ROLE OF IRON IN THE MECHANISM OF ASBESTOS-INDUCED
APOPTOSIS IN HUMAN LUNG AND PLEURAL TARGET CELLS

by

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ABSTRACT

The Role of Iron in the Mechanism of Asbestos-Induced Apoptosis in Human Lung and Pleural Target Cells

by

Aleksander Baldys, Doctor of Philosophy

Utah State University, 2004

Occupational exposure to asbestos has been associated with increased incidence of pulmonary interstitial fibrosis, mesothelioma of the pleura, and bronchogenic carcinoma. Although the mechanism by which asbestos causes cancer remains unknown, iron associated with asbestos is thought to play a role in the pathogenic effects of fibers.

The aim of this research was to examine and compare the asbestos-induced signaling phenomena in relevant human lung and pleural target cells, and to determine the role of iron from asbestos fibers in these events. Exposure of human airway epithelial (A549) cells, human pleural mesothelial (MET5A) cells, and normal human small airway epithelial (SAEC) cells to asbestos resulted in a significant dephosphorylation and inactivation of epidermal growth factor receptor (EGFR). The effects of three types of asbestos, i.e. crocidolite, amosite and chrysotile, on the EGFR phosphorylation state in A549 cells appeared to be directly related to the amount of iron mobilized from these fibers. These results strongly suggest that iron plays an important role in asbestos-
induced inactivation of EGFR.

We observed that exposure of A549 and SAEC cells to crocidolite, but not inert titanium dioxide, led to a significant time- and dose-dependent inactivation of the main EGFR signaling pathways, including Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) pathways. Crocidolite also initiated apoptosis via pathways involving activation of p38 mitogen-activated protein kinase (MAPK), caspase -3 and -9, and cleavage of poly(ADP-ribose) polymerase (PARP). Prevention of these effects with an iron chelator or endocytosis inhibitors strongly suggests that iron mobilized from fibers inside the cells initiates the observed events. Inhibition of p38 MAPK with SB203580 prevented inactivation of EGFR, inactivation of EGFR-associated survival pathways, and initiation of apoptosis. Our results also suggest that p38 MAPK-dependent protein serine/threonine phosphatase activation plays an important role in the observed phenomena. Taken together, it appears that iron-dependent p38 MAPK activation, through a serine/threonine phosphatase-mediated mechanism, regulates asbestos-induced apoptosis in human lung epithelial cells. We speculate that apoptosis of human lung target cells induced by asbestos fibers is a pathologic feature in lung injury and may account for some of the pulmonary toxicity of the fibers.
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I would like to dedicate this dissertation to my mother and wife. To my mother
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<td>A549</td>
<td>human airway epithelial cells</td>
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<tr>
<td>C10</td>
<td>murine alveolar type II epithelial cells</td>
</tr>
<tr>
<td>CB</td>
<td>cytochalasin B</td>
</tr>
<tr>
<td>CD</td>
<td>cytochalasin D</td>
</tr>
<tr>
<td>CFA</td>
<td>coal fly ash</td>
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<tr>
<td>$\Delta \psi_m$</td>
<td>mitochondrial membrane potential changes</td>
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<td>DF</td>
<td>desferrioxamine B</td>
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<td>DSPases</td>
<td>dual specificity phosphatases</td>
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<td>ECL</td>
<td>chemiluminescent detection reagent</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<td>ERK1/2</td>
<td>extracellular signal-regulated kinase 1/2</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>GSH</td>
<td>glutathione</td>
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<td>HER2</td>
<td>human epidermal growth factor receptor 2</td>
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<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
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<td>I-1</td>
<td>inhibitor 1</td>
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<tr>
<td>I-2</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<td>LMW</td>
<td>low-molecular weight</td>
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<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MAPKK</td>
<td>mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>mitogen-activated protein kinase kinase kinase</td>
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<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase/extracellular signal-regulated kinase</td>
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<tr>
<td>MET5A</td>
<td>human pleural mesothelial cells</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
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<tr>
<td>8-oxodG</td>
<td>8-oxoguanine</td>
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<tr>
<td>O$_2^-$</td>
<td>superoxide radical</td>
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<td>OA</td>
<td>okadaic acid</td>
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<tr>
<td>-OH</td>
<td>hydroxyl radical</td>
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<td>ONOO$^-$</td>
<td>peroxynitrite</td>
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<td>phytic acid</td>
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<td>poly(ADP-ribose) polymerase</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PDK1</td>
<td>3-phosphoinositide-dependent protein kinase 1</td>
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<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
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<td>PIP$_3$</td>
<td>phosphatidylinositol-3,4,5-trisphosphate</td>
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<td>PKB</td>
<td>protein kinase B</td>
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<td>PP1</td>
<td>serine/threonine phosphatase type 1</td>
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<td>protein phosphatases</td>
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<td>PSPases</td>
<td>protein serine/threonine phosphatases</td>
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<td>PTB</td>
<td>phosphotyrosine-binding domain</td>
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<td>protein tyrosine phosphatases</td>
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<td>polyvinyl difluoride</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>RPM</td>
<td>rat pleural mesothelial cells</td>
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<td>SAEC</td>
<td>small airway epithelial cells</td>
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<td>SAGM</td>
<td>small airway growth medium</td>
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<td>SAPK</td>
<td>stress-activated protein kinase</td>
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<td>simian virus 40</td>
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<tr>
<td>TiO₂</td>
<td>titanium dioxide</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>TRX</td>
<td>thioredoxin</td>
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<tr>
<td>VA</td>
<td>sodium orthovanadate</td>
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CHAPTER 1
INTRODUCTION

Asbestos is a naturally occurring mineral that has been used for centuries because of its unique physical and chemical properties, including durability, tensile strength, and heat resistance. However, it was recognized to be a human carcinogen in the late 1950s, which led to a complete cessation of the use of asbestos-containing commercial products. It has been demonstrated that exposure to asbestos increases the risk of both malignant (mesothelioma of the pleura and bronchogenic carcinoma of the lungs) (1) and nonmalignant (asbestosis) diseases (2). Even though it is not clear how asbestos exerts these effects, the carcinogenicity of asbestos has been related to some of its physical properties, such as size and durability of fibers (3). The most carcinogenic forms of asbestos contain as much as 27% iron by weight (4, 5). It has been proposed that iron from asbestos catalyzes the generation of reactive oxygen species (ROS), such as superoxide radical (O$_2^-$) and hydroxyl radical (·OH) (3, 6). In addition, iron has been shown to be responsible for O$_2$ consumption (7), induction of DNA single-strand breaks (8), lipid peroxidation (9), and potentially cancer. There is also significant evidence suggesting that iron from other sources besides asbestos may cause human cancer (10, 11).

Another reactive species nitric oxide (NO), produced enzymatically by nitric oxide synthase (NOS), can react with O$_2^-$ to produce peroxynitrite (ONOO$^-$). Chao et al. (12) reported that the formation of 8-oxoguanine (8-oxodG), one of the major types of oxidative DNA damage, increased in asbestos-treated human lung epithelial (A549) cells. Also, they observed that hydroxylation of DNA could be caused by ·OH or ONOO$^-$. 
implying that both iron and NO may be responsible for the formation of 8-oxodG.

With the exception of mucus cells all airway cells endocytize particles (13). Iron can be mobilized from asbestos in vitro, in cultured cells and in vivo (14-16). This uncontrolled entry of iron may represent “iron overload” in cells endocytizing the fibers. It has been suggested that iron mobilization from asbestos into low-molecular weight (LMW) fraction, non-protein (e.g. citrate or ADP) associated iron in cells might be responsible for asbestos-dependent cytotoxicity. Although the mechanism by which asbestos exerts its pathologic effects is not known, at least two plausible mechanisms should be considered. Asbestos-induced pulmonary toxicity might be initiated by ROS generated from mobilized iron via Haber-Weiss reactions (3), and possibly by interaction of asbestos fibers with the plasma membrane through a receptor-like mechanism (17). Despite a strong body of evidence supporting the involvement of asbestos in lung diseases and the knowledge we have gained throughout these years with respect to asbestos-associated carcinogenicity, the light is yet to be shed on the mechanism by which mineral fibers cause cancer.

The purpose of this dissertation has been to determine whether asbestos-induced pulmonary effects might, in part, have cell surface origins, and to determine the effects of asbestos on the control and coordination of the signal transduction pathways in human lung and pleural cells. More specifically, the objective was to determine whether iron associated with fibers, as well as the process of fiber endocytosis, played any role in the proposed hypotheses in relevant human lung and pleural target cells. Chapter 2 is a review of the literature on the role of iron in the pathological effects of asbestos and the role of ROS in cellular redox signaling and control. It also serves as an introduction to
the results presented in this dissertation.

The results presented in Chapter 3 strongly suggest that iron associated with fibers plays an important role in asbestos-induced inactivation of epidermal growth factor receptor (EGFR). This chapter discusses the effects of asbestos fibers on the EGFR activity in relevant human lung and pleural target cells, *i.e.* epithelial (A549 and small airway epithelial (SAEC) cells) and mesothelial (human pleural mesothelial (MET5A) cells) cells. Our results indicated that in contrast to rodent cells, treatment of human lung epithelial and pleural mesothelial cells with asbestos fibers resulted in dephosphorylation and subsequent inactivation of the ErbB family of receptor tyrosine kinases. Additionally, it appeared that iron was responsible, at least in part, and that the effect on the receptor was mediated by internalized fibers. We have identified a potentially important mechanism by which relevant human lung and pleural target cells respond to asbestos fibers.

In Chapter 4, iron mobilized from fibers inside human lung epithelial cells was found to initiate asbestos-induced signaling events preventing survival, but activating apoptotic pathways. This chapter discusses the effects of asbestos on EGFR-dependent and -independent signal transduction pathways in A549 and SAEC cells. Our findings strongly suggest a central role for the upstream activation of p38 mitogen-activated protein kinase (MAPK) in altering the fine balance between cell survival and death. Most importantly, iron mobilized from fibers inside the cells appeared to initiate the activation of p38 MAPK leading to apoptosis. We speculate that apoptosis may be an important response of relevant human lung target cells and that asbestos pathogenesis may result from altered apoptotic mechanisms.
Finally, in Chapter 5, we investigated the role of protein phosphatases (PPases) in asbestos-induced apoptosis in A549 cells, using okadaic acid (OA) and sodium orthovanadate (VA), specific inhibitors of protein serine/threonine phosphatases (PSPases) and protein tyrosine phosphatases (PTPases), respectively. Our findings demonstrated that OA, but not VA, inhibited asbestos-induced global cellular protein dephosphorylation and apoptosis. Importantly, iron mobilized from fibers inside the cells and p38 MAPK activity appeared to be required for the increased phosphatase activity following asbestos treatment. Taken together, our results strongly suggest a critical role for PSPases in a p38 MAPK-mediated regulation of asbestos-induced apoptosis in human lung epithelial cells.

Chapters 3 through 5 in this dissertation are written in the style of the journal in which they were published or will be published and these chapters include their own abstract, introduction, materials and methods, and discussion, as well as their own references.

Chapter 6 summarizes the results presented in this dissertation and suggests directions that future research in the study of the pathological effects of asbestos may take.

REFERENCES


RELEVANT HUMAN LUNG AND PLEURAL TARGET CELLS

The best way to study the effects of asbestos on human lung is the use of actual lung tissue specimens. However, not only is this approach very expensive, but also is not useful when doing research that requires living systems. A less expensive and more practical approach is the use of model systems, e.g. culture of lung cells. There are two types of cell culture models – primary cultures and cell lines. Primary cultures are obtained by taking the airway of a donor and preparing it to grow on an artificial substrate. The advantages of primary culture are that the cells are not modified in any way. The disadvantages of primary cultures are the mixed nature of each preparation, limited lifespan of the culture and the potential contamination problems. A more convenient and more popular approach is the use of cell lines. Cell lines, which originated from lung tumors or from a primary culture that is immortalized by transfection with a virus, e.g. simian virus 40 (SV40), have an unlimited proliferation capacity. However, the major disadvantage of cell lines is the fact that they are genetically modified as compared to normal cells.

Asbestos displays a variety of biological effects in different relevant lung and pleural target cells, such as mesothelial and epithelial cells. One of the cell lines being used in the laboratory of Dr. Ann Aust is the tumor cell line A549. The A549 cells, human lung epithelial cells, have the same morphology as type II epithelial cells. The epithelial type II cells are chosen for the majority of these studies because not only did
they appear at the crossroads of inflammation, fibrogenesis and carcinogenesis, but also this type of cell is most likely to come into contact with external agents, such as particles or asbestos fibers.

Another relevant human target cell type sensitive to toxic effects of asbestos are pleural mesothelial cells. The mesothelial cells are likely to be a target of asbestos fibers, whether by the direct action of the fiber or by the indirect action of other asbestos-exposed cells, e.g. alveolar epithelial cells. Mesothelial cells are very sensitive to the cytotoxic effects of asbestos (1). Asbestos has been shown to cause both chromosomal aberrations and morphological transformation in human (1) and rodent (2) mesothelial cells. Since human pleural mesothelial cells from pleural effusions are difficult to propagate and show individual variability in cytotoxic and genotoxic responses to asbestos (3, 4), an SV40 T antigen transformed human mesothelial cell line MET5A has been used by many investigators (5-7) to study the effects of asbestos on progenitor cells of mesothelioma.

Since A549 cells were originally derived from an individual with alveolar carcinoma tumor, and MET5A cells were immortalized by transfection with a SV40 virus, there is concern that they may not be a faithful representation of normal cells. One cannot know what changes have occurred in the cells isolated from tumors. Additionally, the effects of transfection on the cell might sometimes be ambiguous. Although little work has been done to directly compare the A549s with normal cells, former students in the laboratory have shown that the A549 cells resembled closely the normal human lung epithelial cells with regard to ferritin induction and glutathione maintenance (8). Based on that one would expect to see similar responses to asbestos fibers with respect to other
experiments. For the purpose of this work, some experiments were performed using primary cultures of SAEC from the distal airspace to show that all cell culture models respond similarly to asbestos fibers and that the responses are not an artifact of a single cell type.

ASBESTOS AND IRON

Epidemiological and animal studies indicate that occupational exposure to asbestos has been associated with the development of pulmonary fibrosis (asbestosis), lung cancer, malignant mesothelioma, and other pulmonary disorders (9). The National Institutes of Health in 1978 estimated that approximately 11 million individuals had been exposed to asbestos in the United States since 1940, of which 2000 die from mesothelioma each year (10). Due to these health concerns, the use of asbestos has been limited or prohibited in the United States and in several countries, but in developing countries, the use of asbestos continues to increase.

Asbestos refers to a group of complex, crystalline mineral fibers that are endogenous to different geographic areas and occur naturally as ores. Because of their useful properties such as thermal insulation, chemical and thermal stability, and high tensile strength, these naturally occurring fibrous silicate minerals have been mined and processed for decades in a number of countries and incorporated into thousands of industrial products. The three most common types of asbestos are chrysotile, amosite and crocidolite. Asbestos differs from other minerals in its crystal development. The crystal formation of asbestos is in the form of long thin fibers and can only be identified under a microscope. Asbestos is divided into two mineral groups - serpentine and amphibole. The division between the two types of asbestos is based upon the crystalline structure.
Serpentines have a sheet or layered structure while amphiboles have a chain-like structure. As the only member of the serpentine group, chrysotile is the most common type of asbestos found in buildings. In the amphibole group, there are five types of asbestos. Amosite is the second most prevalent type of asbestos found in building materials. Amosite is also known as "brown asbestos." Next, there is crocidolite or "blue asbestos," which is an asbestos found in specialized high temperature applications. The other three types (anthophyllite, tremolite, and actinolite) are rare and found mainly as contaminants in other minerals. Asbestos deposits can be found throughout the world and are still mined in Australia, Canada, South Africa, and the former Soviet Union. Asbestos is made up of microscopic bundles of fibers that may become airborne when distributed. These fibers get into the air and may become inhaled into the lungs, where they may cause significant health problems. Researchers have not yet determined a "safe level" of exposure but we know the greater and the longer the exposure, the greater the risk of contracting asbestos-related disease.

Two properties of the asbestos fibers appear to affect their ability to cause cancer: physical dimensions and durability (11). Longer asbestos fibers are more biopersistent, i.e. accumulate more readily in the lung and are less effectively cleared. Generally, the longer the fiber resides in the lung, the more likely it is to be carcinogenic. Amphibole fibers remain in the lung for the lifetime of the individual. Nevertheless, neither size nor durability can fully account for the chemistry of, and the biological reactions to, mineral fibers. The pathogenicity associated with exposure to amphiboles, believed to be more carcinogenic in man than the serpentes, has recently been linked to high iron content present on the surface of the fibers (11). Crocidolite and amosite, the most commercially
used forms of amphibole asbestos, contain high levels of iron (Table 2-1). Table 2-1 shows chemical formulas of different types of asbestos. All types may contain different combinations of ions and metals depending on mining locations. Additionally, complete ion exchange can occur, e.g. magnesium ions in chrysotile can be replaced with either iron or manganese, or both.

Table 2-1 *Chemical Empirical Formula of Asbestos*

<table>
<thead>
<tr>
<th>Asbestos type</th>
<th>Formula</th>
<th>Content of Iron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crocidolite</td>
<td>$\text{Na}_2\text{Fe}^{3+}\cdot(\text{Fe}^{2+},\text{Mg})_3<a href="%5Ctext%7BOH%7D">\text{Si}<em>8\text{O}</em>{22}</a>_2$</td>
<td>27%</td>
</tr>
<tr>
<td>Amosite</td>
<td>$(\text{Fe}^{2+},\text{Mg})_7<a href="%5Ctext%7BOH%7D">\text{Si}<em>8\text{O}</em>{22}</a>_2$</td>
<td>27%</td>
</tr>
<tr>
<td>Tremolite</td>
<td>$\text{Ca}_2\text{Mg}_5<a href="%5Ctext%7BOH%7D">\text{Si}<em>8\text{O}</em>{22}</a>_2$</td>
<td>trace</td>
</tr>
<tr>
<td>Anthophyllaite</td>
<td>$(\text{Fe}^{2+},\text{Mg})_7<a href="%5Ctext%7BOH%7D">\text{Si}<em>8\text{O}</em>{22}</a>_2$</td>
<td>N.D.$^a$</td>
</tr>
<tr>
<td>Chrysotile</td>
<td>$\text{Mg}_3\text{Si}_2\text{O}_5(\text{OH})_4$</td>
<td>2-3%</td>
</tr>
</tbody>
</table>

$^a$ not determined

The most carcinogenic forms of asbestos contain as much as 27% iron by weight (12, 13). It has been shown that iron from asbestos catalyzes the generation of ROS, such as $\text{O}_2^-$ and $-\text{OH}$ (11, 14). In addition, iron has been shown to be responsible for $\text{O}_2$ consumption (15), induction of DNA single-strand breaks (16), lipid peroxidation (17), and potentially cancer. There is also significant evidence suggesting that iron from other sources besides asbestos may cause human cancer (18, 19).

