Stress Genes As Biomarkers of Mineral Dust Exposure

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Grant: 5 K01 OH00146-03

50272-101

REPORT DOCUMENTATION PAGE	1. REPORT NO.	2.	PB98-137763

4. Title and Subtitle: Stress Genes as Biomarkers of Mineral Dust Exposure	5. Report Date: 1997/12/02
	6.
7. Author(s): Timblin, C. R.	8. Performing Organization Rept. No.
9. Performing Organization Name and Address	10. Project/Task/Work Unit No.
	11. Contract (C) or Grant(G) No. (C) (G) K01-OH-00146
12. Sponsoring Organization Name and Address: Department of Pathology, University of Vermont, Burlington, Vermont	13. Type of Report & Period Covered
	14.

15. Supplementary Notes

16. Abstract (Limit: 200 words) An evaluation was conducted of in-vitro and in-vivo models of asbestos (1332214) or silica (14808607) exposure for changes in hsp70, grp78, gadd45, gadd153 steady state mRNA and to relate asbestos or silica induced changes in-vitro to other oxidant stress inducing agents. Characterization of the patterns of gene expression and the functional roles of the encoded proteins provide valuable insights into how a cell responds to injury and how injury can result in the development of disease. The findings showed that crocidolite (12001284) asbestos and other oxidant stress inducing agents elicit different patterns of GRP78, HSP72/73, cJun and MnSOD protein expression. Functional studies examining the role of cJun in the response of epithelial cells to crocidolite asbestos induced injury have demonstrated that asbestos directly activates AP-1-dependent gene expression. Over expression of cJun in tracheal epithelial cells results in increased cell proliferation and cellular transformation, indicating that asbestos induced changes in gene expression can result in changes in cell phenotype that are significant in disease development. The authors indicate that their findings support increasing evidence indicating that environmental agents causing oxidative injury to lung epithelium elicit different patterns of stress protein responses.

17. Document Analysis a. Descriptors

b. Identifiers/Open-Ended Terms: NIOSH-Publication, NIOSH-Grant, Grant-Number-K01-OH-00146, End-Date-09-29-1997, Cancer, Pulmonary-system-disorders, Protein-chemistry, Asbestos-fibers, Fibrous-bodies, Lung-irritants, Dust-exposure, Mineral-dusts

c. COSATI Field/Group

18. Availability Statement: Reproduced from best available copy.	19. Security Class (This Report)	21. No. of Pages: 7
	22. Security Class (This Page)	22. Price

Publications:

Timblin CR, Guthrie G, Janssen YMW, Walsh E, Mossman BT: Patterns of c-*fos* and c-*jun* Proto-oncogene Expression, Apoptosis, and Proliferation in Rat Pleural Mesothelial Cells Exposed to Erionite Fibers or Asbestos. (Submitted)

Timblin CR, Janssen YMW, Goldberg JL, Mossman BT: GRP78, HSP72/73, and c-Jun Stress Protein Levels in Lung Epithelial Cells Exposed to Asbestos, Cadmium, or H_2O_2. Free Radicals in Biology and Medicine, in press, 1997

Mossman BT, Faux S, Janssen Y, Jimenez LA, Timblin C, Zanella C, Goldberg J, Walsh E, Barchowsky A, Driscoll, K: Cell Signaling Pathways Elicited by Asbestos. Environmental Health Perspectives, 105:1121-1125, 1997

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Timblin CR, Janssen YMW, Mossman BT: Free Radical-Mediated Alterations of Gene Expression by Xenobiotics. In: Free Radical Toxicology, (ed. KB Wallace), ~~Raven Press~~ Taylor & Francis, pp 325-348, ~~1996~~ 1997

Janssen YMW, Timblin CR, Zanella CL, Jimenez LA, Mossman BT: Induction of Gene Expression by Environmental Oxidants Associated with Inflammation, Fibrogenesis and Carcinogenesis. In: Oxidative Stress and Signal Transduction, (eds. E Cadenas, H J Forman), Chapman & Hall, pp 387-414, 1996

