 -null mice are protected from bleomycin-induced pulmonary fibrosis [26,27]. As previously described, coating crocidolite

asbestos with vitronectin enhances its internalization via the α_v integrin vitronectin receptor $\alpha_v\beta_5$ in mesothelial cells [8]. Using flow cytometry, the present study showed that LA-4 cells express the α_v integrin subunit. VNR and some other integrins recognize the Arg-Gly-Asp (RGD) motif present in ligands for this receptor, which includes vitronectin. RGD peptides have been used previously as competitive inhibitors for receptors that recognized RGD domain [10,11]. In this study, RGD peptides reduced PGI₂ production by 20% after crocidolite exposure. Although the decrease is statistically significant, it also suggests that other mechanisms that do not depend on the RGD domain recognition are responsible for PGI₂ production after crocidolite exposure. Interestingly, it has been proposed that VNR plays a role in TGF β activation and also that TGF β plays an important role in the development of pulmonary fibrosis [7,27–29]. In this study, when the VNRs were blocked with an antibody specific for the α_v chain, crocidolite-induced PGI₂ production decreased more than 20%, which is consistent with the decrease observed using RGD peptides. These findings are consistent with, and extend, other studies that reported that the VNR $\alpha_v\beta_5$ enhances internalization of vitronectin-coated crocidolite fibers by mesothelial cells [8,14]. In all asbestos exposure experiments detailed in this study, it was assumed that the crocidolite fibers were coated with the ECM proteins present in the FBS of the cell culture medium. Since most of the asbestos exposure is typically via inhalation, it was important to demonstrate similar effects at a physiologically relevant concentration of ECM proteins in the BALF. In this study, when LA-4 cells were exposed to crocidolite in the presence of reduced serum culture medium, PGI₂ production was prevented, and when BALF was added, PGI₂ production was rescued. These findings suggest that one or more factors present in BALF (and in FBS) are necessary to induce PGI₂ release in LA-4 cells exposed to crocidolite. Vitronectin is a multifunctional protein with an RGD motif that is recognized by different members of the integrin family, including VNR [30–32]. Vitronectin coats asbestos fibers, enhancing cell internalization. It is a multifunctional adhesive protein present in large concentrations in blood serum [31]. It diffuses into the extravascular tissue where it could bind some other proteins [33]. It has been found in BALF and pleural liquid, and in higher concentrations during inflammatory states [8]. Although this study did not look for the presence of vitronectin in the BALF or FBS, our findings suggest that VNR ligands coat crocidolite fibers, favoring cell interaction and PGI₂ release.

Surprisingly, crocidolite cytotoxicity was prevented in reduced serum culture medium, and the addition of BALF did not rescue the cytotoxicity. This finding favors the idea that a factor present in FBS (e.g., ECM proteins) and not in BALF is required to induce cytotoxicity, possibly binding to a receptor other than the VNR (e.g., the fibronectin receptor VLA-4). It is also possible that the same factor is present normally in very low amounts in BALF, but during lung injury and inflammatory states, production of the factor increases allowing crocidolite-induced cytotoxicity. Interestingly, LA-4 cells were also not protected from cytotoxicity by RGD peptides or VNR blocking antibodies. It should be noted that the RGD domain is not the only one recognized by integrins (e.g., CS-1 domain) and that the RGD domains of some ECM proteins involved in cytotoxicity have higher affinity for their respective receptors than the RGD peptides, reducing their ability to compete. The finding that VNR inhibitors did not prevent crocidolite-induced cytotoxicity strengthens the idea that LA-4 cells respond in two different pathways after crocidolite exposure: a PGI₂ response, which is COX-2- and at least partly VNR-dependent, and a cytotoxic response, which is COX- and VNR-independent (Fig. 6). Both pathways could be associated with the development of lung fibrosis because they could affect the balance of bound and free LAP. Regardless of the pathway, the free LAP is able to bind other VNRs (such as $\alpha_v\beta_6$ or $\alpha_v\beta_8$) expressed in lung epithelial cells such as alveolar type II cells, inducing TGF β activation and promoting healing and/or fibrosis.

Because of the inherent difficulties associated with studying murine alveolar type II cells in situ, this study modeled responses using a previously characterized murine alveolar type II tumor cell. Although this approach has its limitations arising from differences in growth rate

and contact inhibition, which are important and closely regulated in the alveoli environment, our results provide new insights into the pathogenesis of asbestosis that need to be considered in future studies with primary lung epithelial cells (e.g., alveolar type II cells) or animal studies.