Iron is the most important essential trace element in living systems, occurring in the human body in both Fe (III) and Fe (II) forms (20). It is vital for organisms because it is required for multiple metabolic processes to include electron transport and cellular
respiration, cell proliferation and differentiation, as well as regulation of gene expression (21). However, iron is also potentially deleterious. Its excretion from the body in the absence of bleeding is limited to no more than 4 mg/day, regardless of the quantity of iron in body stores. In excess of cellular needs iron is toxic and dietary overload frequently leads to tissue iron deposition and injury (22). Thus, iron concentration in the body organs must be regulated carefully. Iron must be bound to proteins to prevent tissue damage from free radical formation. The following equations show the modified Haber-Weiss (Eq. 1-4) and Fenton reactions (Eq. 4):

\[
\text{Reductant}^{(n)} + \text{Fe}^{(III)} \rightarrow \text{Reductant}^{(n+1)} + \text{Fe}^{(II)} \quad \text{Eq. 1}
\]
\[
\text{Fe}^{(II)} + O_2 \leftrightarrow \text{Fe}^{(III)} + O_2^- \quad \text{Eq. 2}
\]
\[
\text{HO}_2^- + O_2^- + H^+ \rightarrow O_2 + H_2O_2 \quad \text{Eq. 3}
\]
\[
\text{Fe}^{(II)} + H_2O_2 \rightarrow \text{Fe}^{(III)} + OH^- + .OH \quad \text{(Fenton Reaction)} \quad \text{Eq. 4}
\]

Fe (II) acts as a catalyst in the above reaction. This process can be inhibited by free radical scavengers as well as by iron chelators such as EDTA, formaldehyde, and citric acid. Iron will continue to redox cycle in the above reactions as long as there is sufficient O_2 or H_2O_2 and reductant, such as ascorbate or cysteine.

A free radical is any species capable of independent existence that contains one or more unpaired electrons. Radicals can be formed by the loss of a single electron from a non-radical or by the gain of a single electron by a non-radical. Also, radicals can be formed when a covalent bond is broken if one electron from each of the pair shared remains with each atom, a process known as homolytic fission. Once the hydroxyl free radical is created, it reacts virtually immediately. The hydroxyl radical is one of the most
reactive chemical species known. In contrast to other ROS, such as $O_2^-$ and $H_2O_2$, -OH can react with virtually any biological molecule. It reacts within 10 Å of the site of its generation, indicating that it must be produced at the site of the damage (11). ROS generated by Fenton chemistry can deplete intracellular antioxidants and cause cellular damage including lipid peroxidation, DNA adducts and protein modifications (11). Also, they may contribute to major pathological processes such as cancer, arteriosclerosis, and neurodegenerative diseases.

It has been well established that an oxidative environment produces DNA damage through the mediation of iron ions present in the nucleus (23). Since it has been shown that iron can be mobilized from asbestos fibers (24) and is capable of inducing DNA single-strand breaks (16), one can suspect asbestos fibers to be a potential source of the nuclear iron. However, the fibers have rarely been observed in the nucleus. Other findings suggest the presence of iron in the nucleus including the detection of ferritin, the iron storage protein, in this organelle (25, 26), and the discovery of both DNA repair enzymes and transcription factors containing iron-sulfur clusters (27-29). The iron pool in the nucleus could be a direct consequence of the cytosolic iron control if iron would diffuse freely through the nuclear membrane pores. Recently it has been proposed that a P-type ATPase, distinct from other known P-type ATPases, supports iron accumulation in the nucleus (30). Even though newer information appeared during recent years that provided greater insights into factors involved in iron transport into the nucleus, the exact mechanism and players implicated in it still remain obscure.

There is significant evidence demonstrating the notion that iron was mobilized from asbestos fibers and was responsible for $O_2$ consumption, DNA oxidation and lipid
peroxidation (15-17). It has been proposed that iron mobilized from asbestos into a LMW fraction, not associated with proteins, led to pathological phenomena, including cancer. Importantly, it has been found that the reactivity of iron was dependent upon its electronic environment (11). For instance, binding of LMW chelators, including citrate or ADP, renders iron redox active. In contrast, desferrioxamine B (DF) and phytic acid (PA) that bind tightly to all of the iron coordination sites making it inert, can prevent iron-derived ROS production within cells (31, 32).

Taken together, evidence is accumulating to suggest that iron mobilization in the cells is of critical importance in asbestos-induced DNA damage and toxicity.

**UPTAKE OF ASBESTOS FIBERS**

With the exception of mucus cells all airway cells endocytize particles (33). It has been shown that endocytosis of asbestos fibers and other types of iron-containing particles is important for particulate-induced toxicity in both mesothelial and epithelial cells (34-36). Liu et al. (34) have demonstrated an important role for asbestos endocytosis in a wide range of asbestos pathogenic effects, such as intracellular oxidation, DNA strand breakage and apoptosis. Furthermore, it has been shown that endocytosis of crocidolite asbestos in A549 cells was required for iron mobilization from asbestos fibers into a LMW fraction, ferritin induction and asbestos-dependent biological effects (35, 36). Nevertheless, the mechanism by which fibers are internalized is not well understood. Recently, Boylan et al. (37) observed that asbestos fibers could be internalized into the cells through association with the extracellular glycoprotein vitronectin via integrin membrane receptors.
EPIDERMAL GROWTH FACTOR RECEPTOR

Although the mechanism by which asbestos exerts its pathologic effects is not known, it is possible that asbestos-induced pulmonary toxicity might be initiated by interaction of asbestos fibers with the plasma membrane through a receptor-like mechanism (38).

Epidermal growth factor receptor belongs to the ErbB family of four closely related cell membrane receptors: EGFR (HER1 or ErbB1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4). These receptors are transmembrane glycoproteins that consist of three main regions: an extracellular ligand-binding part, a hydrophobic membrane-spanning region, and a cytoplasmic portion containing the tyrosine kinase. Activation of the EGFR occurs when a ligand, such as epidermal growth factor (EGF), binds to its extracellular domain. This causes the receptor to dimerize with either another EGFR monomer or with another member of the ErbB family (39). Following receptor dimerization, activation of the intrinsic protein tyrosine kinase activity and tyrosine autophosphorylation occur. The carboxy terminal tyrosine residues on EGFR, tyrosine 1068 and tyrosine 1173, are the major sites of autophosphorylation. All phosphorylated tyrosines serve as docking sites for signaling molecules containing Src homology 2 (SH2) domains, e.g. Grb2, or phosphotyrosine-binding (PTB) domains, e.g. Shc. Cytoplasmic mediators which bind to EGFR phosphotyrosine residues through SH2- or PTB-domains may either be adaptor proteins or enzymes. EGFR mediates the binding through direct interactions with phospho-tyrosine 1068 and phospho-tyrosine 1086 and through indirect interactions with phospho-tyrosine 1173. Tyrosine 1173 of EGFR also functions as a
kinase substrate (40). Phosphorylation of tyrosine 992, tyrosine 1068 and tyrosine 1086 is required for conformational change in the C-terminal tail of the EGF receptor (41). The events of recruitment and phosphorylation of various signaling proteins lead to subsequent activation of many different downstream signaling pathways. It has been shown that a variety of stresses, including both chemical and physical agents, can activate EGFR. These include ultraviolet rays (42), hydrogen peroxide (H$_2$O$_2$) (43) and asbestos fibers (38, 44-47). Activation of the EGFR has been shown to enhance processes responsible for tumor growth and progression, including the promotion of proliferation, angiogenesis, invasion/metastasis, and inhibition of apoptosis. These effects are mediated by a complex series of signaling mechanisms, such as engagement of the MAPK and phosphatidylinositol-3 kinase (PI3K) pathways. Inactivation of the EGFR can lead to G1 arrest (48) and apoptosis (49). Both activation and inactivation of the EGFR are associated with pathological effects.

Activation of the receptor by EGF also leads to rapid internalization of the receptor through clathrin-coated pits (reviewed in (50)). After internalization, EGF-receptor complexes appear sequentially in early endosomes and multivesicular bodies. During passage through the endosomal compartments, these complexes are either recycled back to cell surface or sorted to late endosomes and lysosomes, where both EGF and EGFR are proteolytically degraded. The accelerated internalization and efficient lysosomal targeting of EGF-occupied receptors result in a dramatic degradation of the EGFR protein in the presence of EGF. Interaction of the receptor with the ubiquitin ligase c-Cbl has been shown to be essential for efficient removal of the activated receptors from the cell surface ("down-regulation") (51). Recently this interaction has also been shown
to depend upon receptor phosphorylation at tyrosine 1045, the major docking site for c-Cbl (52). This binding results in the activation of the ubiquitin ligase activity of c-Cbl. The latter of these events promote the recruitment of the ubiquitin-conjugating enzyme UbcH7 (53) and the subsequent receptor monoubiquitination, which targets the receptor to lysosomal degradation. In addition to lysosomal degradation, several other mechanisms of receptor degradation have been proposed, e.g. by proteasome (54), calpain-mediated pathways (55), and caspase-mediated EGFR degradation during the early phase of cell death (56). Importantly, Opresko et al. (57) have shown that endocytosis and lysosomal targeting of EGFR are mediated by distinct sequences independent of the tyrosine kinase domain.

The majority of studies of interaction between asbestos and EGFR have been done in rodent cells or rodents, i.e. rat pleural mesothelial (RPM) cells (38, 45, 58), murine alveolar type II epithelial (C10) cells and transgenic mice (47). The treatment of rodent cells led to increased expression, phosphorylation and subsequent activation of the EGFR, leading to cell injury (apoptosis) and/or proliferation. Inhibition of the asbestos-induced phosphorylation of EGFR blocked the development of apoptosis in RPM cells. Studies with human cells are very limited and involve investigation of EGFR on the plasma membrane using only microscopy (44). In an immunohistochemical study of EGFR after treatment of MET5A cells and A549 cells with long (≥ 60 µm) asbestos fibers, MET5A cells showed patterns of aggregation and increases in EGFR protein while no effects were observed in A549 cells. It was concluded that this might be due to some differences in sensitivity to cytotoxic effects of asbestos and/or different mechanisms of response between mesothelial cells and epithelial cells.
Protein kinases are enzymes that covalently attach phosphate to the side chain of either serine, threonine, or tyrosine of specific proteins inside cells. Such phosphorylation of proteins can control their enzymatic activity, their interaction with other proteins, their location in the cell, and their susceptibility for degradation by proteases. Less than fifteen years ago, researchers discovered a group of novel protein kinases, now known as MAPKs. From that time, studies of the MAPK superfamily have grown explosively and have significantly contributed to our understanding of the regulation of growth and development and the mechanisms by which cells respond to external stresses. MAPKs have crucial roles in cellular responses to various extracellular signals (59). Their function and regulation have been conserved during evolution from unicellular organisms to complex organisms including humans (60). They regulate cellular activities ranging from gene expression, mitosis, movement, metabolism, and programmed death. They belong to a large family of proline-directed serine/threonine kinases that are the terminal kinases of three-tiered kinase modules consisting of MAPKKK (MAP kinase kinase kinase), MAPKK, and MAPK (61). Full activation of MAP kinases requires dual phosphorylation of a tyrosine and a threonine within a Thr-X-Tyr (where X is glutamate, proline and glycine in extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 MAPKs, respectively) motif in the activation loop. This is performed by dual specificity kinases, the MAPKK, which are themselves activated by phosphorylation by MAPKKK. The MAPKKK are activated by either phosphorylation or
by interaction with a small GTPase of the Ras or Rho family. There are three well-characterized subfamilies of MAPKs. These MAPKs include the extracellular-regulated kinases, ERK1 and ERK2, the c-Jun kinases, JNK 1, JNK 2, and JNK 3, and the five p38 enzymes, p38α, p38β, p38γ, p38δ, and p38-2. A fourth MAPK, ERK5, is a relatively recently identified MAPK and is being currently studied (62). Additionally, there are several possible MAPK candidates that are still not well characterized, e.g. ERK7 (63). The ERK module responds primarily to growth factors and mitogens and stimulates transcriptional responses in the nucleus. ERK1 and ERK2 are activated by MAPK/ERK kinase (MEK) 1 and MEK2, which phosphorylate at the threonine-glutamate-tyrosine motif (64). The MEKs, in turn, are activated by c-Raf, the MAPKKK of this signaling pathway that is in turn regulated by growth factor receptors and tyrosine kinases activating through Ras (65). Upon translocation to the nucleus, ERKs are responsible for the phosphorylation of multiple substrates, including activators of transcription, e.g. p90Rsk kinase, as well as transcription factors, e.g. Elk-1 (66). Generally, activation of ERK signaling pathways has a role in mediating cell division, migration, and survival.

There are five p38 isoforms (α, β, γ, δ and p38-2) that differ based on their substrate specificity. The p38α enzyme is the best characterized and perhaps the most physiologically relevant kinase involved in inflammatory responses. p38 MAPK is activated by dual phosphorylation on Thr180 and Tyr182 by an upstream MAPKK termed MAP2K6 (also known as MKK6). Other MAPKKs, such as MAP2K3 (also known as MKK3), have also been suggested to activate p38 MAPK. Recently, a MAPKK-independent mechanism of p38 MAPK activation has been described (67). The p38 MAPKs are generally activated by environmental stresses, including heat, osmotic...
and oxidative stresses, ionizing radiation, as well as inflammatory cytokines and tumor necrosis factor (TNF) receptor signaling (68). Downstream of p38 MAPK there is a diversification and extensive branching of signaling pathways. The substrates of p38 MAPKs include other kinases, cytosolic proteins and transcription factors (69, 70). The p38 MAP kinases regulate the expression of many cytokines, have an important role in activation of the immune response, and are involved in affecting cell motility, transcription and chromatin remodeling (70). Also, p38 MAPK is required for the induction of apoptosis by diverse stimuli such as UV irradiation, osmotic shock, and oxidants (71-73). Although the precise mechanism by which p38 MAPK induces cell death in unclear, blockade of this kinase has been shown to be protective against a variety of apoptotic stimuli (74). Finally, p38 MAPKs appear to be involved in human diseases such as asthma and autoimmunity.

In recent years, it has been shown that survival signals induced by several membrane receptors are mediated mainly by PI3K/Akt pathway. The Akt, or protein kinase B (PKB), has recently been a focus of intense research. Akt is a serine/threonine kinase, which in mammals comprises three highly homologous members known as PKBα (Akt1), PKBβ (Akt2), and PKBγ (Akt3). Akt is activated in cells exposed to diverse stimuli such as hormones, growth factors, and extracellular matrix components. The activation mechanism remains to be fully characterized but occurs downstream of PI3K. PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PIP3), a lipid second messenger essential for the translocation of Akt to the plasma membrane where it is phosphorylated and activated by phosphoinositide-dependent kinase 1 (PDK1) and possibly other kinases. Akt phosphorylates and regulates the function of many cellular
proteins involved in processes that include metabolism, cell cycle progression, cellular growth, cell survival and apoptosis. It appears that Akt lies in the crossroads of multiple cellular signaling pathways and acts as a transducer of many functions initiated by growth factor receptors. In addition, the recent discovery of the tumor suppressor PTEN as an antagonist of PI3K and Akt kinase activity suggests that Akt is a critical factor in the genesis of cancer. Studies conducted over the last years show that Akt is critical for cell survival triggered by growth factors, extracellular matrix, and other stimuli (75). Generally, in contrast to p38 MAPK signaling, phosphorylation of Akt substrates results in antiapoptotic effects (76).

Several independent research groups have provided evidence for the involvement of some signaling cascades in rodent cells following asbestos exposure (38, 77, 78). For instance, Jimenez et al. (79) have observed that asbestos-induced RPM cell toxicity was associated with activation of ERK, but not JNK/SAPK pathways. However, the effects of asbestos on the control and coordination of the signal transduction pathways in human lung cells have not been investigated. The results reported herein (Chapter 3 and 4) reveal not only important species differences but also the complexity and diversity of regulatory mechanisms in asbestos-induced signaling events.

PROTEIN PHOSPHATASES

It has been estimated that one third of all proteins in an organism, i.e. close to 10,000 proteins in humans, undergo reversible phosphorylation, often at multiple sites (80). Target proteins are phosphorylated at specific serine, threonine, or tyrosine residues (there is also evidence for histidine phosphorylation) by protein kinases and the
phosphate group is removed by the action of specific PPases. Over 98% of all phosphorylations occur on serine and threonine residues, whereas the remaining less than 2% affects tyrosines. Phosphorylation and dephosphorylation of proteins mediate signal transduction events that control a multitude of cellular processes including metabolism, transport, cell division, differentiation and development, learning and memory (81, 82).

PPases are classified based on their substrate specificity, dependence on metal ions, and sensitivity to inhibitory agents. There are two main classes of PPases according to their substrate specificity, *i.e.* PTPases and PSPases, which remove phosphate from proteins containing phosphotyrosine or phosphoserine/phosphothreonine, respectively. Interestingly, PTPase family includes both tyrosine-specific and dual specificity phosphatases (DSPases) that remove phosphate from proteins containing all three phosphoamino acid residues.