Timblin CR, Janssen YMW, Mossman BT: Transcriptional Activation of the Proto-oncogene, c-*jun*, by Asbestos and H_2O_2 is Directly Related to Increased Proliferation and Transformation of Tracheal Epithelial Cells. Cancer Research 55:2723-2726, 1995

Significant Findings:

Asbestos causes persistent increases in c-*jun* mRNA and AP-1 DNA binding activity in hamster tracheal epithelial (HTE) cells, the progenitor cell type of asbestos-induced bronchogenic carcinoma. Studies in this grant were designed to determine mechanisms of c-*jun* induction by asbestos and the phenotypic consequences of Jun expression in HTE cells. Our results demonstrate that asbestos and the oxidant, hydrogen peroxide, directly activate AP-1-dependent gene transcription. Furthermore, overexpression of c-*jun* in HTE cells leads to increased proliferation and changes in cell phenotype that are consistent with cellular transformation. The ability of c-*jun* alone to alter the phenotype of tracheal epithelial cells towards a more malignant state is significant since asbestos persistently increases c-*jun* mRNA and AP-1 DNA-binding activities in this cell type. The observation that hydrogen peroxide also activates AP-1-dependent gene expression suggests that asbestos-mediated transcriptional activation of AP-1 may be associated with the production of AOS from asbestos fibers directly or during phagocytosis of fibers by cells. These results suggest that persistent induction of c-*jun* may contribute to asbestos-induced proliferation, a feature of associated lung cancers and mesotheliomas.

Tumor promotion and progression are characterized by unregulated cell proliferation, an event that may be mediated by both alterations in the regulation of cell death, i.e., apoptosis, or in the control of cell proliferation. The proto-oncogenes, c-*jun* and c-*fos*, encode subunits of the AP-1 transcription factor that is important in the regulation of cell proliferation and cell death. Persistent induction of c-*jun* and c-*fos* in rat pleural mesothelial cells exposed to crocidolite asbestos or the erionite, a more potent inducer of mesothelioma than crocidolite asbestos, correlate with changes in cell proliferation and apoptosis. However, the patterns of proto-oncogene expression, cell proliferation and apoptosis are different in mesothelial cells exposed to crocidolite asbestos or erionite fibers. Erionite induces increases in DNA synthesis at lower concentrations of fiber numbers than asbestos and is also a less potent inducer of apoptosis in comparison to crocidolite asbestos. These observations suggest that the balance between apoptosis and cell proliferation may be critical in mineral-induced responses.

Mammalian cells respond to a diversity of environmental insults, both physical and chemical, by inducing the expression of a group of genes referred to as stress response genes. These include *grp*78, *hsp*72/73, and c-*jun*. The induction of these genes and the functions of their encoded protein products define a defense mechanism(s) whereby cells detect damaged cellular components and activate appropriate scavenging and repair pathways. Although a number of environmental oxidants affect lung, studies have not focused on alterations of stress proteins by these agents or agent-specific effects in lung epithelium. Studies in this grant were designed to examine protein expression of GRP78, HSP72/73, cJun, and MnSOD, an indication of oxidant stress, in target epithelial cells of lung after exposure to asbestos and other agents inducing oxidant injury. Our results demonstrate that crocidolite asbestos and the oxidant, hydrogen peroxide, do not alter GRP78 or HSP72/73 protein levels in rat lung epithelial cells, but do increase levels of cJun protein and levels of the antioxidant enzyme, manganese-containing superoxide dismutase (MnSOD), an indirect indicator of oxidant stress. The oxidant-stress inducing agent, cadmium chloride, elicits a different pattern of stress protein expression: levels of MnSOD remain unchanged while levels of GRP78, HSP72/73 and cJun proteins increased. Our observations support increasing evidence indicating that environmental agents causing oxidative injury to lung epithelium elicit different patterns of stress protein responses.