The cause of most ILDs is still unknown. In this study, the environmental/occupational agent crocidolite was studied. This agent can activate inflammation and produce direct injury to the resident pulmonary cells [34]. Crocidolite alone could not induce either inflammation (PGI₂ production) or cytotoxicity. The former required the presence of an RGD protein coating the fibers, and the latter required one or more factors present in the fetal bovine serum. Other factors such as ECM proteins (e.g., vitronectin), different cell surface receptors (e.g., integrins), and the cell's activation status may be required to initiate and maintain the response required to produce ILD.

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References

1. Mossman BT, Bignon J, Corn M, Seaton A, Gee JB. Asbestos: scientific developments and implications for public policy. *Science* 1990;247(4940):294–301. [PubMed: 2153315]
2. Kamp DW, Weitzman SA. Asbestosis: clinical spectrum and pathogenic mechanisms. *Proc Soc Exp Biol Med* 1997;214(1):12–26. [PubMed: 9012357]
3. Faust, RA. Toxicity summary for asbestos . 1995 [Accessed 9 June 2009]. <http://www.cira.ornl.gov/documents/Asbestos.pdf>
4. Huaringa, AJ.; Leyva, FJ. Diffuse parenchymal lung disease: a practical approach. 2000 [Accessed 6 Sept 2009]. <http://www.ispub.com/ostia/index.php?xmlFilePath=journals/ijpm/vol1n1/dpld.xml>
5. Leyva, FJ. Asbestos-associated lung disease. 2009 [Accessed 6 Sept, 2009]. http://www.ispub.com/journal/the_internet_journal_of_toxicology/volume_6_number_1_35/article/asbestos_associated_lung_disease.html
6. Clement A, Henrion-Caude A, Fauroux B. The pathogenesis of interstitial lung diseases in children. *Paediatr Respir Rev* 2004;5(2):94–97. [PubMed: 15135117]
7. Gross TJ, Hunninghake GW. Idiopathic pulmonary fibrosis. *N Engl J Med* 2001;345(7):517–525. [PubMed: 11519507]
8. Boylan AM, Sanan DA, Sheppard D, Broaddus VC. Vitronectin enhances internalization of crocidolite asbestos by rabbit pleural mesothelial cells via the integrin alpha v beta 5. *J Clin Invest* 1995;96(4):1987–2001. [PubMed: 7560092]
9. Converse, PJ. Integrin, Alpha-V; ITGAV . [Accessed 2 May 2009]. (updated 21 March 2008) <http://www.ncbi.nlm.nih.gov/entrez/dispmim.cgi?id=193210>
10. Roberts K, Yokoyama WM, Kehn PJ, Shevach EM. The vitronectin receptor serves as an accessory molecule for the activation of a subset of gamma/delta T cells. *J Exp Med* 1991;173(1):231–240. [PubMed: 1702138]
11. Ruoslahti E. The RGD story: a personal account. *Matrix Biol* 2003;22(6):459–465. [PubMed: 14667838]
12. Sheppard D. Integrin-mediated activation of latent transforming growth factor beta. *Cancer Metastasis Rev* 2005;24(3):395–402. [PubMed: 16258727]
13. Liu W, Ernst JD, Broaddus VC. Phagocytosis of crocidolite asbestos induces oxidative stress, DNA damage, and apoptosis in mesothelial cells. *Am J Respir Cell Mol Biol* 2000;23(3):371–378. [PubMed: 10970829]