PSPases are divided into two groups, type 1 and type 2, depending on whether they dephosphorylate the β subunit of phosphorylase kinase specifically and are inhibited by nanomolar concentrations of two small heat- and acid-stable proteins, termed inhibitor 1 (I-1) and inhibitor 2 (I-2) (type 1, PP1), or whether they dephosphorylate the α subunit of phosphorylase kinase preferentially and are insensitive to I-1 and I-2 (type 2, PP2). The type 2 phosphatases could, in turn, be subdivided into three distinct enzymes, PP2A, PP2B, PP2C, in a number of ways, but most simply by their dependence on divalent cations. PP2B and PP2C had absolute requirements for Ca (II) and Mg (II), respectively, while PP2A (like PP1) was at least partially active toward most substrates in the absence of divalent cations (83, 84).

PSPases have been shown to regulate apoptosis. Although inhibitors of PP1 and
PP2A, such as OA and calyculin A, induce apoptosis in some cell lines (85, 86), inhibition of apoptosis by these agents has also been reported (87-90). Inhibition of PP2A has been shown to block poly(ADP-ribose) polymerase (PARP) cleavage, a caspase-3 substrate (91). In addition, PP1 inhibition suppressed the ceramide-induced alternative splicing of specific apoptotic factors, caspase-9 and Bcl-x (89).

Like protein tyrosine kinases, PTPases constitute a large family of enzymes (92). PTPases can exert both positive and negative effects on a signaling pathway and play crucial physiological roles in a variety of mammalian tissues and cells (93). Unlike protein tyrosine kinases, however, which share sequence identity with protein serine/threonine kinases, the PTPases show no sequence similarity with the PSPases. PTPases are characterized by the presence of the active site signature motif (H/V)C(X)₃R(S/T) in the conserved catalytic domain (94). In addition to the catalytic domain, PTPases are decorated with a wide range of structural elements including SH2 domains, extracellular ligand binding domains, and many others (95). A recent estimate from the nearly completed human genome sequence suggests that humans have 112 PTPases (96), which include both the tyrosine-specific and dual-specificity phosphatases. The tyrosine-specific phosphatases, such as PTPlB, hydrolyze phosphotyrosine-containing proteins, while the dual-specific phosphatases, such as Cdc25, can utilize protein substrates that contain phosphotyrosine, as well as phosphoserine and phosphothreonine. The tyrosine-specific PTPases can be further categorized into two groups: receptor-like and intracellular. The receptor-like PTPases, exemplified by CD45, generally have an extracellular putative ligand-binding domain, a single transmembrane region, and one or two cytoplasmic PTP domains. The intracellular PTPases, exemplified
by PTP1B, contain a single catalytic domain and various amino or carboxyl terminal extensions including SH2 domains that have targeting or regulatory functions. Examples of dual-specificity phosphatases include the MAP kinase phosphatases, the cell cycle regulator cdc25 phosphatases, and the tumor suppressor PTEN. All PTPases are characterized by their sensitivity to vanadate, ability to hydrolyze p-nitrophenyl phosphate, insensitivity to okadaic acid, and lack of metal ion requirement for catalysis.

Several findings suggest that PTPases could be actively involved in the induction of apoptosis. Activation of PTPases inhibited cellular growth of human renal and breast carcinoma cells (97). Meredith et al. (98) found that apoptosis was blocked by treating cells in suspension with the tyrosine phosphatase inhibitor sodium orthovanadate, and this effect was reversed by the tyrosine kinase inhibitor herbimycin A. Microinjection of MAP kinase phosphatase 1 has also been shown to inhibit ras-induced DNA synthesis in rat fibroblasts (99), and several other factor inhibited cell proliferation by stimulating PTPases (100).

**REDOX SIGNALING**

Growing evidence indicates that reduction-oxidation (redox) status can regulate various aspects of cellular function. It affects cell growth and functions via modulating or utilizing signaling pathways from cell surface receptors. In addition, in several signaling cascades ROS themselves may act as an intracellular signaling system (101). In addition to direct damage, generation of ROS may lead to excessive signals to the cells resulting in cell death or pathological processes in which ROS are involved. The redox state of the cell is primarily a consequence of the precise balance between the levels of oxidizing...
ROS and reducing equivalents. To counteract the effects of ROS, cells have developed two important defense systems mechanisms: a thiol reducing buffer consisting of small proteins with redox-active sulfhydryl moieties, e.g. glutathione (GSH) and thioredoxin (TRX), and enzymatic systems, e.g. superoxide dismutase, catalase, and glutathione peroxidase (102). In recent years it has become clear that this finely tuned cellular redox state can be modulated under several conditions, e.g. oxidative stress. The term oxidative stress has rarely been defined in a universally accepted way. One accepted definition by Sies et al. (103) is a disturbance in the pro-oxidant - antioxidant balance in favor of the former, leading to potential damage. Oxidative stress has been implicated in a variety of human diseases including atherosclerosis, diabetes, arthritis, cancer, and neurodegenerative disorders (104). In higher eukaryotes, ROS can be generated when oxygen is partially reduced as electrons leak out of the electron transport chain during respiration in mitochondria. Oxidative stress can also originate from other tissues or from neighboring cells, as well as from the extracellular stimuli, e.g. asbestos fibers. The most common forms of ROS include $O_2^-$, $H_2O_2$, and the highly reactive $-OH$. ROS have been implicated in the regulation of diverse cellular functions including defense against pathogens, proliferation and apoptosis. Additionally, several lines of evidence have indicated that the cellular redox state plays an essential role in many cellular signaling systems (105-108). Cells respond to their environment and carry out specific functions through highly organized and tightly regulated networks of signaling pathways that encompass kinases, phosphatases, adapter and scaffold proteins, and others that produce or respond to small diffusible molecules called second messengers. There is growing body of evidence that supports a role for ROS as second messengers and critical
modulators of protein phosphorylation and gene transcription. Exposure of cysteine residues to ROS can give rise to a variety of different reaction products, and the stability of a given product depends on the local environment of the thiol involved (109).

The distinctive characteristic of EGFR is the presence of two-cysteine rich sequence repeat regions within the extracellular domains of these receptors. Strict conservation of cysteine residue spacing and similarity of interspersed sequences suggest functional significance for a disulfide-linked structural motif (110). It is believed that the function of EGFR depends on intra- and intermolecular S-S bridges (111). It has been suggested that oxidation of the reactive SH-groups enhances receptor activation while reduction of relevant S-S bridges leads to its inactivation (112, 113). Interestingly, it has been observed that iron-derived ROS, directly or indirectly, led to receptor dephosphorylation (see Chapter 3). Since \( \text{H}_2\text{O}_2 \) activates, rather than inactivates, EGFR activity (43, 114), this observation may seem somewhat controversial. However, \( \text{H}_2\text{O}_2 \) and iron-derived ROS differ markedly in their reactivity with cellular molecules, which might explain that inconsistency. Hydrogen peroxide is only mildly reactive and thus reacts sluggishly with biomolecules (115). On the other hand, iron can generate reactive oxygen species via the sequential, one electron reductions of molecular oxygen to produce the highly reactive hydroxyl radical that can react with almost any biological molecule. In addition, iron as a catalyst in these reactions may continue to cycle indefinitely through a series of Haber-Weiss reactions (11). One plausible mechanism by which iron-derived ROS may lead to EGFR dephosphorylation is through activation and/or recruitment of protein phosphatases that dephosphorylate and inactivate the EGFR.
Although cells have antioxidant enzymes and some reducing systems, e.g. GSH and Trx, ROS can produce transient changes in the cellular redox state, particularly, in the redox state of cysteinyi thiols, which can affect the activity, the protein-protein and DNA-protein interactions of enzymes and transcription factors. Evidence has recently accumulated in support of a role of ROS in the modulation of PPase activity. PTPases contain in their active site a cysteine (CX5R) that is essential for their activity and can be modified by oxidation. This cysteine is particularly reactive with $\text{H}_2\text{O}_2$ because the surrounding residues in the active site allow for its ionization to a thiolate. Reaction of the thiolate with $\text{H}_2\text{O}_2$ leads to inactivation of the PTPases. This intermediate quickly reacts with a thiol (-SH) such a GSH to produce a disulfide in the PTPases, a form that is also catalytically inactive. Data obtained in vivo and in vitro indicate that $\text{H}_2\text{O}_2$ can specifically and reversibly inactivate PTPases (116, 117). Interestingly, it has recently been demonstrated that a PPase regulated the dephosphorylation of tyrosine residues, directly or indirectly, in the oxidative stress-mediated inactivation of EGFR (56). This finding was very unusual since, as mentioned above, oxidation of critical cysteine residues within PPases catalytic sites leads to their inactivation rather than activation (118). However, it has been speculated that a sphingolipid ceramide could be a possible intermediate between singlet oxygen and activation of PPases (56). It has been shown that singlet oxygen induces rapid ceramide production, and moreover, that ceramides activate PPases (119). Additionally, oxidative stress has been shown to activate p38 MAPK (120), which in turn may activate PPases (121). Thus, p38 MAPK could also link production of ROS and activation of PPases.

Many studies have shown that exposure to oxidative stress leads to activation of
the MAP kinases (106, 120, 122-124). The mechanisms by which exogenous or endogenous produced ROS activate the MAP kinases are not well defined. Several mechanisms have been proposed for p38 MAPK activation that involve ROS-dependent dissociation of a signalosome that maintains the pathway in an inactive state. ASK1, a MAPKKK for p38 MAPK associates with reduced Trx in non-stressed cells. Oxidation of Trx by ROS releases ASK-1, and leads to p38 MAPK activation (125).

Activation of some MAP kinases could also be the result of activation of the Src kinase family. c-Src was found to be required for the H2O2-induced activation of ERK5 (126) while Fyn, another member of the Src family, appears to be involved in the H2O2-induced activation of ERK1/2 (127). Additionally, the DSPases that control the activity of the MAP kinases also contain the cysteine signature motif, although the role of ROS in modulating their activity has not yet been extensively explored (128). Taken together, despite a great deal of work that has been done in this field, the exact mechanisms for the redox sensitivity of these kinases are not known.

**APOPTOSIS AND CANCER**

Programmed cell death, or apoptosis, is recognized as a critical element during normal development and in the removal or elimination of cells following exposure to toxic compounds, without affecting neighboring cells or eliciting an inflammatory response. This process of programmed cell death is characterized by marked changes in cell morphology including chromatin condensation, membrane blebbing, nuclear breakdown, appearance of membrane-associated apoptotic bodies, as well as by internucleosomal DNA fragmentation and cleavage of many housekeeping proteins, e.g.
PARP. In the last decade, much progress has been made towards elucidating the various signal transduction pathways that can ultimately lead to cell death. Many apoptotic cascades have been described, such as mitochondrial and death receptor as well as p53-dependent and -independent pathways, in association with initiation, commitment, and execution phases. It has become clear that apoptosis is not a series of clearly defined pathways, but rather, a multitude of highly regulated, interconnected pathways. The intrinsic cell death pathway involves the initiation of apoptosis as a result of a disturbance of intracellular homeostasis. In this pathway, mitochondria are critical for the execution of cell death and therefore this pathway has also been referred to as the mitochondrial cell death pathway. The extrinsic pathway involves the initiation of apoptosis through ligation of plasma membrane death receptors and therefore this pathway is also referred to as the death receptor pathway. While the initiation mechanisms of these pathways are different, both converge to result ultimately in cellular morphological and biochemical alterations characteristic of apoptosis.

Caspases, cysteine aspartate-specific proteases, are family of intracellular proteins involved in the initiation and execution of apoptosis. To date, at least 14 mammalian caspases have been identified. Caspases are synthesized as pro-caspases that are then proteolytically processed, at critical aspartate residues, to their active forms. The induction of apoptosis through extrinsic or intrinsic death mechanisms results in the activation of initiator caspases, i.e. caspases 2, 8, or 10 (extrinsic pathway) and caspase 9 (intrinsic pathway). Activation of initiator caspases is the first step of a highly regulated, irreversible, self-amplifying proteolytic pathway. Initiator caspases are able to cleave pro-caspases, and thus, are able to activate effector caspases (caspases 3, 6, and 7).
Effector caspases are common to both the extrinsic and intrinsic pathways, and that is why the ultimate morphological and biochemical hallmarks of apoptosis are relatively independent of the apoptotic inducer.

PARP, a 116 kDa nuclear poly(ADP-ribose) polymerase, appears to be involved in DNA repair predominantly in response to environmental stress (129). PARP is thought to be one of the earliest targets for cleavage by caspase-3-like proteases during apoptosis. The cleavage of PARP between Asp214 and Gly215 results in separation of the two DNA-binding motifs in the NH₂-terminal region of the enzyme and renders PARP inactive. With the use of a caspase-3-resistant PARP mutant, it has been shown that cleavage of PARP plays an important role in the normal progression of apoptosis (130). The cleavage of PARP has been suggested to occur to prevent depletion of the energy reserves, i.e. ATP, that are thought to be required for the later stages of apoptosis, and to prevent futile repair of DNA strand breaks during the death program. Cleavage of PARP has also been suggested to facilitate cellular disassembly and serves as a marker of cells undergoing apoptosis (131).

Upon binding its receptor, EGF activates EGFR and initiates signal transduction cascades including the cellular components such as PI3K/Akt and MAPK. Generally, EGF-like growth factors concomitantly activate these two pathways, whereas EGFR inhibition suppresses both signaling cascades (48). Depending on the cell type and the duration and strength of the stimulus, these pathways are often activated simultaneously with apparently conflicting responses such as apoptosis and proliferation (132). Generally, however, activation of PI3K/Akt and MAPK signaling pathways plays a role in mediating cell survival. The cytosolic serine/threonine protein kinase Akt is considered
the focal point of a survival pathway known to protect cells from apoptosis during cytokine and growth factor stimulation. Activated Akt has also been shown to delay the onset of p53-mediated transcriptionally dependent apoptosis (133). It has become evident that ErbB receptor blockade results in interruption of constitutive MAPK and PI3K/Akt signaling, reversible G1 arrest, and apoptosis (134, 135). Recent evidence has pointed to a relationship between the Akt and MAPK survival pathways and the structurally similar stress-activated members of the MAPK family, which includes p38 MAPK pathway. It has been shown that activation of ERK functions to protect cells from oxidative stress-induced apoptosis, while activation of p38 MAPK signaling plays a proapoptotic role in this process. There is growing evidence suggesting cross-talk between p38 MAPK pathway and ERK and Akt pathways in a variety of eukaryotic cells. It seems as if the dynamic balance among PI3K/Akt, ERK1/2 and p38 MAPK pathways may govern the ability of cells to survive or undergo apoptosis (136). It has been observed that blockade of PI3K/Akt and ERK1/2 pathways with concurrent activation of the p38 MAPK pathway suppressed cell survival and subsequently stimulated apoptosis in HeLa cells (137). The exact cellular mechanism underlying the cross-talk between p38 MAPK and ERK and Akt pathways remains unknown. It is clear though that regulation of these proteins involves a dynamic interplay between kinases and phosphatases.

Growing evidence suggests that apoptosis is regulated by the active PPases. Activation of PPases has been implicated in dephosphorylation of a number of survival proteins including Akt and ERK1/2 (138, 139), and furthermore, inhibition of PPases led to activation of MEK and ERK1/2 (140). Recent studies have also demonstrated that activation of p38 MAPK by arsenite blocked the activation of survival ERK1/2 via a
PSPase in NIH 3T3 fibroblasts (121). In addition, caspase-3 has been shown to be associated with altered phosphatase activity. Caspase-3 directly cleaves the PP2A regulatory A subunit thereby increasing phosphatase activity (141), and inhibition of PP2A blocks PARP cleavage (91). Finally, PPase inhibition has been shown to suppress the ceramide-induced alternative splicing of specific apoptotic factors, caspase-9 and Bcl-x (89).

It is now widely recognized that cancer is not simply a proliferative disease, but the manifestation of an imbalance between cell growth and cell death. In light of the critical importance of apoptosis in the regulation of tissue growth, one may speculate that alterations in this pathway may be an important factor in tumorigenesis. It is not clear, however, what role, if any, apoptosis may play in the early stages of carcinogenesis. One possibility is that an active pathway leading to apoptotic cell death may simply be overcome. Also, some cells may exhibit preexisting genetic insensitivity to induction of apoptosis. Furthermore, the carcinogen-induced apoptosis could play a passive role in carcinogenesis by indirectly stimulating cells in the area around the site of apoptosis (which may contain sublethal but mutagenic levels of damage) to proliferate. In this way apoptosis would not differ from necrosis or wounding in facilitating tissue regeneration and expansion of potentially mutagenized cells. Another possibility is that defective apoptosis may permit the persistence of damaged, mutated cell that would otherwise have been deleted. Importantly, for neoplastic growth to occur, an imbalance between proliferation and apoptosis must be established such that cell growth predominates.

Studies by others have demonstrated asbestos-induced apoptosis in both rodent (38, 77, 142-144) and human (142, 145-147) lung cells. The mechanism of asbestos-
induced apoptosis in the lung cells is not entirely known. Studies in rodent cells and rodents provided some insights into the signal transduction pathways involved in asbestos-induced apoptosis. Mossman and coworkers have shown that in addition to mitochondrial-regulated pathways (77), apoptosis was associated with signaling through EGFR and subsequent activation of the ERK1/2 cascades (45). Additionally, Swain et al. (78) have suggested an important role for p38 MAPK in asbestos-induced cytotoxicity in RPM cells. Since some differences were reported in the way rodent and human cells responded to asbestos fibers (Chapter 3 and 4), it is of great importance to examine and compare the asbestos-induced signaling events in human lung epithelial cells. Kamp and coworkers have established that asbestos induces apoptosis in human lung epithelial cells (145-147). It was proposed that apoptosis resulted from mitochondrial dysfunction and was, in part, due to iron-derived ROS. Nevertheless, the signaling cascades involved in asbestos-induced apoptosis in human cells have not been thoroughly explored.