Usefulness of Findings:

The molecular response of cells to carcinogenic and fibrogenic mineral dusts is complex and involves increased expression of a number of genes with different functions. Characterization of the patterns of gene expression and the functional roles of the encoded proteins provide valuable insights into how a cell responds to injury and how injury can lead to development of disease. Since pulmonary disease induced by mineral dust may progress in the absence of additional exposure, i.e. after the worker leaves the workplace, knowledge of the molecular events which leads to disease may be essential to designing preventative and therapeutic approaches.

Abstract:

Occupational exposure to asbestos or silica is associated with the development of both non-malignant and malignant pulmonary disease. Considerable evidence indicates that the mechanism of mineral dust toxicity involves the production of active oxygen species (AOS) catalyzed directly on the mineral surface or by phagocytic cells within the lung. Production of AOS in excess of cellular defenses creates an environment of oxidative stress for the cell. The molecular response of cells to stress is a reprogramming of gene expression to meet the new challenges of its environment. Oxidant-induced genes include: genes encoding antioxidant enzymes, proto-oncogenes (e.g., c-*jun*), *hsp*72/73, *grp*78, *gadd*45, and *gadd*153. Detailed characterization of the molecular stress responses to asbestos or silica will aid in the understanding of the mechanisms of mineral dust mediated disease formation and may identify biomarkers of exposure and/or disease development. The focus of this proposal was to characterize the molecular stress response within target cells of asbestos or silica exposed lung, to compare this response to that elicited by other oxidants, and to relate this response to known markers of disease in well-characterized inhalation models. Our results demonstrate that crocidolite asbestos and other oxidant stress-inducing agents elicit different patterns of GRP78, HSP72/73, cJun and MnSOD protein expression. Functional studies examining the role of cJun in the response of epithelial cells to crocidolite asbestos-induced injury have shown that asbestos directly activates AP-1-dependent gene expression. Furthermore, overexpression of c-*jun* in tracheal epithelial cells leads to increased cell proliferation and cellular transformation. These results demonstrate that asbestos-induced alterations in gene expression (i.e., c-*jun*) can lead to changes in cell phenotype (i.e., proliferation, apoptosis, transformation) that are important in the development of disease.

Report:

Occupational exposure to mineral dusts is associated with the development of both non-malignant and malignant pulmonary disease. This research has focused on characterizing the molecular response of target cells of the lung to crocidolite asbestos and other oxidant stress-inducing agents. The central hypothesis of this research is that oxidative stress imposed by mineral dusts elicits a molecular stress response that can be characterized by changes in gene expression.

Specific Aim #1 is to evaluate *in vitro* and *in vivo* models of asbestos and/or silica exposure for changes in *hsp*70, *grp*78, *gadd*45, *gadd*153 steady state mRNA. Specific Aim #2 is to relate asbestos- or silica-induced changes *in vitro* to other oxidant stress-inducing agents. These approaches will allow us to identify components of an asbestos-specific response, and to determine if individual target cells of the lung respond differently to oxidative stress.