14. Pande P, Mosleh TA, Aust AE. Role of alphavbeta5 integrin receptor in endocytosis of crocidolite and its effect on intracellular glutathione levels in human lung epithelial (A549) cells. *Toxicol Appl Pharmacol* 2006;210(1-2):70-77. [PubMed: 16169567]
15. Wu J, Liu W, Koenig K, Idell S, Broaddus VC. Vitronectin adsorption to chrysotile asbestos increases fiber phagocytosis and toxicity for mesothelial cells. *Am J Physiol Lung Cell Mol Physiol* 2000;279(5):L916-L923. [PubMed: 11053028]
16. Garcia JG, Dodson RF, Callahan KS. Effect of environmental particulates on cultured human and bovine endothelium. Cellular injury via an oxidant-dependent pathway. *Lab Invest* 1989;61(1):53-61. [PubMed: 2747217]
17. Garcia JG, Gray LD, Dodson RF, Callahan KS. Asbestos-induced endothelial cell activation and injury. Demonstration of fiber phagocytosis and oxidant-dependent toxicity. *Am Rev Respir Dis* 1988;138(4):958-964. [PubMed: 3202471]
18. Stoner GD, Kikkawa Y, Kniazeff AJ, Miyai K, Wagner RM. Clonal isolation of epithelial cells from mouse lung adenoma. *Cancer Res* 1975;35(8):2177-2185. [PubMed: 167947]
19. Kalgutkar AS, Marnett AB, Crews BC, Remmel RP, Marnett LJ. Ester and amide derivatives of the nonsteroidal anti-inflammatory drug, indomethacin, as selective cyclooxygenase-2 inhibitors. *J Med Chem* 2000;43(15):2860-2870. [PubMed: 10956194]
20. Finkelstein, JN.; Barrett, EG. Alterations in gene expression in pulmonary cells following particle interactions. In: Gehr, P.; Heyder, J., editors. *Particle-lung interactions*. Marcel Dekker, Inc; New York: 2000. p. 379-399.
21. Belton O, Byrne D, Kearney D, Leahy A, Fitzgerald DJ. Cyclooxygenase-1- and -2-dependent prostacyclin formation in patients with atherosclerosis. *Circulation* 2000;102(8):840-845. [PubMed: 10952950]
22. Caughey GE, Cleland LG, Penglis PS, Gamble JR, James MJ. Roles of cyclooxygenase (COX)-1 and COX-2 in prostanoic acid production by human endothelial cells: selective up-regulation of prostacyclin synthesis by COX-2. *J Immunol* 2001;167(5):2831-2838. [PubMed: 11509629]
23. Lovgren AK, Jania LA, Hartney JM, Parsons KK, Audoly LP, Fitzgerald GA, Tilley SL, Koller BH. COX-2-derived prostacyclin protects against bleomycin-induced pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2006;291(2):L144-L156. [PubMed: 16473862]
24. Murakami S, Nagaya N, Itoh T, Kataoka M, Iwase T, Horio T, Miyahara Y, Sakai Y, Kangawa K, Kimura H. Prostacyclin agonist with thromboxane synthase inhibitory activity (ONO-1301) attenuates bleomycin-induced pulmonary fibrosis in mice. *Am J Physiol Lung Cell Mol Physiol* 2006;290(1):L59-L65. [PubMed: 16155090]
25. Murphy JF, Steele C, Belton O, Fitzgerald DJ. Induction of cyclooxygenase-1 and -2 modulates angiogenic responses to engagement of alphavbeta3. *Br J Haematol* 2003;121(1):157-164. [PubMed: 12670347]
26. Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, Pittet JF, Kaminski N, Garat C, Matthay MA, Rifkin DB, Sheppard D. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* 1999;96(3):319-328. [PubMed: 10025398]
27. Pittet JF, Griffiths MJ, Geiser T, Kaminski N, Dalton SL, Huang X, Brown LA, Gotwals PJ, Koteliansky VE, Matthay MA, Sheppard D. TGF-beta is a critical mediator of acute lung injury. *J Clin Invest* 2001;107(12):1537-1544. [PubMed: 11413161]
28. Toews, GB. Interstitial lung disease. In: Goldman, L.; Bennett, JC., editors. *Cecil textbook of medicine*. 21. W. B. Saunders Company; Philadelphia: 2000. p. 409-419.
29. Wang L, Scabilloni JF, Antonini JM, Rojanasakul Y, Castranova V, Mercer RR. Induction of secondary apoptosis, inflammation, and lung fibrosis after intratracheal instillation of apoptotic cells in rats. *Am J Physiol Lung Cell Mol Physiol* 2006;290(4):L695-L702. [PubMed: 16299056]
30. McKusick, VA. Vitronectin; VTN. [Accessed 2 May 2009]. (edited 18 July 1994) <http://www.ncbi.nlm.nih.gov/entrez/dispmim.cgi?id=193190>
31. Schvartz I, Seger D, Shaltiel S. Vitronectin. *Int J Biochem Cell Biol* 1999;31(5):539-544. [PubMed: 10399314]
32. Takagi J. Structural basis for ligand recognition by RGD (Arg-Gly-Asp)-dependent integrins. *Biochem Soc Trans* 2004;32(Pt 3):403-406. [PubMed: 15157147]

33. Hayman EG, Pierschbacher MD, Ohgren Y, Ruoslahti E. Serum spreading factor (vitronectin) is present at the cell surface and in tissues. *Proc Natl Acad Sci USA* 1983;80(13):4003–4007. [PubMed: 6191326]
34. Rom WN, Travis WD, Brody AR. Cellular and molecular basis of the asbestos-related diseases. *Am Rev Respir Dis* 1991;143(2):408–422. [PubMed: 1990961]

Fig. 1.