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ABSTRACT

Occupational exposure to asbestos has been associated with increased incidence of pulmonary interstitial fibrosis, mesothelioma of the pleura, and bronchogenic carcinoma. Although the mechanism by which asbestos causes cancer remains unknown, iron associated with asbestos is thought to play a role in the pathogenic effects of fibers. Here, we examined the effects of asbestos on the epidermal growth factor receptor (EGFR) in human lung epithelial (A549) cells, human pleural mesothelial (MET5A) cells, and normal human small airway epithelial (SAEC) cells. Treatment of A549, MET5A and SAEC cells with asbestos caused a significant reduction of EGFR tyrosine phosphorylation. This was both time- (15 min to 24 h) and concentration-dependent (1.5, 3 and 6 µg/cm²) in A549 cells. Also, treatment with 6 µg/cm² crocidolite for 24 h diminished the phosphorylation levels of human epidermal growth factor receptor 2 (HER2). In contrast to treatment with 100 ng/ml epidermal growth factor for 15 min, exposure of A549 cells to 6 µg/cm² crocidolite for 3 to 24 h resulted in no detectable Y1045 phosphorylation and no apparent degradation of the EGFR. Inhibition of fiber endocytosis resulted in a considerable inhibition of EGFR dephosphorylation. Most importantly, removal of iron from asbestos by desferrioxamine B or phytic acid inhibited asbestos-induced decreases in EGFR phosphorylation. The effects of crocidolite, amosite,
and chrysotile on the EGFR phosphorylation state appeared to be directly related to the amount of iron mobilized from these fibers. These results strongly suggest that iron plays an important role in asbestos-induced inactivation of EGFR. This inactivation may be involved in events leading to apoptosis.

INTRODUCTION

Asbestos is a naturally occurring mineral that has been used for centuries because of its unique physical and chemical properties, including durability, tensile strength, and heat resistance. However, it was recognized to be a human carcinogen in the late 1950s. Exposure to asbestos increases the risk of mesothelioma of the pleura and bronchogenic carcinoma of the lungs. Even though the molecular mechanism by which asbestos exerts these effects is not fully understood, the carcinogenicity of asbestos has been related to some of its physical properties, such as size and durability of fibers (1). The most carcinogenic forms of asbestos contain as much as 27% iron by weight. It has been shown that iron from asbestos catalyzes the generation of reactive oxygen species (ROS), such as superoxide radical \( \left( O_2^- \right) \) and hydroxyl radical \( (-\cdot OH) \). In addition, iron has been shown to be responsible for \( O_2 \) consumption, induction of DNA single-strand breaks, lipid peroxidation, and potentially cancer. There is also significant evidence suggesting that iron from other sources besides asbestos may cause human cancer.

Although the mechanism by which asbestos exerts its pathologic effects is not known, at least two plausible mechanisms should be considered. Asbestos-induced pulmonary toxicity might be initiated by ROS generated from mobilized iron via Haber-Weiss reactions (1), and possibly by interaction of asbestos fibers with the plasma...
membrane through a receptor-like mechanism (2).

Epidermal growth factor receptor (EGFR/ErbB1) is a transmembrane protein with intrinsic tyrosine kinase activity that is stimulated upon ligand binding. EGFR activation involves homo- and heterodimerization with other members of ErbB receptor family, such as ErbB2 (HER2), transphosphorylation of receptors, recruitment of various signaling proteins, and subsequently activation of many different downstream signaling pathways. It has been shown that a variety of stresses, including both chemical and physical agents, can activate EGFR. These include ultraviolet rays (3), hydrogen peroxide (H$_2$O$_2$) (4) and asbestos fibers (2, 5-8). Inactivation of the EGFR can lead to G1 arrest (9) and apoptosis (10). Both activation and inactivation of the EGFR are associated with pathological effects.

The majority of studies of interaction between asbestos and EGFR have been done in rodent cells or rodents, i.e. rat pleural mesothelial (RPM) cells (2, 6, 11), murine alveolar type II epithelial (C10) cells and transgenic mice (8). The treatment of rodent cells led to increased expression, phosphorylation and subsequent activation of the EGFR, leading to cell injury (apoptosis) and/or proliferation. Inhibition of the asbestos-induced phosphorylation of EGFR blocked the development of apoptosis in RPM cells. Studies with human cells are very limited and involve investigation of EGFR on the plasma membrane using only microscopy (5). In an immunohistochemical study of EGFR after treatment of human pleural mesothelial (MET5A) cells and human lung epithelial (A549) cells with long (≥ 60 µm) asbestos fibers, MET5A cells showed patterns of aggregation and increases in EGFR protein while no effects were observed in A549 cells. It was concluded that this might be due to some differences in sensitivity to
cytotoxic effects of asbestos and/or different mechanisms of response between mesothelial cells and epithelial cells.

The purpose of this study was to investigate and compare the effects of asbestos fibers on the EGFR activity in relevant human lung and pleural target cells, i.e. epithelial (A549 and small airway epithelial cells (SAEC)) and mesothelial (MET5A) cells, and to determine if iron associated with fibers plays some role in this process. Our results indicated that in contrast to rodent cells, treatment of human lung epithelial and pleural mesothelial cells with asbestos fibers resulted in dephosphorylation and subsequent inactivation of the ErbB family of receptor tyrosine kinases. Additionally, it appeared that iron was responsible, at least in part, and that the effect on the receptor was mediated by internalized fibers. We have identified a potentially important mechanism by which relevant human lung and pleural target cells respond to asbestos fibers.

MATERIALS AND METHODS

**Asbestos and Reagents.** Crocidolite, amosite and chrysotile fibers were obtained from Dr. Richard Griesemer, National Institute for Environmental Health Sciences/National Toxicology Program (Research Triangle Park, NC) and contained 27, 27, and 2% iron by weight, respectively. The crocidolite sample used had a mean length of 10 µm and mean width of 0.27 µm, determined by scanning electron microscopy.

Sodium bicarbonate and the reagents to prepare phosphate buffered saline (PBS) were obtained from Fisher Scientific (Pittsburgh, PA). A custom preparation of Ham's F-12 tissue culture medium (free of added iron salts) and 0.5% trypsin (with 0.2% EDTA) were obtained from Invitrogen (Carlsbad, CA). Gentamicin (50 mg/ml) was obtained
From Biowhittaker Inc. (Walkersville, MD). Fetal bovine serum (FBS) was obtained from HyClone Laboratories Inc. (Logan, UT).

Desferrioxamine B (DF), phytic acid (PA), titanium dioxide (TiO₂), cytochalasin B and D (CB and CD), and epidermal growth factor (EGF) were purchased from Sigma-Aldrich (St. Louis, MO). Biotin-N-hydroxysuccinimide ester, Biotin-X-NHS, and horseradish peroxidase-conjugated streptavidin were purchased from Calbiochem (San Diego, CA). Antibodies were purchased from either Santa Cruz Biotechnology Inc. (Santa Cruz, CA) or Cell Signaling Technology (Beverly, MA). Protein A/G plus-agarose beads were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-mouse and anti-rabbit enzyme-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA). Chemiluminescent Western blotting detection reagent (ECL) was purchased from Amersham Biosciences, Inc. (Piscataway, NJ), and X-ray film was purchased from Kodak (Kodak, Rochester, NY). Radiolabel [γ-³²P] ATP was purchased from ICN Biomedicals Inc. (Irvine, CA). All remaining reagents were purchased in the highest purity possible.

**Cell Culture.** A human lung epithelial cell line, A549, with characteristics of alveolar epithelial type II cells, was used for the majority of these studies. The A549 cells (ATCC CCL185) were purchased from American Type Culture Collection (Rockville, MD). The cells were cultured as previously described by Chao et al. (12). Briefly, A549 cells were cultivated in complete growth medium in a Forma Model 315B water-jacked incubator (Forma Scientific, Marietta, OH) at 37 ± 1°C in an atmosphere of 5 ± 0.5% CO₂ and 95% humidity. Complete growth medium was composed of iron-free Ham’s F12 cell culture medium, 50 μg/ml gentamicin, 10% FBS, and 1.176 g NaHCO₃/l
medium to obtain a final pH of 7.4. For maintenance of stock cultures, cells were dislodged with 0.5% trypsin and 0.2% EDTA before reaching confluence, resuspended in complete growth medium, and plated.

A human pleural mesothelial cell line immortalized by transformation with SV40 T antigen (MET5A) was kindly provided from Dr. Brenda Gerwin and Dr. Curtis Harris (Laboratory of Human Carcinogenesis, NCI, Bethesda, MD). The cells were cultured as previously described by Pache \textit{et al.} (5), with some modifications. Briefly, MET5A cells were maintained in a complete iron-free Ham’s F12 cell culture medium containing hydrocortisone (100 ng/ml), insulin (2.5 µg/ml) and transferrin (2.5 µg/ml). For maintenance of stock cultures, cells were dislodged with 0.5% trypsin and 0.2% EDTA before reaching confluence, resuspended in a complete growth medium, and plated.

Primary cultures of human small airway epithelial cells (SAEC) (Clonetics, Walkersville, MA) from the distal airspace were used for some experiments. The SAEC cells were cultured in small airway growth medium (Clonetics, Walkersville, MA), as directed by the manufacturer.

\textbf{Preparation of Asbestos for Treatment of Cells.} Cells were treated with crocidolite, amosite, or chrysotile. Fibers were suspended in sterile 14 mM NaHCO$_3$ (pH 7.4) at a concentration of 1 mg/ml immediately before treatment of cells, vortexed for 1 min, and diluted to the appropriate concentration with the growth medium.

\textbf{Treatment of Cells with Asbestos Fibers.} The cells were cultured until ~75% confluent, rinsed with PBS, then dislodged with trypsin-EDTA and plated at a density of 20,000 cells/cm$^2$. Twenty-four hours prior to and during treatment, cells were cultured in
0.5% FBS, referred to as serum-deprived. After the indicated treatment time intervals, the medium, containing the fibers that were not associated with cells and/or phagocytized, was removed. The cells were rinsed with ice-cold PBS, pH 7.4, and dislodged with 0.5% trypsin. The cell pellets were stored at -80°C.

**Lysate Preparation and Western Blotting.** Cell lysates were prepared for Western blotting, as described by Simeonova et al. (13), with some modifications. Briefly, after treatments, cells were lysed in RIPA buffer containing protease inhibitors (30 µl/ml aprotonin, 4 µg/ml leupeptin, 4 µg/ml soybean trypsin inhibitor, 0.1 M PMSF, and 1 µM benzamidine), and tyrosine phosphatase inhibitor (1 mM sodium orthovanadate). Lysates were cleared by centrifugation at 15,000 × g for 30 min at 4°C, and the protein concentrations were determined by the Bradford method. Lysates were then subjected to electrophoresis in 8-12% SDS-PAGE, and transferred to polyvinyl difluoride (PVDF) membranes. The membranes were incubated with blocking solution (5% milk) to reduce nonspecific binding. Then, the appropriate primary antibodies (1:1000 dilution) were applied (1 h at room temperature or overnight at 4°C), followed by incubation with the secondary enzyme-conjugated antibodies (1:10,000 dilution) for 2 h at room temperature. Immune complexes were visualized on the PVDF membranes by ECL, and detected by exposure to X-ray film, as directed by the manufacturer.

**Immunoprecipitation and In Vitro Kinase Assay.** Immunoprecipitation was performed, as described by Simeonova et al. (13), with some modifications. Briefly, after treatments, cells were washed with ice-cold PBS, and lysed with 1.0 ml of solubilization buffer [1% Triton, 5 mM EDTA, 150 mM NaCl, 50 mM NaF, 10 mM Tris/HCl, pH 7.6] containing protease inhibitors and tyrosine phosphatase inhibitor, as described in Lysate
Preparation and Western Blotting. Lysates were cleared by centrifugation at 15,000 x g for 30 min at 4°C, and the protein concentrations were determined by the Bradford method. Lysates were then immunoprecipitated by overnight incubation with the indicated antibody at 4°C, followed by protein A/G plus-agarose precipitation for 2 h. The beads bearing the immunoprecipitates were washed three times with ice-cold solubilization buffer for the *in vitro* kinase assay.

*In vitro* kinase phosphorylation assays were performed, as described by Zanella *et al.* (6), with some modifications. Briefly, the immunoprecipitated proteins were washed twice with the kinase buffer [20 mM HEPES, pH 7.5, 0.015% Triton, 0.1 mM EDTA] and resuspended in the kinase reaction buffer [0.15 mM ATP, 30 mM MgCl₂] containing poly (Glu, Tyr, 4:1) (Sigma-Aldrich, St. Louis, MO) as an artificial substrate, and radiolabel [γ-³²P] ATP (10 μCi/ml). The kinase reactions were carried out for 10 min at 30°C, and were terminated by adding 4 x SDS sample buffer. Samples were separated on 10% SDS-PAGE. Gels were dried and exposed to X-ray film at -80°C for autoradiography.

**Biotin Labeling of EGFR.** Methods for biotin labeling of cell surface receptors have been described by Ravid *et al.* (4). Briefly, 24 h prior to treatment cells were cultured in 0.5% FBS. The cells were then incubated with 0.5 mg/ml Biotin X-NHS for 45 min at 4°C. Washing the cells with ice-cold PBS containing 15 mM glycine ended biotinylation of primary amine groups of proteins. Then, after washing with room temperature PBS the cells were treated as indicated. Following treatment, proteins were lysed and immunoprecipitated, as described in Immunoprecipitation and *In Vitro* Kinase Assay. Protein labeling was detected with horseradish peroxidase-conjugated
streptavidin, a biotin-binding protein with four high affinity-binding sites. Immune complexes were visualized on the PVDF membranes with ECL, and detected by exposure to X-ray film, as directed by the manufacturer.

**Incubation of Cells with DF-Crocidolite, Phytic Acid or Cytochalasins.** In some experiments, the role of iron was examined by treating the cells with crocidolite from which the redox active iron (bioavailable iron) had been removed with desferrioxamine B (DF-crocidolite). DF-crocidolite was prepared, as previously described (14). This treatment removed approximately 8% of the total iron from the fibers and the majority of the bioavailable iron. The fibers were resuspended immediately before use, as described in Treatment of Cells with Asbestos Fibers. In some experiments, another iron chelator, phytic acid (PA), was added to the A549 cells 1 h before treatment. Crocidolite fibers were then added to PA-containing culture medium on the cells.

To inhibit endocytosis of fibers, 1 h prior to and during treatment, cells were cultured in the presence of 5 µg/ml cytochalasin B or D.

**Statistical Analysis.** The results are expressed as the mean ± SE. A student's t test was used to assess the significance of differences between two groups. Probability values < 0.05 were considered significant. The *in vitro* kinase assay and Western blotting experiments were repeated two and three times, respectively.

**RESULTS**

**Effects of EGF on EGFR Phosphorylation.** To investigate if A549 cells, originally obtained from an alveolar carcinoma, respond to EGF as do normal cells,
serum-deprived A549 cells were treated with EGF at 100 ng/ml. Consistent with previous findings by Goldkorn et al. (15) the addition of EGF stimulated a rapid autophosphorylation of the EGFR, as measured by Western blotting analysis using antibodies recognizing either the total EGFR or the EGFR phosphorylated on Y1068 and Y1173 (Fig. 3-1A). The tyrosine phosphorylation peaked between 15 and 30 min after the addition of EGF and then rapidly declined. We also investigated the effects of EGF on kinase activation of the EGFR by means of a traditional in vitro kinase assay using \([\gamma-^{32}\text{P}]\) ATP and the universal protein tyrosine kinase substrate poly (Glu, Tyr, 4:1) (Fig. 3-1B). Consistent with Western blotting results, EGF markedly increased the phosphorylation level of both the receptor (data not shown) and the substrate above that of the control, untreated cells. A 4.7-fold increase was observed with the peak response after 30 min. Taken together, these results demonstrated that A549 cells showed normal responses to EGF.

**Effects of Three Types of Asbestos Fibers on EGFR Phosphorylation.** We comparatively examined the ability of three types of asbestos fibers to modulate the phosphorylation state of Y1068 and Y1173 in EGFR. In contrast to the positive control, all three types of asbestos fibers significantly decreased constitutive phosphorylation of Y1068 and Y1173 (Fig. 3-2). The amount of decrease in phosphorylation observed after crocidolite, amosite or chrysotile treatment appeared to be directly related not only to the percentage of iron by weight present in the different forms, 27, 27, and 2%, respectively, but also to the amount of iron mobilized from these fibers (16).

Since Zanella et al. (6) have shown that asbestos induces EGFR phosphorylation rather than dephosphorylation in RPM cells, we analyzed if different types of F12 media
Fig. 3-1. Effects of EGF on EGFR Phosphorylation. Serum-deprived A549 cells were incubated with or without 100 ng/ml EGF for the indicated time intervals. A, cellular lysates were subjected to Western blotting analysis, as described under Materials and Methods. Phosphorylation of Y1068 and Y1173 of the EGFR was detected using the phospho-specific antibodies. EGFR levels were detected using an anti-EGFR antibody (the upper and lower bands represent phosphorylated and not phosphorylated EGFR forms, respectively). B, one mg protein aliquots from untreated or EGF-treated A549 cells were subjected to immunoprecipitation with anti-EGFR, and to traditional in vitro kinase assays with the substrate poly (Glu, Tyr, 4:1), as described under Materials and Methods. The in vitro kinase assay and Western blotting experiments were repeated two and three times, respectively, and the representative autoradiograms are shown. The optical densities (OD) of the bands were quantified. The results are shown as the mean ± SE, and are expressed as fold increase relative to the untreated control. Error bars represent the SE. * P <0.05 compared with control, untreated groups.
Fig. 3-2. Effects of Three Types of Asbestos Fibers on EGFR Phosphorylation. Serum-deprived A549 cells were treated with 6 µg/cm² crocidolite (CRO), amosite (A) or chrysotile (short and intermediate range; CSR and CIR, respectively) for 24 h, or with 100 ng/ml EGF for 15 min (positive control). Cellular lysates were subjected to Western blotting analysis, as described under Materials and Methods. Phosphorylation of Y1068 and Y1173 of the EGFR was detected using the indicated antibodies. EGFR levels were detected using an anti-EGFR antibody (the upper and lower bands represent phosphorylated and not phosphorylated EGFR forms, respectively). The two upper bands in EGFR are due to low percentage SDS-PAGE gels. Data presented is representative of three separate experiments.

(Kaighn’s modification, with or without Fe) or serum (dialyzed or normal FBS) could change the effects of asbestos on EGFR in A549 cells. We observed that neither medium nor serum had an impact on asbestos-induced EGFR dephosphorylation in A549 cells (data not shown).