Previous work in our laboratory has demonstrated that exposure to asbestos causes increased mRNA levels of antioxidant enzymes (i.e., manganese superoxide dismutase (MnSOD), catalase, and glutathione peroxidase) and the proto-oncogenes, c-*jun* and c-*fos* in target cells of asbestos-induced disease. To further characterize the molecular responses of pulmonary cells to asbestos, we examined the expression of the stress response proteins GRP78, and HSP72/73 (HSP70) in rat lung epithelial cells (RLE) by Western blot analysis. Epithelial cells are primary targets of asbestos-induced disease giving rise to bronchogenic carcinoma. In addition, we also examined the levels of oxidized and reduced glutathione and protein levels of MnSOD in order to relate changes in stress protein levels to patterns of oxidative stress. In comparative studies, we also examined GRP78, HSP72/73, and cJun expression in RLE cells exposed to equitoxic concentrations of cadmium chloride and the oxidant, hydrogen peroxide. Cadmium chloride has been implicated as an agent inducing oxidative injury as a mechanism of toxicity. Our results demonstrate that asbestos and hydrogen peroxide do not alter GRP78 or HSP72/73 protein levels in RLE cells, but do increase levels of cJun protein. Increases by asbestos and hydrogen peroxide were not accompanied by alterations in cellular glutathione levels in this cell type but asbestos caused elevations in protein levels of MnSOD, an indirect indicator of oxidative stress. In contrast, exposure of cells to cadmium chloride led to no changes in MnSOD protein levels, but increases in GRP78, HSP72/73, and cJun proteins as well as significant increases in glutathione pools. These results suggest that environmental agents causing oxidative injury to lung epithelium elicit different patterns of stress responses. This work is published in Free Radicals in Biology and Medicine (in press).

Alterations in the expression of stress genes *gadd*45 and *gadd*153 were also examined in RLE cells and rat pleural mesothelial cells exposed to asbestos, however, mRNA levels for these genes were too low to be characterized by Northern blot analysis. Characterization of these genes in target cells of asbestos-induced disease was not pursued further.

Mesothelial cells lining the pleural cavity are also targets of asbestos-induced disease. In this cell type, asbestos is thought to act as a complete carcinogen initiating DNA damage and promoting the formation of malignant mesothelioma. The mineral fiber, erionite, is also a potent inducer of mesothelioma and like crocidolite asbestos, induces oxidative stress in exposed cells. In rat pleural mesothelial cells (RPM) exposed to crocidolite asbestos, the levels of stress proteins HSP72/73 are decreased while levels of GRP78 remain unchanged. In contrast, in cells exposed to erionite fibers, the opposite pattern is observed: HSP72/73 protein levels are unchanged and GRP78 protein levels are strikingly decreased (Unpublished observations). Erionite fibers are more potent inducers of mesothelioma than crocidolite asbestos, an observation that may be related to the differential molecular responses of mesothelial cells to the two fiber types.

In an attempt to correlate our *in vitro* observation with changes in *grp*78 and *hsp*72/73 gene expression in the lungs of rats exposed to asbestos, we analyzed total lung RNA from control and exposed animals by Northern blot analysis. The levels of *grp*78 and *hsp*72/73 mRNAs in rat lung (control and exposed animals) was too low to detect by Northern blot analysis. This observation precludes us from making any direct comparisons to our *in vitro* results on *grp*78 and *hsp*72/73 expression in cells exposed to asbestos.

In work recently submitted for publication, we further characterized the molecular and cellular

responses of mesothelial cells to crocidolite asbestos and erionite fibers. Patterns of early response proto-oncogenes (i.e., c-*fos* and c-*jun*) expression , Activator Protein-1 (AP-1) binding to DNA, and changes in cell proliferation and apoptosis were examined in RPM cells exposed to crocidolite asbestos and erionite fibers. Increased expression and transactivation of c-*fos* and c-*jun* (genes encoding the subunits of the AP-1 transcription factor) have been linked to the development of cell proliferation or apoptosis in a number of cell types. Apoptosis, a genetically programmed process of cell death, when balanced with cell proliferation, is important in the maintenance of normal tissue homeostasis. An imbalance between cell growth and cell death is thought to be involved in the pathogenesis of a number of diseases including cancer. More specifically, suppression of apoptosis is associated with the establishment or maintenance of the transformed cellular phenotype, an early event in carcinogenesis.

In RPM cells exposed to crocidolite asbestos or erionite fibers, different patterns of proto-oncogene expression are observed. At equal weight concentrations of both fiber types, c-*fos* mRNA levels are increased comparably. In contrast, erionite fibers caused significantly increased levels of c-*jun* mRNA at lower mass concentrations than crocidolite asbestos, but comparable levels of AP-1 binding to DNA. AP-1 DNA binding activity is examined as an indicator of transcription factor activity.