a Crocidolite-induced cytotoxicity of lung epithelial cells LA-4, dose response. Crocidolite was added at a final concentration of 5, 10, or 20 $\mu\text{g}/\text{cm}^2$. After 24 h of exposure, viability was evaluated as described in the Methods section. **b** LDH release by LA-4 cells after crocidolite exposure. Crocidolite was added at a final concentration of 20 $\mu\text{g}/\text{cm}^2$. After 24 h of exposure, cytotoxicity was evaluated by detecting LDH release. **c** 6-Keto PGF_{1α} production in LA-4 cells after crocidolite exposure. Crocidolite was added at a final concentration of 10 $\mu\text{g}/\text{cm}^2$. After 24 h of exposure, 6-keto PGF_{1α} release was measured as described in the Methods section. **d** 6-Keto PGF_{1α} production in LA-4 cells after crocidolite exposure in the presence of COX inhibitors. COX-1/COX-2 inhibitor indomethacin and specific COX-2 inhibitor indomethacin heptyl ester were added at a dose 10 times their respective IC₅₀ simultaneously with crocidolite (10 $\mu\text{g}/\text{cm}^2$). After 24 h of exposure, 6-keto PGF_{1α} release was measured as described in the Methods section.

Fig. 2.

a Cyclooxygenase-2 mRNA expression in LA-4 cells after crocidolite exposure. Crocidolite was added at a final concentration of 10 μg/cm². After 2 and 4 h of crocidolite exposure, cells were lysed and total RNA isolated. COX-2 mRNA levels were measured by quantitative real-time PCR. **b** Crocidolite-induced cytotoxicity of LA-4 cells in the presence of indomethacin, a COX-1/COX-2 inhibitor. Indomethacin was added at doses 10 and 100 times its IC₅₀ simultaneously with crocidolite (5, 10, 20 μg/cm²). After 24 h of exposure, viability was evaluated. **c** Crocidolite-induced cytotoxicity of LA-4 cells in the presence of indomethacin heptyl ester, a COX-2 inhibitor. Indomethacin heptyl ester was added at doses 10 and 100 times its IC₅₀ simultaneously with crocidolite (5, 10, 20 μg/cm²). After 24 h of exposure, viability was evaluated. **d** Integrin receptors expression in LA-4 cells. Three hundred thousand LA-4 cells were labeled with anti-α_v fluorescent-labeled antibody. After 20 min of incubation at 4° C, fluorescence was detected by flow cytometry

Fig. 3. 6-Keto PGF_{1α} production in LA-4 cells after crocidolite exposure in the presence of VNR inhibitors. **a** RGDS peptide (a VNR inhibitor) or RGES peptide (nonspecific binding control) at a final concentration of 10 μg/ml was added simultaneously with crocidolite (20 μg/cm²). After 24 h of exposure, 6-keto PGF_{1α} release was measured as described in the Methods section. **b** VNR blocking antibody (anti-α_v) was added at a final dose of 2 μg/ml at 1 h before crocidolite exposure (20 μg/cm²). After 24 h of exposure, 6-keto PGF_{1α} release was measured as described in the Methods section

Fig. 4.

Crocidolite-induced response of LA-4 cells in the presence of bronchoalveolar lavage fluid (BALF). Cell culture medium was changed for reduced serum medium and BALF at a final concentration of 25% with crocidolite (10 and 15 $\mu\text{g}/\text{cm}^2$). **a** 6-Keto $\text{PGF}_{1\alpha}$ production in LA-4 cells after crocidolite exposure in the presence of BALF. After 24 h of exposure, 6-keto $\text{PGF}_{1\alpha}$ release was measured as described in the Methods section. **b** Crocidolite-induced cytotoxicity in the presence of reduced serum medium and BALF. After 24 h of exposure, viability was evaluated as described in the Methods section

Fig. 5.

a Crocidolite-induced cytotoxicity of LA-4 cells in the presence of RGDS peptide inhibitor. RGDS peptide (a VNR inhibitor) or RGEs peptide (nonspecific binding control) at a final dose of 10 and 100 $\mu\text{g}/\text{ml}$ was added simultaneously with crocidolite (5, 10, 20 $\mu\text{g}/\text{cm}^2$). After 24 h of exposure, viability was measured as described in the Methods section. **b** LDH release by LA-4 cells after crocidolite exposure (20 $\mu\text{g}/\text{cm}^2$) in the presence of RMV-7, a VNR inhibitor. RMV-7 blocking antibody (anti- α_v) was added at a final dose of 2 $\mu\text{g}/\text{ml}$ at 1 h before crocidolite exposure (20 $\mu\text{g}/\text{cm}^2$ per well). After 24 h of exposure, cytotoxicity was measured by detecting LDH release as described in the Methods section

Fig. 6.

Lung epithelial cells respond in two different pathways after crocidolite exposure. First, an RGD protein present in fetal bovine serum and BALF coats crocidolite and binds VNR. This interaction induces COX-2 expression and activity promoting PGI₂ biosynthesis. Second, crocidolite induces cytotoxicity independently from RGD protein, VNR, COX-1, or COX-2