Effects of Crocidolite on ErbB2/HER2 Phosphorylation. Since HER2, one of the 4 members of the ErbB receptor family, acts synergistically with EGFR and both are thought to interact with each other by transphosphorylation via heterodimer formation, we examined the effect of asbestos fibers on HER2 membrane receptors (Fig. 3-3). Consistent with the results with EGFR, crocidolite treatment decreased the constitutive
phosphorylation levels on Y877, the site involved in regulation of HER2 biological activity, and Y1248, one of the major autophosphorylation sites that couples HER2 to the Ras-Raf-MAP kinase signal transduction pathway. Interestingly, we also observed a minor, time-dependent decrease in the total level of HER2 protein, suggesting that HER2 inactivation could be coupled to its degradation. Overall, it appears that crocidolite treatment is able to affect not only EGFR, but also other members of the ErbB family of receptor tyrosine kinases.

**Effects of Crocidolite on EGFR Activation. Time and Dose Dependences.**

Because asbestos decreased the constitutive EGFR phosphorylation, it was necessary to determine whether this phenomenon was time and/or concentration dependent. For the remainder of the study only crocidolite was used. EGFR dephosphorylation after crocidolite treatment occurred within the first 3 h with a peak response at 24 h (almost
total disappearance of phosphorylation bands) (Fig. 3-4A). Furthermore, we also investigated the ability of crocidolite to modulate the \textit{in vitro} kinase activity of the EGFR (Fig. 3-4B). Crocidolite treatment decreased the phosphorylation of the receptor (data not shown) and decreased the phosphorylation activity of EGFR on the substrate below that of the control, untreated cells.

Others have observed that TiO$_2$ particles do not cause cell apoptosis (17) or a change in mitochondrial membrane potential ($\Delta\psi_m$) (18, 19). Thus, we also investigated the effects of TiO$_2$ on the EGFR activity. In contrast to crocidolite, TiO$_2$ had no effect on the EGFR phosphorylation state (Fig. 3-4C). This is consistent with previous findings that TiO$_2$ did not affect the cells.

Treatment of cells with three different concentrations of crocidolite resulted in a dose-dependent decrease in the EGFR phosphorylation (Fig. 3-5A). A strong association between crocidolite concentration and the receptor dephosphorylation was observed. Importantly, MET5A and normal human small airway epithelial cells showed similar responses to crocidolite, suggesting that the responses observed in A549 cells were representative of transformed and normal human cells (Fig. 3-5B and C). Next, we investigated if exposure of A549 cells to three concentrations of crocidolite for 24 h could affect the \textit{in vitro} kinase activity of the EGFR (Fig. 3-5D). In comparison to untreated control cells exhibiting constitutive levels of phosphorylation, crocidolite treatment (1.5, 3, 6 $\mu$g/cm$^2$) decreased the phosphorylation activity of EGFR on the substrate below that of the control by approximately two- to fourfold in a dose-dependent manner.

Interestingly, we also found that the inhibitory effects of asbestos were not
Fig. 3-4. Effects of Crocidolite and TiO₂ on EGFR Activation. Time Dependence.

A, serum-deprived A549 cells were incubated with 6 µg/cm² crocidolite (CRO) for the indicated time intervals, or with 100 ng/ml EGF for 15 min (positive control). Cellular lysates were subjected to Western blotting analysis, as described under Materials and Methods. Phosphorylation of Y1068 and Y1173 of the EGFR was detected using the indicated antibodies. EGFR levels were detected using an anti-EGFR antibody (the upper and lower bands represent phosphorylated and not phosphorylated EGFR forms, respectively). B, one mg protein aliquots from untreated or crocidolite-treated A549 cells were subjected to immunoprecipitation with anti-EGFR, and to traditional in vitro kinase assays with the substrate poly (Glu, Tyr, 4:1), as described under Materials and Methods. C, serum-deprived A549 cells were incubated with 6 µg/cm² crocidolite (CRO) or TiO₂ for the indicated time intervals. Cellular lysates were subjected to Western blotting analysis, as described above. The in vitro kinase assay and Western blotting experiments were repeated two and three times, respectively, and representative autoradiograms are shown. The optical densities (OD) of the bands were quantified. The results are shown as the mean ± SE, and are expressed as fold increase relative to the untreated control. Error bars represent the SE. * P <0.05 compared with control, untreated groups.
Fig. 3-5. Effects of Crocidolite on EGFR Activation. Dose Dependence. A, serum-deprived A549 cells were incubated with three different concentrations of crocidolite (CRO) (1.5, 3, 6 µg/cm²) for 24 h. Cellular lysates were subjected to Western blotting analysis, as described under Materials and Methods. Phosphorylation of Y1068 and Y1173 of the EGFR was detected using the indicated antibodies. EGFR levels were detected using an anti-EGFR antibody (the upper and lower bands represent phosphorylated and not phosphorylated EGFR forms, respectively). B, MET5A cells were incubated with or without crocidolite (1.5, 3, 6 µg/cm²) for 24 h, and cellular lysates were subjected to Western blotting analysis, as described above. C, SAEC cells were incubated with or without crocidolite (3 and 6 µg/cm²) for 24 h, and cellular lysates were subjected to Western blotting analysis, as described above. D, one mg protein aliquots from untreated or crocidolite-treated A549 cells were subjected to immunoprecipitation with anti-EGFR, and to traditional in vitro kinase assays with the substrate poly (Glu, Tyr, 4:1), as described under Materials and Methods. The in vitro kinase assay and Western blotting experiments were repeated two and three times, respectively, and representative autoradiograms are shown. The optical densities (OD) of the bands were quantified. The results are shown as the mean ± SE, and are expressed as fold increase relative to the untreated control. Error bars represent the SE. * P <0.05 compared with control, untreated groups.
suppressed by actinomycin D or cycloheximide, indicating that the effects of crocidolite were independent of transcription and translation (data not shown).

**Effects of Crocidolite on EGFR Degradation.** Since lysosomal targeting of EGFR has been shown to be independent of the tyrosine kinase activity (20), we investigated if asbestos-induced EGFR dephosphorylation was coupled to receptor degradation. Phosphorylation of Y1045 in the EGFR creates a major docking site for c-Cbl, which in turn enables receptor ubiquitination and degradation. Thus, we analyzed the phosphorylation status of Y1045 and the levels of EGFR before and after asbestos treatment. Additionally, since Zhuang et al. (21) showed that oxidative stress led to a caspase-mediated EGFR degradation during the early phase of cell death, we followed the fate of the surface EGFR more specifically with Biotin-X-NHS. Consistent with observations by Ravid et al. (4), 15-min EGF treatment resulted in significant Y1045 phosphorylation (Fig. 3-6A). Also, in the presence of 100 ng/ml EGF for 9 h, the levels of biotinylated EGFR were dramatically decreased (Fig. 3-6B). In contrast, crocidolite exposure from 3-24 h resulted in negligible Y1045 phosphorylation with no apparent decrease in biotinylated EGFR (Fig. 3-6A and B). Thus, crocidolite treatment did not lead to EGFR degradation.

**Effects of Cytochalasins, Desferrioxamine B, and Phytic Acid on Crocidolite-Induced EGFR Inactivation.** To determine whether asbestos-induced EGFR inactivation required the uptake of fibers, we blocked actin polymerization, which prevents endocytosis, with cytochalasins. As shown in Fig. 3-7A, cytochalasin B or D (CB and CD), alone, had negligible effect on EGFR phosphorylation. However, treatment of A549 cells with crocidolite in the presence of cytochalasin B or D (CB/CRO or
Fig. 3-6. Effects of Crocidolite on EGFR Degradation. A, serum-deprived A549 cells were incubated with or without 6 µg/cm² crocidolite (CRO) for the indicated time intervals, or with 100 ng/ml EGF for 15 min (positive control). Cellular lysates were subjected to Western blotting analysis, as described under Materials and Methods. Phosphorylation of Y1045 of the EGFR was detected using the indicated antibody. B, cell surface proteins of serum-deprived A549 cells were biotinylated, followed by incubation with 6 µg/cm² crocidolite for the indicated time intervals, or with 100 ng/ml EGF for 9 h (positive control). Biotin protein labeling was detected with horseradish peroxidase-conjugated streptavidin, as described under Materials and Methods. The experiments were repeated three times and representative autoradiograms are shown.
Fig. 3-7. Effects of Cytochalasins, Desferrioxamine B and Phytic Acid on Crocidolite-Induced EGFR Inactivation. 

A, serum-deprived A549 cells were pretreated with or without 5 µg/ml cytochalasin D or B (CD and CB) for 1 h, followed by incubation with 6 µg/cm² crocidolite for 24 h (CRO). Cellular lysates were subjected to Western blotting analysis, as described under Materials and Methods. Phosphorylation of Y1068 and Y1173 of the EGFR was detected using phospho-specific antibodies. EGFR levels were detected using an anti-EGFR antibody (the upper and lower bands represent phosphorylated and not phosphorylated EGFR forms, respectively). B, serum-deprived A549 cells were incubated with three different concentrations of crocidolite (CRO), 1.5, 3, 6 µg/cm², for 24 h, or fibers from which the redox active iron had been removed with desferrioxamine B (DF-CRO). Cellular lysates were subjected to Western blotting analysis, as described above. C, one mg protein aliquots from crocidolite- or DF-CRO-treated A549 cells were subjected to immunoprecipitation with anti-EGFR, and to traditional in vitro kinase assays with the substrate poly (Glu, Tyr, 4:1), as described under Materials and Methods. D, serum-deprived A549 cells were treated with 6 µg/cm² DF-CRO for 24 h, or the cells were pretreated with or without 500 µM phytic acid (PA) for 1 h, followed by incubation with 6 µg/cm² crocidolite for 24 h (PA/CRO). Cellular lysates were subjected to Western blotting analysis, as described above. The in vitro kinase assay and Western blotting experiments were repeated two and three times, respectively, and representative autoradiograms are shown. The optical densities (OD) of the bands were quantified. The results are shown as the mean ± SE, and are expressed as fold increase relative to the untreated control. Error bars represent the SE. * P <0.05 compared with 6 µg/cm² crocidolite.
B

EGFR

P-Tyr 1068

P-Tyr 1173

Control 1.5 µg 3 µg 6 µg

CRO 24 h

DF-CRO 24 h

C

poly (Glu, Tyr, 4:1)

Control 1.5 µg 3 µg 6 µg

CRO 24 h

DF-CRO 24 h

D

EGFR

P-Tyr 1068

P-Tyr 1173

Control CRO DF-CRO PA PA/CRO

24 h
CD/CRO) resulted in a considerable inhibition of EGFR dephosphorylation. Notably, cytochalasin D treatment resulted in a much higher inhibition of receptor dephosphorylation than respective treatment with cytochalasin B. Nevertheless, co-treatment of A549 cells with fibers and either cytochalasin did not result in a full recovery of receptor phosphorylation levels. Taken together, the results presented here strongly suggest that fiber endocytosis is an important step for EGFR dephosphorylation.

We have previously shown that the pretreatment of asbestos fibers (22) and coal fly ash (23) with an iron chelator desferrioxamine B (DF-crocidolite and -CFA, respectively) greatly reduced the ability of these particulates to exert their pathological effects. To determine if iron associated with crocidolite was involved in EGFR inactivation, serum-deprived A549 cells were treated with three concentrations of DF-crocidolite (1.5, 3, 6 µg/cm²) for 24 h. Iron removal from crocidolite was able to greatly inhibit the amount of the EGFR dephosphorylation, compared with that seen with fibers not treated with a chelator (Fig. 3-7B). Furthermore, we also investigated the ability of DF-crocidolite to modulate the in vitro kinase activity of the EGFR (Fig. 3-7C). Consistent with the Western blotting analysis, incubation of cells with DF-crocidolite radically inhibited dephosphorylation levels of the receptor (data not shown) and inhibited EGFR phosphorylation activity on the substrate compared with that seen with fibers not treated with a chelator. Further evidence implying an important role for iron in the EGFR inactivation was that fibers incubated with PA, another iron chelator, also decreased the levels of receptor dephosphorylation (Fig. 3-7D). PA alone had no effect on the EGFR phosphorylation level. Noteworthy is the fact that DF-pretreated fibers inhibited crocidolite-induced EGFR inactivation to a much greater extent than crocidolite co-
incubated with PA. Taken together, the correlation between iron removal from crocidolite fibers and inhibition of receptor dephosphorylation clearly supports the involvement of iron in the inactivation of EGFR.

DISCUSSION

In the present study we observed that asbestos treatment of human lung epithelial and pleural mesothelial cells resulted in EGFR dephosphorylation and inactivation. This phenomenon may represent a potentially important mechanism by which relevant human lung and pleural target cells respond to asbestos fibers. The results presented here strongly suggest that the fibers must be internalized for the EGFR to be inactivated, and that the iron associated with fibers was directly or indirectly responsible for some of this effect. The inhibitory effects of asbestos were not suppressed by actinomycin D or cycloheximide, showing that these phenomena were independent of transcription and translation. We also found that treatment with crocidolite decreased the phosphorylation levels of the HER2 two major tyrosine residues. Based on these results we conclude that asbestos fibers modulate not only EGFR, but also other members of the ErbB family of receptor tyrosine kinases, suggesting a global membrane receptor inactivation rather than a unique inhibition of EGFR. Understanding the role of ErbB receptor family inactivation in asbestos-induced multiple effects on target cells may provide new insights into the process of carcinogenesis.

The interaction between asbestos and EGFR has mostly been investigated in rodent cells and rodents. Two research groups have observed that asbestos treatment of RPM cells induced EGFR phosphorylation (2, 6, 11). Increases in EGFR mRNA and
protein synthesis have also been noted (2). Additionally, Manning et al. (8) have observed EGFR-mediated proto-oncogene expression and cell proliferation in transgenic mice and murine C10 cells. The results presented here support other studies in the literature showing differences between rodent and human tissue in terms of resistance to genotoxicity and/or cytotoxicity by asbestos (24).

On the other hand, the interaction between asbestos and EGFR has not been thoroughly explored in humans. The studies were limited to microscopic work, as reported by Pache et al. (5). Interestingly, they observed that MET5A cells showed patterns of aggregation and increases in EGFR protein on the surface of cells phagocytizing long asbestos fibers (≥ 60 µm), while no changes were observed in A549 cells. Our observations reported herein are inconsistent with that work. A comparative examination of human A549 and MET5A cells showed that patterns of EGFR dephosphorylation caused by asbestos were similar. In both cell lines the amount of decrease in phosphorylation correlated with dose and time.

A549 cells are one of the three most common cultured human lung epithelial cell lines being used as a tissue culture model of lung epithelial cells in research. However, since A549 cells were originally obtained from an alveolar carcinoma, there is concern that they may not be a faithful representation of normal cells. However, in the studies presented here, A549 cells responded in the same way to crocidolite as normal human small airway epithelial cells (SAEC). We have previously shown that A549 cells responded similarly to particles (glutathione efflux, ferritin induction) when compared with SAEC and that the responses were not an artifact of a single cell type (25). This was complemented by our findings that crocidolite treatment of both SAEC and A549 cells
resulted in a decrease in the EGFR phosphorylation levels in a dose-dependent manner. We conclude that the responses observed in A549 cells are representative of not only mesothelial and epithelial cells, but also of normal and transformed human lung cells.

We have also observed that in contrast to EGF-treated cells, EGFR remains at the plasma membrane throughout the 24-h exposure time to crocidolite, suggesting that receptor dephosphorylation is not coupled to its degradation. Since there are several pathways leading to receptor degradation, *i.e.* lysosome or proteasome, we used two distinctive approaches to investigate this. We observed that asbestos failed to initiate not only ubiquitin-mediated lysosomal targeting but also alternative mechanisms of receptor downregulation.

Inert particulates, such as TiO$_2$ or glass beads, do not cause any pathological effects in either mesothelial (26, 27) or epithelial cells (17-19). Consistent with that, we observed that significant decreases in the EGFR phosphorylation levels occurred with crocidolite, but not with TiO$_2$. The fact that iron on the asbestos fibers appears to be related to EGFR dephosphorylation may explain the differences observed between TiO$_2$ and asbestos.

There is significant evidence demonstrating that iron was mobilized intracellularly from asbestos fibers and was responsible for O$_2$ consumption, lipid peroxidation, and DNA oxidation. It has been proposed that iron mobilized from asbestos into a low-molecular-weight fraction led to pathological phenomena, including cancer. DF and PA, which bind tightly to all of the iron coordination sites making it inert, can prevent iron-derived reactive oxygen species production within cells. We found that iron chelators had inhibitory effects on crocidolite-induced EGFR dephosphorylation.
Noteworthy is the fact that the long-term removal of bioavailable iron from crocidolite by DF prior to exposure to cultured cells inhibited EGFR inactivation to a much greater extent than fibers co-incubated with PA. We also observed that the amount of EGFR dephosphorylation in A549 cells by two types of asbestos, crocidolite and amosite, was significantly greater than that by the third type, chrysotile, either short or intermediate length fibers. Also, direct comparison of short and intermediate length chrysotile fibers revealed that intermediate fibers seemed to be somewhat more toxic to A549 cells than short fibers, which could be due to differences in the fiber dimensions and/or iron bioavailability. Most importantly, the observed effects of crocidolite, amosite and chrysotile on the EGFR phosphorylation state appeared to be related to the amount of iron mobilized from these fibers (16). Thus, there appeared to be a correlation between iron mobilization from asbestos and the degree of EGFR dephosphorylation.

Since the EGFR is an integral membrane protein with crucial domains on both sides of the plasma membrane, we examined whether the process of fiber endocytosis was necessary for its inactivation. We observed that the endocytosis inhibitor cytochalasin considerably inhibited EGFR dephosphorylation. Interestingly, analysis of the results revealed that CD treatment resulted in a much greater inhibition of receptor dephosphorylation caused by crocidolite than respective treatment with CB. This phenomenon could be simply explained by the fact that CD is a much more potent inhibitor of actin polymerization than CB. Endocytosis is also important for other asbestos-mediated effects in lung epithelial cells (14) and mesothelial cells (28, 29). We have previously observed that endocytosis of crocidolite in A549 cells was required for iron mobilization (14). This uncontrolled entry of iron may represent "iron overload" in
cells endocytizing the fibers, which could be responsible for many asbestos-dependent biological effects. These observations, taken together, suggest that the effects of asbestos on EGFR are mediated intracellularly, not extracellularly.