Different patterns of cell proliferation and apoptosis are also observed in RPM cells exposed to crocidolite asbestos or erionite fibers. An indicator of cell proliferation is the incorporation of the thymidine analog, 5'-bromodeoxyuridine (BrdU), into the DNA of cells in the S phase of the cell cycle. Apoptosis is visualized using a nuclear dye diamidino-2-phenylindole (DAPI) and examining changes in nuclear morphology. The combined techniques of BrdU incorporation and DAPI staining provide a sensitive, *in situ* protocol for examining cellular responses to carcinogenic mineral dusts and other toxic agents. In comparison to untreated controls, numbers of RPM cells incorporating BrdU were increased dramatically after exposure to asbestos or erionite fibers. Most interestingly, although significant dose-dependent increases were observed with asbestos at all time points examined, erionite fibers failed to induce apoptosis at earlier time points (8 and 24 hours of exposure), and higher concentrations than asbestos were required to elicit apoptosis at 48 hours. (This dual labeling technique and its application to studies on the response of cells to toxicants has recently been published in the American Journal of Respiratory Cell and Molecular Biology.)

These data show that erionite fibers and crocidolite asbestos fibers cause proto-oncogene expression, and BrdU incorporation at comparable weight concentrations in mesothelial cells. Most importantly, we demonstrate that erionite is a less potent inducer of apoptosis in comparison to crocidolite asbestos. Erionite also induces increases in DNA synthesis at lower concentrations of fiber numbers than asbestos. Data in concert suggest that the balance between apoptosis and cell proliferation may be critical in mineral-induced responses and provide a possible mechanistic explanation for the increased tumorigenicity of erionite in comparison to crocidolite asbestos after inhalation.

Specific Aim #3 is to modulate expression of c-*jun* in target cells using transfection techniques and to determine its role in mineral dust-mediated disease.

Previously, our laboratory has demonstrated that asbestos causes persistent increases in mRNA levels of c-*jun* and AP-1-DNA binding activity in hamster tracheal epithelial cells (HTE), the progenitor cell type of asbestos-induced bronchogenic carcinoma. Subsequently, we demonstrated that asbestos causes transcriptional activation of the c-*jun* through an oxidative mechanism. To further characterize the role of c-*jun* and AP-1 in cellular responses to asbestos, we examined the ability of crocidolite asbestos to directly activate AP-1-dependent gene expression and whether c-*jun* over-expression is involved in epithelial cell proliferation. HTE cells were transiently transfected using the $CaPO_4$ co-precipitation technique with a plasmid containing a fragment of the c-*jun* promoter coupled to a luciferase reporter gene. HTE cells transfected with the *jun*-luciferase construct showed increased luciferase activity when exposed to crocidolite asbestos or hydrogen peroxide. These results demonstrate that asbestos and the oxidant, hydrogen peroxide, activate AP-1-dependent gene transcription.

The role of c-*jun* in epithelial cells proliferation and transformation was examined by transiently transfecting HTE cells with a plasmid that constitutively over-expresses c-*jun* . Over-expression of c-*jun* led to increased cell proliferation and an enhanced ability of HTE cells to grow in soft agar, an indication of cellular transformation. Our working model of asbestos-induced disease suggests that persistent induction of the early response pathway (i.e., c-*jun*) may lead to chronic cell proliferation and changes in cell phenotype indicative of neoplastic transformation. We have shown that asbestos can directly activate transcription of the c-*jun* gene and that over-expression of c-*jun* alone is sufficient to phenotypically change tracheal epithelial cells. Our results suggest that persistent induction of c-*jun* may contribute to asbestos-induced proliferation, a feature of associated lung cancers and mesotheliomas. This work has been published in Cancer Research (1995).