It is apparent that iron associated with crocidolite was required, at least in part, for EGFR dephosphorylation. The primary mechanism by which iron from asbestos fibers leads to receptor inactivation is not yet clear. Iron can act as both an oxidant and a reductant, directly or indirectly through generation of ROS. Kamata et al. (30) have shown that redox-sensitive cellular processes could inactivate EGFR. Direct treatment of cells with H₂O₂ activates, rather than inactivates, EGFR activity (4). These treatments could have had extracellular, as well as intracellular effects and certainly would expose cells to much higher levels of H₂O₂ than would ever be generated by asbestos. Also, as discussed earlier, it appears that asbestos exerts its effects intracellularly (14). It is possible that asbestos may be activating protein phosphatases that dephosphorylate and inactivate the EGFR. Zhuang et al. (21) have recently observed that exposure of human keratinocytes to singlet oxygen resulted in rapid decreases in EGFR phosphorylation through activation of protein phosphatases.

Activation of EGFR and its signaling is believed to be an important contributor to the growth and survival of cells in a variety of conditions. Thus, the dephosphorylation and inactivation of EGFR observed after asbestos treatment may be one step in the pathway leading to apoptosis. Stimuli other than asbestos have also been shown to inhibit EGFR phosphorylation resulting in G1 arrest, up-regulation of p27 (9), and subsequent apoptosis (10). Recent evidence strongly suggests a relationship between inactivation of EGFR-associated survival pathways and stimulation of the stress-activated protein
kinases in terms of a balance between cell death and proliferation. The emerging role of EGFR inactivation in relevant human lung and pleural target cells may be of critical importance in the process of apoptosis. Several groups have independently provided strong evidence that asbestos triggers apoptosis in mesothelial (2, 26, 27, 31-33) and epithelial cells (34), including A549 cells (17-19). Apoptosis represents an important mechanism critical for elimination of defective cells without stimulating an inflammatory response. However, extensive cell death due to apoptosis can lead to compensatory proliferation of surrounding cells. If these replicating cells have already sustained critical mutations, then replication could promote formation of a tumor. We are currently investigating the signaling events responsible for asbestos-induced EGFR dephosphorylation and apoptosis in human lung epithelial cells.

In conclusion, we observed that crocidolite treatment of A549, MET5A and SAEC cells resulted in EGFR dephosphorylation and inactivation. This effect appeared to be mediated, at least in part, by the presence of bioavailable iron, and appeared to be mediated intracellularly. Inactivation of EGFR may be an important step in the pathway leading to apoptosis. Elucidation of molecular mechanisms underlying asbestos-induced DNA damage and apoptosis may lead to a better understanding of asbestos-related pulmonary toxicity.

REFERENCES


ABSTRACT

Despite intensive investigation the mechanism of asbestos-induced carcinogenicity is not well understood. We have previously observed that asbestos induced dephosphorylation and inactivation of epidermal growth factor receptor (EGFR) in relevant human lung target cells. Here, we investigated the role of signal transduction events in crocidolite-induced apoptosis of human lung epithelial (A549) cells and normal human small airway epithelial (SAEC) cells. We observed that exposure of A549 and SAEC cells to crocidolite, but not inert TiO₂, led to a significant time- (15 min to 24 h) and dose- (1.5, 3, and 6 µg/cm²) dependent inactivation of the main EGFR signaling pathways, including Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) pathways. Crocidolite also initiated apoptosis via pathways involving activation of p38 mitogen-activated protein kinase (MAPK), caspase -3 and -9, and cleavage of poly(ADP-ribose) polymerase (PARP). Prevention of these effects with an iron chelator or endocytosis inhibitors strongly suggests that iron mobilized from fibers inside the cells initiates the observed events. Inhibition of p38 MAPK with SB203580 prevented inactivation of EGFR, inactivation of EGFR-associated survival pathways, and initiation of apoptosis. Taken together, it appears that iron-dependent p38 MAPK activation plays a central role in crocidolite-induced apoptosis of human lung epithelial cells via inhibition
Asbestos-induced apoptosis in target human lung cells may account for some of the pulmonary toxicity of the fibers.

INTRODUCTION

Although asbestos was recognized to be a human carcinogen in the late 1950s, the mechanism by which it causes mesothelioma of the pleura and bronchogenic carcinoma of the lung is still not well understood. It has been shown that iron from asbestos catalyzes the generation of reactive oxygen species (ROS), such as superoxide radical \( O_2^- \) and hydroxyl radical \( -\cdot OH \) (1). In addition, iron has been shown to be responsible for \( O_2^- \) consumption (2), DNA oxidation (3), lipid peroxidation (4), human lung epithelial cell apoptosis (5), and potentially cancer. There is also significant evidence suggesting that iron from sources other than asbestos may cause human cancer.

It is now believed that the imbalance between apoptosis and proliferation plays a critical role in the process of carcinogenesis. Apoptosis is a physiological process critical for organ development, tissue homeostasis and elimination of defective cells without stimulating an inflammatory response. It might also play an important role in the development of diseases (6, 7), such as cancer, by leading to compensatory proliferation of cells with damaged DNA (8). Studies by others have demonstrated asbestos-induced apoptosis in both rodent (9-13) and human (5, 9, 14, 15) lung cells. The mechanism of asbestos-induced apoptosis in the lung cells is not entirely known. Studies in rodent cells and rodents provided some insights into the signal transduction pathways involved in asbestos-induced apoptosis. Mossman and coworkers have shown that in addition to
mitochondrial-regulated pathways (11), apoptosis was associated with signaling through epidermal growth factor receptor (EGFR) and subsequent activation of the extracellular-regulated protein kinase (ERK1/2) cascades (12). Additionally, Swain et al. (16) have suggested an important role for p38 mitogen-activated protein kinase (MAPK) in asbestos-induced cytotoxicity in rat pleural mesothelial (RPM) cells. Since some differences were reported in the way rodent and human cells responded to asbestos fibers (unpublished observations), it is of great importance to examine and compare the asbestos-induced signaling events in human lung epithelial cells. Kamp and coworkers have established that asbestos induces apoptosis in human lung epithelial cells (5, 14, 15). It was proposed that apoptosis resulted from mitochondrial dysfunction and was, in part, due to iron-derived ROS. Nevertheless, the signaling cascades involved in asbestos-induced apoptosis in human cells have not been thoroughly explored.

The ErbB receptor superfamily, of which EGFR is an important member, is one of the major systems participating in the transduction of signals through the highly conserved MAPK cascade and the phosphatidylinositol 3-kinase (PI3K/Akt) pathway. It has been shown that EGF-like growth factors activate both pathways (17), while inhibition of the ErbB receptors suppress them (18). MAPKs phosphorylate specific serines and threonines of target protein substrates and regulate cellular activities ranging from gene expression, mitosis, movement, metabolism, and programmed death. It is now recognized that the MAPK superfamily is made up of three main and distinct signaling pathways: the ERKs, the c-Jun N-terminal kinases or stress-activated protein kinases (JNK/SAPK), and the p38 family of kinases. The ERKs are strongly activated by growth factors, whereas various cell stresses, such as hyperosmotic shock, metabolic stress or
protein synthesis inhibitors, UV radiation, heat shock, cytokines, and ischemia can activate JNK/SAPKs and p38 MAPK (19). Signaling by PI3K/Akt is believed to be one of the pivotal pathways regulating cell growth and proliferation. Phosphorylation of Akt substrates results in antiapoptotic effects (20). It seems as if the dynamic balance among PI3K/Akt, ERK1/2 and JNK/SAPK and p38 pathways may govern the ability of cells to survive or undergo apoptosis (21). It has been observed that blockade of PI3K/Akt and ERK1/2 pathways with concurrent activation of the p38 pathway suppressed cell survival and subsequently stimulated apoptosis in HeLa cells (22). As reviewed earlier, several independent research groups have provided evidence for the involvement of some signaling cascades in apoptosis of rodent cells following asbestos exposure (11, 12, 16). However, the effects of asbestos on the control and coordination of the signal transduction pathways in human lung cells have not been investigated.

We have recently observed that treatment of human lung epithelial (A549), normal human small airway epithelial (SAEC) and human pleural mesothelial (MET5A) cells with asbestos resulted in dephosphorylation and subsequent inactivation of EGFR (unpublished observations). This led us to investigate the effects of asbestos on EGFR-dependent and -independent signal transduction pathways in A549 cells and SAEC cells. Our findings strongly suggest a central role for the upstream activation of p38 MAPK in altering the fine balance between cell survival and death. Most importantly, iron mobilized from fibers inside the cells appeared to initiate the activation of p38 MAPK leading to apoptosis. We speculate that apoptosis may be an important response of relevant human lung target cells and that asbestos pathogenesis may result from altered apoptotic mechanisms.
MATERIALS AND METHODS

Asbestos and Reagents. Crocidolite was obtained from Dr. Richard Griesemer, National Institute for Environmental Health Sciences/National Toxicology Program (Research Triangle Park, NC) and contained 27% iron by weight. The crocidolite sample used had a mean length of 10 \( \mu \text{m} \) and mean width of 0.27 \( \mu \text{m} \), determined by scanning electron microscopy.

Sodium bicarbonate and the reagents to prepare phosphate buffered saline (PBS) were obtained from Fisher Scientific (Pittsburgh, PA). A custom preparation of Ham’s F-12 tissue culture medium (free of added iron salts) and 0.5% trypsin (with 0.2% EDTA) were obtained from Invitrogen (Carlsbad, CA). Gentamicin (50 mg/ml) was obtained from Biowhittaker Inc. (Walkersville, MD). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories Inc. (Logan, UT).

Desferrioxamine B, titanium dioxide (TiO\(_2\)), cytochalasin B and D, and epidermal growth factor were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies were purchased from either Santa Cruz Biotechnology Inc. (Santa Cruz, CA) or Cell Signaling Technology (Beverly, MA). Anti-mouse and anti-rabbit enzyme-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA). Chemiluminescent Western blotting detection reagent (ECL) was purchased from Amersham Biosciences, Inc. (Piscataway, NJ), and x-ray film was purchased from Kodak (Kodak, Rochester, NY). All remaining reagents were purchased in the highest purity possible.

Cell Culture. A human lung epithelial cell line, A549, with characteristics of alveolar epithelial type II cells, was used for the majority of these studies. The A549 cells
(ATCC CCL185) were purchased from American Type Culture Collection (Rockville, MD). A549 cells were cultivated in complete growth medium in a Forma Model 315B water-jacked incubator (Forma Scientific, Marietta, OH) at $37 \pm 1^\circ C$ in an atmosphere of $5 \pm 0.5\% CO_2$ and 95\% humidity. Complete growth medium was composed of iron-free Ham's F12 cell culture medium, 50 µg/ml gentamicin, 10\% FBS, and 1.176 g NaHCO_3/l medium to obtain a final pH of 7.4. For maintenance of stock cultures, cells were dislodged with 0.5\% trypsin and 0.2\% EDTA before reaching confluence, resuspended in complete growth medium, and plated.

Primary cultures of normal human small airway epithelial cells (SAEC) (Clonetics, Walkersville, MA) from the distal airspace were used for some experiments. The SAEC cells were cultured in small airway growth medium (SAGM) (Clonetics, Walkersville, MA), as directed by the manufacturer.

**Treatment of Cells.** The cells were cultured until ~75% confluent, rinsed with PBS, then dislodged with trypsin-EDTA and plated at a density of 20,000 cells/cm^2. Twenty-four h prior to and during treatment, cells were cultured in 0.5\% FBS, referred to as serum-deprived. Cells were treated with crocidolite fibers. Fibers were suspended in sterile 14 mM NaHCO_3 (pH 7.4) at a concentration of 1 mg/ml immediately before treatment of cells, vortexed for 1 min, and diluted to the appropriate concentration with the growth medium. After the indicated treatment time intervals, the medium, containing the fibers that were not associated with cells and/or phagocytized, was removed. The cells were rinsed with ice-cold PBS, pH 7.4, and dislodged with 0.5\% trypsin. The cell pellets were stored at –80 \degree C until use.

In some experiments, the role of iron was examined by treating the cells with
crocidolite from which the redox active iron (bioavailable iron) had been removed with desferrioxamine B (DF-crocidolite). DF-crocidolite was prepared, as previously described (23). This treatment removed approximately 8% of the total iron from the fibers and the majority of the bioavailable iron. The fibers were resuspended immediately before use, as described above.

In some experiments, the cells were treated with various inhibitors. To inhibit EGFR kinase activity the cells were treated with quinazoline AG-1478 for 2 h. To inhibit endocytosis of fibers or to inhibit p38 MAPK, the cells were treated for 1 h prior to and during treatment with 5 µg/ml cytochalasin B and D or 20 µM SB203580, respectively. The stock solutions of the inhibitors were made in dimethyl sulfoxide and diluted to the appropriate concentrations in culture medium.

**Lysate Preparation and Western Blotting.** Cell lysates were prepared for Western blotting, as described by Simeonova et al. (24), with some modifications. Briefly, after treatments, cells were lysed in RIPA buffer containing protease inhibitors (30 µl/ml aprotinin, 4 µg/ml leupeptin, 4 µg/ml soybean trypsin inhibitor, 0.1 M PMSF, and 1 µM benzamidine), and tyrosine phosphatase inhibitor (1 mM sodium orthovanadate). Lysates were cleared by centrifugation at 15,000 x g for 30 min at 4°C, and the protein concentrations were determined by the Bradford method. Lysates were then subjected to electrophoresis in 8-12 % SDS-PAGE, and transferred to polyvinyl difluoride (PVDF) membranes. The membranes were incubated with blocking solution (5% milk) to reduce nonspecific binding. Then, the appropriate primary antibodies (1:1000 dilution) were applied (1 h at room temperature or overnight at 4°C), followed by incubation with the secondary enzyme-conjugated antibodies (1:10,000 dilution) for 2 h.
at room temperature. Immune complexes were visualized on the PVDF membranes by ECL, and detected by exposure to X-ray film, as directed by the manufacturer.

**RESULTS**

**Effects of Crocidolite on Akt and ERK1/2 Pathways.** We have previously observed that exposure of A549 cells to crocidolite caused dose- and time-dependent dephosphorylation and inactivation of EGFR (unpublished observations). Since major downstream signaling routes of the EGFR are thought to proceed via Ras-Raf-MAP-kinase and PI3K/Akt pathways (20), we investigated whether one or more of these kinases were affected in response to crocidolite in A549 cells. We employed Western blotting analysis using specific phospho-antibodies to look at phosphorylation levels of Raf, MEK 1,2, ERK1/2, p90Rsk, Elk-1 (ERK1/2 signaling pathway), as well as PTEN, PDK1, Akt, FKHR, GSK-3β (Akt signaling pathway) (Fig. 4-1A and B). Crocidolite treatment blocked phosphorylation of all these proteins, except for PTEN, which remained phosphorylated throughout the treatment. PTEN, a dual-specificity phosphatase that is activated upon dephosphorylation, has been shown to be a major negative regulator of the PI3K/Akt pathway. The effects were observed within the first 3 h with a dramatic decrease in phosphorylation after 24 h. We observed that crocidolite caused inactivation of the Akt pathway independently of PTEN activation. In contrast to ERK1/2, the long-term dephosphorylation of Akt was accompanied by its subsequent degradation (Fig. 4-1C).

Epidermal growth factor (EGF) was used as a positive control in these experiments. Consistent with findings by others (17), treatment with EGF induced time-
Fig. 4-1. Effects of Crocidolite and EGFR Kinase Inhibitor, AG1478, on Akt and ERK1/2 Pathways. Serum-deprived A549 cells were treated with 6 μg/cm² crocidolite (CRO), 100 ng/ml EGF for the indicated time intervals, or with 1 μM EGFR kinase inhibitor AG1478 for 2 h; Cellular lysates were subjected to Western blotting analysis, as described under Materials and Methods. Phosphorylation of EGFR, PTEN, PDK1, Akt, FKHR, GSK-3β, Raf, MEK1/2, ERK1/2, p90Rsk and Elk-1 was detected using the indicated phospho-antibodies specific to activated forms of proteins. Akt and ERK1/2 protein contents were analyzed with antibodies to Akt or ERK1/2. Data presented is representative of three separate experiments.
C

![Image of protein expression levels over time with proteins labeled: P-Akt Thr308, P-Akt Ser473, Akt, P-ERK1/2 Thr202/Tyr204, ERK1/2.](image)

Control 0.25 3 12 24 CRO incubation (h)

D

![Image of protein expression levels over time with proteins labeled: P-Akt, Akt, P-ERK1/2, ERK1/2.](image)

Control 5 15 30 60 120 EGF incubation (min)

E

![Image of protein expression levels with proteins labeled: P-EGFR Tyr1068, P-EGFR Tyr1173, P-Akt, P-ERK1/2.](image)

Control CRO AG 1478

24 h 2 h
dependent increases in the phosphorylation level of Akt and ERK1/2 (Fig. 4-1D). The level of total ERK1/2 and Akt proteins remained unchanged during this treatment.

A highly selective inhibitor of the EGFR tyrosine kinase activity, tyrphostin AG-1478, was used to determine whether EGFR inhibition affected the phosphorylation states of Akt and ERK1/2 in these cells. Consistent with observations by others (25), exposure to AG1478 resulted in a blockage of constitutive EGFR phosphorylation, as well as MAPK and Akt phosphorylation (Fig. 4-1E). Taken together, crocidolite treatment of A549 cells resulted in an efficient blockade of the main EGFR signal transduction pathways, including the MAPK and Akt pathways.

**Effects of Crocidolite on Akt and ERK1/2 Phosphorylation States in Human Lung Epithelial A549 and Normal Human Small Airway Epithelial SAEC Cells.** We have previously observed that crocidolite treatment of A549 cells or SAEC cells resulted in very similar responses (26). Our recent data showed that crocidolite treatment of both A549 and normal SAEC cells resulted in a reduction in the EGFR phosphorylation levels in a dose-dependent manner. Consistent with these findings, crocidolite treatment led to a dose-dependent blockage of phosphorylation of ERK1/2 and Akt in both A549 and SAEC cells (Fig. 4-2A and B). It has been previously observed that TiO₂ particles do not cause EGFR dephosphorylation (unpublished observations), human lung cell death (15), or a change in mitochondrial membrane potential (ΔΨₘ) (5, 14). Thus, we also investigated the effects of TiO₂ on the ERK1/2 and Akt phosphorylation states (Fig. 4-2C). TiO₂ did not affect protein expression, nor did it change the phosphorylation states of ERK1/2 or Akt. This is consistent with previous findings that TiO₂ did not affect the cells.
Fig. 4-2. Effects of Crocidolite and TiO$_2$ on Akt and ERK1/2 Phosphorylation States in Human Lung Epithelial Cells. A and B, serum-deprived A549 and SAEC cells were incubated with or without 1.5, 3 and 6 µg/cm$^2$, or 3 and 6 µg/cm$^2$ crocidolite (CRO), respectively, for 24 h. C, serum-deprived A549 cells were incubated with 6 µg/cm$^2$ crocidolite (CRO) or TiO$_2$ for the indicated time intervals; Cellular lysates were subjected to Western blotting analysis, as described under Materials and Methods. Phosphorylation of Akt and ERK1/2 was determined using antibodies specific to activated Akt and ERK1/2. Data presented is representative of three separate experiments.
Effects of Crocidolite on Total Cellular Threonine, Serine and Tyrosine

Phosphorylation States. Because crocidolite blocked the phosphorylation of proteins in two major survival pathways, it was of great interest to determine whether the observed phenomena were representative of effects on proteins throughout the cell. Thus, we next examined effects of crocidolite on the phosphorylation status of proteins in the whole cell lysates. Exposure of A549 cells to crocidolite caused a significant, time-dependent blockage of most cellular phosphothreonine, phosphoserine and phosphotyrosine below the control, untreated cells (Fig. 4-3). Dephosphorylation was observed as early as 0.25 h with dramatic effects after 24 h. Noteworthy is the fact that the phosphorylation levels of some proteins remained unchanged and the levels of some proteins were increased above those of the control.

Effects of Crocidolite on p38 MAPK. Time and Dose Dependences. It has been shown that p38 MAPK is strongly activated by a variety of environmental stresses (19). Additionally, asbestos was shown to activate p38 MAPK in rat pleural mesothelial cells (16). Since some differences were reported in the way rodent and human cells responded to asbestos fibers (unpublished observations), we examined whether crocidolite treatment affected the phosphorylation state of p38 MAPK in A549 cells, and whether p38 MAPK could play a role in the crocidolite-induced injury in human lung epithelial cells. When cells were treated with crocidolite, a transient increase in p38 MAPK phosphorylation was observed, as measured by Western blotting with an antibody specific to activated p38 MAPK (Fig. 4-4A). Levels of p38 MAPK phosphorylation began to increase at 0.25 h and reached a peak at 12 h. In addition, treatment of cells with three different concentrations of crocidolite (1.5, 3 and 6 µg/cm²) resulted in a dose-
Fig. 4-3. Effects of Crocidolite on Total Cellular Phosphothreonine, Phosphoserine and Phosphotyrosine Levels. Serum-deprived A549 cells were incubated with 6 µg/cm² crocidolite (CRO) for the indicated time intervals. Cellular lysates were subjected to Western blotting analysis, as described under Materials and Methods. Phosphorylation of the total cellular phosphothreonine (A), phosphoserine (B) and phosphotyrosine (C) levels were determined using the indicated phospho-specific antibodies. Data presented is representative of three separate experiments.
Fig. 4-4. Effects of Crocidolite on p38 MAPK. Time and Dose Dependences. Serum-deprived A549 cells were incubated with 6 µg/cm² crocidolite (CRO) for the indicated time intervals (A), or with three concentrations of crocidolite (CRO) (1.5, 3, 6 µg/cm²) for 24 h (B). Cellular lysates were subjected to Western blotting analysis, as described under Materials and Methods. Phosphorylation of p38 MAPK was determined with antibody specific to activated p38 MAPK. Total p38 MAPK protein content was analyzed with an antibody to p38 MAPK. Data presented is representative of three separate experiments.

dependent increase in the p38 MAPK phosphorylation state (Fig. 4-4B). Regardless of treatment, the level of p38 MAPK protein remained unchanged.
Effects of Crocidolite on Caspases and Poly(ADP-ribose) Polymerase.

Since we observed that crocidolite treatment resulted in inactivation of some of the most important survival pathways, i.e. PI3K/Akt and ERK1/2 pathways, we examined whether crocidolite induced apoptosis under these conditions. Caspase-3 and -9, which are important mediators of apoptosis, are synthesized as inactive precursors that are proteolytically cleaved to generate active enzymes. Crocidolite, but not inert particulate TiO₂, caused a time- and dose-dependent cleavage of caspase-3 and -9 (Fig. 4-5), as has been observed by others (11, 14, 15, 27). Poly(ADP-ribose) polymerase (PARP) is one of the main cleavage targets of caspase-3. Consistent with caspase-3 activation, crocidolite treatment induced cleavage of PARP (Fig. 4-5). Taken together, these results support the involvement of bioavailable iron from endocytized crocidolite fibers in the activation of p38 MAPK and subsequent apoptosis.

Effect of p38 MAPK Inhibitor on Crocidolite-Induced Events. The data to this point suggested a role for p38 MAPK in crocidolite-induced cellular events. However, it did not address the question of where in this series of events p38 MAPK functioned. To determine whether p38 MAPK was involved in the initiation of dephosphorylation of kinases in survival cascades, a pyridylimidazole derivative SB203580, a potent inhibitor of p38 MAPK activity, was used before treatment of cells with crocidolite. We observed that blockade of p38 MAPK activity abolished crocidolite-induced EGFR, Akt and ERK1/2 dephosphorylation (Fig. 4-6A).

The above results indicated a central role for the upstream activation of p38 MAPK in altering the survival pathways. We further examined the role of p38 MAPK activation in crocidolite-induced apoptosis. One-hour pretreatment with SB203580
Fig. 4-5. Effects of Crocidolite and TiO$_2$ on Caspases and Poly(ADP-ribose) Polymerase States. Serum-deprived A549 cells were incubated with 6 µg/cm$^2$ crocidolite (CRO) (A and C) or TiO$_2$ for the indicated time intervals (C), or with three concentrations of crocidolite (1.5, 3, 6 µg/cm$^2$) for 24 h (B). Cellular lysates were subjected to Western blotting analysis, as described under Materials and Methods. Activation of caspase-3 and -9 was determined with anti-caspase-3 or -9 antibodies. PARP cleavage analysis was performed with anti-PARP antibody. Data presented is representative of three separate experiments.
Fig. 4-6. Effects of p38 MAPK Inhibitor on Crocidolite-Induced Events. Serum-deprived A549 cells were pretreated with or without 20 μM p38 MAPK inhibitor, SB203580, for 1 h (Control), followed by incubation with 6 μg/cm² crocidolite for 24 h (p38 I/CRO). Cellular lysates were subjected to Western blotting analysis, as described under Materials and Methods. A, phosphorylation of EGFR, Akt, ERK1/2 and p38 MAPK was determined using antibodies specific to activated Akt, ERK1/2 and p38 MAPK. EGFR protein content was analyzed with an antibody to EGFR (the upper and lower bands represent phosphorylated and not phosphorylated EGFR forms, respectively). B, activation of caspase-3 and -9 was determined with anti-caspase-3 or -9 antibodies. PARP cleavage analysis was performed with anti-PARP antibody. Data presented is representative of three separate experiments.
significantly blocked crocidolite-induced caspase-3 and -9 cleavage (Fig. 4-6B). Consistent with this result, SB203580 also inhibited cleavage of PARP. The inhibitor itself had negligible effect on the levels of caspase-3, -9 and PARP and the phosphorylation states of the ERK1/2, Akt and p38 MAPK.

**Effects of Cytochalasins and Desferrioxamine on Crocidolite-Induced Events.**

To determine whether crocidolite-induced changes in signal transduction events required the uptake of fibers and the intracellular release of iron, we blocked actin polymerization, which prevents endocytosis, with cytochalasins. As shown in Fig. 4-7A, cytochalasin B or D (CB or CD, respectively), alone, had negligible effect on the phosphorylation states of the ERK1/2, Akt and p38 kinases. However, treatment of A549 cells with crocidolite in the presence of CB or CD resulted in a considerable blockage of the dephosphorylation patterns. Notably, CD appeared to be a much more potent inhibitor than CB.

Previously we observed that pretreatment of asbestos fibers (3) or coal fly ash (28) with an iron chelator desferrioxamine (DF) greatly reduced the ability of these particulates to exert their biological effects. Consistent with that, we have recently shown that DF pretreatment of crocidolite blocked crocidolite-induced EGFR dephosphorylation and subsequent inactivation (unpublished observations). Thus, we examined the role of crocidolite-associated iron in the observed signaling events. When serum-deprived A549 cells were treated with three concentrations of DF-crocidolite (1.5, 3, 6 µg/cm²) for 24 h, we observed a significant dose-dependent inhibition of crocidolite-induced dephosphorylation of Akt and ERK1/2 (Fig. 4-7B). Additionally, chelation of iron from crocidolite fibers was also able to greatly block the amount of p38 MAPK phosphorylation, compared with that seen with unchelated fibers.
Fig. 4-7. Effects of Cytochalasins and Desferrioxamine on Crocidolite-Induced Events. 

A and C, serum-deprived A549 cells were pretreated with or without 5 µg/ml cytochalasin D or B (CD and CB) for 1 h, followed by incubation with 6 µg/cm² crocidolite for 24 h (CD/CRO and CB/CRO). B and D, serum-deprived A549 cells were incubated with three concentrations of crocidolite (CRO), 1.5, 3, 6 µg/cm², for 24 h, or fibers from which the redox active iron had been removed with desferrioxamine B (DF-CRO). Cellular lysates were subjected to Western blotting analysis, as described under Materials and Methods. Phosphorylation of Akt, ERK1/2 and p38 MAPK was determined using antibodies specific to activated Akt, ERK1/2 and p38 MAPK. Activation of caspase-3 and -9 was determined with anti-caspase-3 or -9 antibodies. PARP cleavage analysis was performed with anti-PARP antibody. Data presented is representative of three separate experiments.
Consistent with the above results, pretreatment of A549 cells with cytochalasins as well as removal of the majority of the bioavailable iron from fibers with DF, significantly blocked activation of caspase-3 and -9, and cleavage of PARP (Fig. 4-7C and D).

**DISCUSSION**

We have previously reported that exposure of relevant human lung target cells to asbestos fibers resulted in dephosphorylation and subsequent inactivation of EGFR
(unpublished observations). Results presented here strongly suggest that p38 MAPK controls the molecular survival/death switch that directs the cells away from survival/proliferation to apoptosis. Most importantly, we observed that iron mobilized from fibers inside the cells initiated, in part if not entirely, the observed phenomena.

The effects of asbestos on signal transduction pathways controlling survival and apoptosis have only been investigated in rodent cells (27, 29-31) and rodents (32, 33). Two research groups have observed that asbestos treatment of RPM cells induced EGFR phosphorylation (12, 34). Additionally, Jimenez et al. (29) have observed that asbestos-induced RPM cell apoptosis was associated with activation of ERK, but not JNK/SAPK pathways. The results reported herein reveal not only important species differences but also the complexity and diversity of regulatory mechanisms in asbestos-induced apoptosis.

EGFR is an important signal transducer that activates many signaling pathways, e.g. PI3K/Akt and ERK1/2 pathways, resulting in increased proliferation and cell survival, as well as inhibition of apoptosis. We observed that in contrast to EGF treatment, crocidolite asbestos, but not inert particulates (TiO2), caused a dose- and time-dependent inactivation of EGFR-associated survival pathways, including Akt and ERK1/2 pathways. We observed that the Akt phosphorylation state was not influenced by PTEN, a dual specificity phosphatase, even though PTEN has been shown to negatively regulate the PI3K/Akt pathway. This suggests an alternative mechanism of Akt dephosphorylation after crocidolite exposure, which has also been observed in some malignant tumors (35). Treatment of A549 cells with crocidolite also caused a time-dependent decrease in cellular phosphothreonine, phosphoserine and phosphotyrosine,
suggesting major protein dephosphorylation events rather than unique effects of crocidolite on Akt and ERK1/2 pathways. Additionally, a potent EGFR inhibitor, AG1478, inhibited activation of Akt and ERK1/2, as has also been observed by others (25). Consistent with our previous observation that EGFR was inhibited by crocidolite treatment, it appears that the observed inactivation of the survival pathways depended on the inhibition of EGFR kinase activity.

We have previously observed that A549 cells showed similar responses to particles (glutathione efflux, ferritin induction) as normal human small airway epithelial (SAEC) cells and that the responses were not an artifact of the tumor origin of the A549 cells (26). Our recent findings showed that crocidolite treatment of A549, MET5A and SAEC cells resulted in EGFR dephosphorylation in a dose-dependent manner (unpublished observations). Consistent with that, in the studies presented here, we observed that exposure of A549 and SAEC cells to crocidolite led to interruption of Akt and ERK1/2 signaling. We conclude that the responses observed in A549 cells are representative of human lung epithelial cells in these respects.

Independent studies have provided strong evidence that asbestos triggers apoptosis in both rodent (9-13) and human (5, 9, 14, 15) cells. It appears that asbestos-induced apoptosis in lung target cells may account for the pathogenic effects of the fibers. However, in contrast to rodent cells, the signaling cascades involved in asbestos-induced apoptosis in human cells have not yet been identified. Our results presented here showed that exposure to crocidolite, but not inert particulates (TiO₂), resulted in a dose- and time-dependent activation of the pro-apoptotic p38 MAPK, and subsequently initiation of apoptosis in human lung epithelial cells. The characteristic features of apoptosis provided
by our studies include a dose- and time-dependent activation of caspase-3, -9, and subsequent cleavage of PARP. The observed degradation of the survival protein Akt may also accelerate apoptosis, as was reported by others (36). Interestingly, a potent inhibitor of p38 MAPK activity, SB203580, was able to prevent not only activation of caspases and cleavage of PARP, but also inactivation of EGFR and survival pathways. Thus, it appears that p38 MAPK plays a central role in crocidolite-induced apoptosis in A549 cells. Stimuli other than asbestos, i.e. nitric oxide (36), arsenite (37), phorbol esters (38), have also been shown to induce apoptosis in a p38 MAPK-dependent manner. The precise mechanism by which p38 MAPK leads to apoptosis is unclear. It has been proposed that activated p38 MAPK may facilitate cytochrome c release and subsequent activation of caspases (39). It has also been suggested that protein phosphatases may be direct targets for p38 MAPK-mediated phosphorylation. Westermarck et al. (40) have observed that activation of p38 MAPK resulted in increased protein phosphatase activity that directly inhibited ERK1/2 and MEK1,2 phosphorylation. Additionally, activated phosphatases may regulate the alternative splicing of specific apoptotic factors, i.e. caspase 9 and Bcl-x (41).

Cytochalasins, the endocytosis inhibitors, blocked asbestos-induced injury to mesothelial and lung epithelial cells (9). Also, it has been observed that iron mobilized from asbestos fibers was responsible for DNA oxidation (42) and mitochondrial dysfunction (5, 14, 15). Consistent with that, our results presented here showed that decreasing fiber uptake or removal of bioavailable iron with DF prevented the observed cellular events and apoptosis. Cytochalasin D appeared to be a more potent inhibitor of actin polymerization than cytochalasin B. This phenomenon could be simply explained
by the fact that CD is a much more potent inhibitor of actin polymerization than CB.

Taken together, we propose a model of crocidolite-induced apoptosis in human lung epithelial cells involving p38 MAPK control of cell survival/apoptosis (Fig. 4-8). This may involve iron-dependent p38 MAPK-mediated activation of protein phosphatases and subsequent dephosphorylation of EGFR and EGFR-associated survival pathways (Akt and ERK1/2), as well as, activation of apoptotic pathways (casapase-3, -9). Apoptosis may represent an important mechanism of eliminating cells that have asbestos-induced DNA alterations during the process of carcinogenesis. Even though apoptosis is responsible for normal tissue homeostasis, it might also play an important role in the development of diseases, such as cancer (7). It has been observed that excessive death of epithelial cells, which appeared to overwhelm the clearing mechanism, led to persistent inflammation (6). There is a body of evidence suggesting a direct correlation between alveolar epithelial cell apoptosis and lung pathological disorders (43). In addition, some reports suggest that a primary increase in apoptosis can stimulate compensatory proliferation (8). Increased cell proliferation without a compensatory increase in apoptosis may be associated with tumor promotion and progression. Finally, we speculate that some cells may bypass the protective mechanism of apoptosis, i.e. resistance to and/or defects in apoptosis, which could allow the abnormal survival of the target lung cells with asbestos-induced mutations. Development of malignant tumors in humans is a multi-staged process, which may require 40 years or more after initial exposure to asbestos. During this time, DNA damage, mutagenesis, apoptosis and compensatory cell proliferation may represent successive and/or coexistent phenomena.

In summary, our results strongly suggest that iron mobilized from fibers inside
Fig. 4-8. Model of Crocidolite-Induced p38 MAPK-Mediated Apoptosis in Human Lung Epithelial Cells. The signaling events appear to be initiated by iron mobilized from crocidolite fibers inside the cells. Additionally, p38 MAPK appears to control the molecular survival/death switch that directs the cells away from survival/proliferation to apoptosis. This may involve p38 MAPK-mediated activation of protein phosphatases and subsequently dephosphorylation of EGFR and EGFR-associated survival pathways (Akt and ERK1/2), as well as, p38 MAPK-mediated activation of apoptotic pathways (casapase-3, -9, PARP). Decreasing fiber uptake (cytochalasins) and removal of bioavailable iron with iron chelator desferrioxamine prevented the observed cellular events and apoptosis.

The human lung epithelial cells initiates the crocidolite-induced signaling events preventing survival, but activating apoptosis pathways. It is of great importance to understand the
molecular events by which iron initiates activation of p38 MAPK and by which p38 MAPK induces the subsequent cellular responses. Better understanding of the molecular mechanisms underlying the balance between proliferation and apoptosis in relevant human lung target cells may promote more effective strategies for asbestos-related pulmonary toxicity.

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ABSTRACT

Despite intensive investigation the mechanism of asbestos-induced carcinogenicity is unknown. We have previously demonstrated that asbestos induced dephosphorylation and inactivation of epidermal growth factor receptor (EGFR). Also, we found that p38 mitogen-activated protein kinase (MAPK) played a central role in asbestos-induced apoptosis in human lung epithelial (A549) cells via inhibition of extracellular signal-regulated kinase (ERK1/2) and Akt survival pathways, and concurrent activation of caspase-3 and -9. Exposure of A549 cells to asbestos also caused a significant, time-dependent blockage of cellular phosphoserine and phosphotyrosine below the control, untreated cells. Here, we investigated the role of protein phosphatases (PPases) in asbestos-induced apoptosis in A549 cells. Treatment with okadaic acid (OA) (200 nM), an inhibitor of protein phosphatase 2A (PP2A) and protein phosphatase-1 (PP1) at the concentrations used, but not with sodium orthovanadate (VA) (50 µM), an inhibitor of protein tyrosine phosphatases, protected the cells from asbestos-induced apoptosis. Importantly, inhibition of p38 MAPK by SB203580 (20 µM), selective decreases in fiber uptake and removal of the redox active iron from the fibers resulted in significant attenuation of asbestos-induced dephosphorylation of cellular proteins. Our
results demonstrate that p38 MAPK, through a serine/threonine phosphatase-mediated mechanism, regulates asbestos-induced apoptosis in human lung epithelial cells. We speculate that the intracellular imbalance between protein phosphorylation and dephosphorylation caused by asbestos is a critical determinant of both cell survival and death.

INTRODUCTION

Asbestos is a naturally occurring mineral that has been used for centuries because of its unique physical and chemical properties, including durability, tensile strength, and heat resistance. However, it was recognized to be a human carcinogen in the late 1950s, which led to a complete cessation of the use of asbestos-containing commercial products. It has been demonstrated that exposure to asbestos increases the risk of both malignant (1) and nonmalignant (2) diseases. Even though it is not clear how asbestos exerts these effects, the carcinogenicity of asbestos has been related to some of its physical properties, such as size and durability of fibers (3). The most carcinogenic forms of asbestos contain as much as 27% iron by weight (4, 5). It has been proposed that iron from asbestos catalyzes the generation of reactive oxygen species (ROS), such as superoxide radical ($O_2^-$) and hydroxyl radical (-OH) (3, 6). In addition, iron has been shown to be responsible for $O_2$ consumption (7), induction of DNA single-strand breaks (8), lipid peroxidation (9), and potentially cancer. There is also significant evidence suggesting that iron from other sources besides asbestos may cause human cancer (10, 11).

Protein phosphorylation and dephosphorylation are central mechanisms that mediate signal transduction events that control a variety of cellular processes (12, 13).
Target proteins are phosphorylated at specific serine, threonine, or tyrosine residues by protein kinases and the phosphate group is removed by the action of specific protein phosphatases (PPases). Over 98% of all phosphorylations occur on serine and threonine residues, whereas the remaining less than 2% affects tyrosine (14). PPases are present in all eukaryotic cells and regulate numerous cellular processes including cell cycle progression, transcriptional regulation, cell growth, differentiation and apoptosis.

Abnormal PPase activity has been implicated in diseases such as diabetes, inflammation, and cancer (15, 16). There are two main classes of PPases according to their substrate specificity, *i.e.* protein tyrosine phosphatases (PTPases) and protein serine/threonine phosphatases (PSPases), which remove phosphate from proteins containing phosphotyrosine or phosphoserine/phosphothreonine, respectively (17). Interestingly, PTPase family includes both tyrosine-specific and dual specificity phosphatases (DSPases) that remove phosphate from proteins containing all three phosphoamino acid residues. Biochemical studies have shown that PTPases and DSPases share a common catalytic mechanism (18). PSPases are usually classified into type 1 (PP1) or type 2 (PP2), depending on their substrate specificity and sensitivity to inhibitors. Type 2 phosphatases are subdivided into three major groups; PP2A, calcium-dependent calcineurin PP2B and magnesium-dependent PP2C (19).

Okadaic acid (OA) has been shown to be a potent inhibitor of protein phosphatase 1 (IC$_{50} = 10 - 15$ nM) and protein phosphatase 2A (IC$_{50} = 0.1$ nM) (20). Because of its very hydrophobic structure OA has been extensively used as a protein phosphatase inhibitor in intact cells (21). It is now apparent that OA is an extremely valuable tool useful to test the physiological role of protein phosphorylation and to study protein...
phosphatases in cell extracts as well as intact cells. Although OA induces apoptosis in some cell lines (22, 23), inhibition of apoptosis by this agent has also been reported (24-26).

Vanadate (VA) and its analogues have been widely used as inhibitors of PTPases by mimicking the phosphorous group of phosphotyrosine (27). As a result a general increase in cellular protein tyrosine phosphorylation has been observed (28), which in turn led to activation of a variety of signaling pathways (29, 30). VA has also been used as an inhibitor of DSPases. VA is known to prevent apoptosis in some cell lines (31-36) but has been found in other studies to potentiate growth inhibition and apoptosis (34, 37). Yang et al. (33) showed that VA prevented cell detachment, greatly enhanced cell viability and blocked apoptosis of endothelial cells. They demonstrated that addition of VA at low concentration (up to 50 µM) was protective, while higher concentrations did not enhance cell survival. Prolonged incubation with higher concentrations of VA (>50 µM) with intact cells in culture appears to be toxic.

We have recently observed that asbestos induced dephosphorylation and inactivation of epidermal growth factor receptor (EGFR) (see Chapter 3). Further, we found that asbestos induced apoptosis in human lung epithelial (A549) cells in a p38 mitogen-activated protein kinase (MAPK)-dependent manner via inhibition of extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt survival pathways, and concurrent activation of caspase-3 and -9 (see Chapter 4). Exposure of A549 cells to asbestos also caused a significant, time-dependent blockage of cellular phosphoserine and phosphotyrosine below the control, untreated cells. Phosphorylation and dephosphorylation of proteins mediate signal transduction events that control a multitude
of cellular processes that include metabolism, transport, cell division, differentiation and development, learning and memory (12, 13). Recent studies strongly suggest that PTPases as well as PSPases are essential components critically involved in the control of apoptosis (33, 38). We therefore investigated the role of PPases in asbestos-induced apoptosis in A549 cells, using OA and VA, specific inhibitors of PSPases and PTPases, respectively. Our findings demonstrated that OA, but not VA, inhibited asbestos-induced global cellular protein dephosphorylation and apoptosis. Importantly, iron mobilized from fibers inside the cells and p38 MAPK activity appeared to be required for the increased phosphatase activity following asbestos treatment. Taken together, our results strongly suggest a critical role for PSPases in a p38 MAPK-mediated regulation of asbestos-induced apoptosis in human lung epithelial cells.

MATERIALS AND METHODS

Asbestos and Reagents. Crocidolite was obtained from Dr. Richard Griesemer, National Institute for Environmental Health Sciences/National Toxicology Program (Research Triangle Park, NC) and contained 27% iron by weight. The crocidolite sample used had a mean length of 10 µm and mean width of 0.27 µm, determined by scanning electron microscopy.

Sodium bicarbonate and the reagents to prepare phosphate buffered saline (PBS) were obtained from Fisher Scientific (Pittsburgh, PA). A custom preparation of Ham’s F-12 tissue culture medium (free of added iron salts) and 0.5% trypsin (with 0.2% EDTA) were obtained from Invitrogen (Carlsbad, CA). Gentamicin (50 mg/ml) was obtained from Biowhittaker Inc. (Walkersville, MD). Fetal bovine serum (FBS) was obtained from
Hyclone Laboratories Inc. (Logan, UT).

Desferrioxamine B and cytochalasin D were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies were purchased from either Santa Cruz Biotechnology Inc. (Santa Cruz, CA) or Cell Signaling Technology (Beverly, MA). Anti-mouse and anti-rabbit enzyme-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA). Chemiluminescent Western blotting detection reagent (ECL) was purchased from Amersham Biosciences, Inc. (Piscataway, NJ), and x-ray film was purchased from Kodak (Kodak, Rochester, NY). All remaining reagents were purchased in the highest purity possible.

Cell Culture. A human lung epithelial cell line, A549, with characteristics of alveolar epithelial type II cells, was used for the majority of these studies. The A549 cells (ATCC CCL185) were purchased from American Type Culture Collection (Rockville, MD). A549 cells were cultivated in complete growth medium in a Forma Model 315B water-jacked incubator (Forma Scientific, Marietta, OH) at 37 ± 1°C in an atmosphere of 5 ± 0.5% CO₂ and 95% humidity. Complete growth medium was composed of iron-free Ham’s F12 cell culture medium, 50 µg/ml gentamicin, 10% FBS, and 1.176 g NaHCO₃/l medium to obtain a final pH of 7.4. For maintenance of stock cultures, cells were dislodged with 0.5% trypsin and 0.2% EDTA before reaching confluence, resuspended in complete growth medium, and plated.

Treatment of Cells. The cells were cultured until ~75% confluent, rinsed with PBS, then dislodged with trypsin-EDTA and plated at a density of 20,000 cells/cm². Twenty-four h prior to and during treatment, cells were cultured in 0.5% FBS, referred to as serum-deprived. Cells were treated with crocidolite fibers. Fibers were suspended in
sterile 14 mM NaHCO₃ (pH 7.4) at a concentration of 1 mg/ml immediately before treatment of cells, vortexed for 1 min, and diluted to the appropriate concentration with the growth medium. After the indicated treatment time intervals, the medium, containing the fibers that were not associated with cells and/or phagocytized, was removed. The cells were rinsed with ice-cold PBS, pH 7.4, and dislodged with 0.5% trypsin. The cell pellets were stored at –80 °C until use.

In some experiments, the role of iron was examined by treating the cells with crocidolite from which the redox active iron (bioavailable iron) had been removed with desferrioxamine B (DF-crocidolite). DF-crocidolite was prepared, as previously described (39). This treatment removed approximately 8% of the total iron from the fibers and the majority of the bioavailable iron. The fibers were resuspended immediately before use, as described above.

In some experiments, the cells were treated with various inhibitors. To inhibit endocytosis of fibers or to inhibit p38 MAPK, the cells were treated for 1 h prior to and during treatment with 5 µg/ml cytochalasin D or 20 µM SB203580, respectively. The stock solutions of the inhibitors were made in dimethyl sulfoxide and diluted to the appropriate concentrations in culture medium.

Lysate Preparation and Western Blotting. Cell lysates were prepared for Western blotting, as described by Simeonova et al. (40), with some modifications. Briefly, after treatments, cells were lysed in RIPA buffer containing protease inhibitors (30 µl/ml aprotonin, 4 µg/ml leupeptin, 4 µg/ml soybean trypsin inhibitor, 0.1 M PMSF, and 1 µM benzamidine), and tyrosine phosphatase inhibitor (1 mM sodium orthovanadate). Lysates were cleared by centrifugation at 15,000 x g for 30 min at 4°C,
and the protein concentrations were determined by the Bradford method. Lysates were then subjected to electrophoresis in 8-12 % SDS-PAGE, and transferred to polyvinyl difluoride (PVDF) membranes. The membranes were incubated with blocking solution (5% milk) to reduce nonspecific binding. Then, the appropriate primary antibodies (1:1000 dilution) were applied (1 h at room temperature or overnight at 4°C), followed by incubation with the secondary enzyme-conjugated antibodies (1:10,000 dilution) for 2 h at room temperature. Immune complexes were visualized on the PVDF membranes by ECL, and detected by exposure to X-ray film, as directed by the manufacturer.

RESULTS

Effects of Cytochalasins, Desferrioxamine and p38 MAPK Inhibitor on Crocidolite-Induced Cellular Serine and Tyrosine Dephosphorylation. We have previously observed that crocidolite blocked the phosphorylation of proteins in two major survival pathways (see Chapter 3). We also demonstrated that the observed phenomena were representative of effects on proteins throughout the cell. Exposure of A549 cells to 6 µg/cm² crocidolite caused a significant, time-dependent blockage of most cellular phosphothreonine, phosphoserine and phosphotyrosine below the control, untreated cells. To determine whether crocidolite-induced changes in the cellular protein phosphorylation required the uptake of fibers and the intracellular release of iron, we blocked actin polymerization, which prevented endocytosis, with cytochalasin D (CD). As shown in Fig. 5-1, CD alone had little effect on the phosphorylation states of cellular serines and tyrosines. However, treatment of A549 cells with crocidolite in the presence of CD
Fig. 5-1. Effects of Cytochalasins, Desferrioxamine and p38 MAPK Inhibitor on Crocidolite-Induced Cellular Serine and Tyrosine Dephosphorylation. Serum-deprived A549 cells were incubated with medium (Control) or treated with either 6 µg/cm² crocidolite (CRO) or fibers from which the redox active iron had been removed with desferrioxamine B (DF-CRO) for 24 h. Alternatively, cells were pretreated with 5 µg/ml cytochalasin D or 20 µM p38 MAPK inhibitor SB203580 for 1 h (Control), followed by incubation with 6 µg/cm² crocidolite for 24 h (CD/CRO and p38 I/CRO, respectively). Cellular lysates were subjected to Western blotting analysis, as described under Materials and Methods. Phosphorylation of the total cellular tyrosine (A) and serine (B) levels were determined using phospho-specific antibodies. Data presented is representative of three separate experiments.
resulted in a considerable blockage of the dephosphorylation patterns.

We have previously observed that pretreatment of asbestos fibers (41) or coal fly ash (42) with an iron chelator desferrioxamine (DF) greatly reduced the ability of these particulates to exert their biological effects. Also, we have recently shown that DF-crocidolite significantly blocked the observed EGFR inactivation and apoptosis. Thus, we examined the role of crocidolite-associated iron on total cellular serine and tyrosine phosphorylation states. When serum-deprived A549 cells were treated with DF-crocidolite for 24 h, we observed a significant inhibition of crocidolite-induced cellular phosphoserine and phosphotyrosine dephosphorylation (Fig. 5-1).

Although most cellular proteins were dephosphorylated upon crocidolite treatment, noteworthy is the fact that the phosphorylation levels of some proteins remained unchanged and the levels of some proteins were increased above those of the control, i.e. p38 MAPK (data not shown). We have recently shown that p38 MAPK activation played a central role in crocidolite-induced apoptosis of A549 cells via inhibition of ERK1/2 and Akt survival pathways and concurrent activation of caspase-3 and -9 (see Chapter 3). Thus, we next examined whether p38 MAPK was involved in the initiation of cellular protein dephosphorylation. A pyridylimidazole derivative SB203580, a potent inhibitor of p38 MAPK activity, was used before treatment of cells with crocidolite. We observed that blockade of p38 MAPK activity decreased crocidolite-induced global phosphoserine and phosphotyrosine dephosphorylation (Fig. 5-1).

**Effects of Okadaic Acid and Sodium Orthovanadate on Crocidolite-Induced Serine and Tyrosine Dephosphorylation.** The data to this point suggested a role for PPases in crocidolite-induced cellular events. However, it did not address the questions of
what type of PPases played the most significant role and where in this series of events PPases functioned. To determine whether PSPases and PTPases were involved in the initiation of dephosphorylation of cellular proteins, OA, a potent inhibitor of protein phosphatase 1 \( (IC_{50} = 10 - 15 \text{ nM}) \) and protein phosphatase 2A \( (IC_{50} = 0.1 \text{ nM}) \) (20), or VA (27), were used before treatment of cells with crocidolite. OA and VA were the most effective at 200 nM and 50 µM, respectively (Fig. 5-2). It should be noted that concentrations greater than 200 nM for OA appeared to be toxic to the A549 cells (data not shown). We observed that blockade of PSPases activity with OA abolished not only crocidolite-induced cellular phosphoserine dephosphorylation but also cellular phosphotyrosine dephosphorylation. In contrast to OA, treatment with VA resulted in a significant inhibition of crocidolite-induced phosphotyrosine dephosphorylation. However, this treatment had no effect on the crocidolite-induced levels of cellular phosphoserine.

**Effects of Okadaic Acid and Sodium Orthovanadate on Crocidolite-Induced Events.** Activation of PPases has been implicated in dephosphorylation of a number of protein kinases, including receptor kinases and protein kinases associated with cell survival (43-46). However, the identity and relative activity of PPases that inactivate EGFR and its downstream signaling (Akt and ERK1/2) are not well established. Hence, we investigated whether PTPases or PSPases played any role in the crocidolite-induced EGFR, Akt and ERK1/2 dephosphorylation. Fig. 5-3A shows that 200 nM OA treatments not only significantly inhibited the observed dephosphorylation of EGFR, Akt and ERK1/2, but also blocked crocidolite-induced Akt degradation. In contrast to OA, VA treatment was able to block only EGFR and ERK1/2 dephosphorylation with no apparent
Fig. 5-2. Effects of Okadaic Acid and Sodium Orthovanadate on Crocidolite-Induced Serine and Tyrosine Dephosphorylation. Serum-deprived A549 cells were incubated with medium (Control) or treated with 6 µg/cm² crocidolite (CRO) for 24 h. Alternatively, cells were pretreated with 200 nM okadaic acid or 50 µM Na₃VO₄ for 1 h (Control), followed by incubation with 6 µg/cm² crocidolite for 24 h (OA/CRO and VA/CRO, respectively). Cellular lysates were subjected to Western blotting analysis, as described under Materials and Methods. Phosphorylation of the total cellular tyrosine (A) and serine (B) levels were determined using phospho-specific antibodies. Data presented is representative of three separate experiments.
Fig. 5-3. Effects of Okadaic Acid and Sodium Orthovanadate on Crocidolite-Induced Events. Serum-deprived A549 cells were incubated with medium (Control) or treated with 6 µg/cm² crocidolite (CRO) for 24 h. Alternatively, cells were pretreated with 200 nM okadaic acid or 50 µM Na₃VO₄ for 1 h (Control), followed by incubation with 6 µg/cm² crocidolite for 24 h (OA/CRO and VA/CRO, respectively). Cellular lysates were subjected to Western blotting analysis, as described under Materials and Methods. Phosphorylation of EGFR, Akt and ERK1/2 was determined using phospho-antibodies specific to activated EGFR, Akt and ERK1/2. EGFR, Akt and ERK1/2 protein contents were analyzed with antibodies to EGFR, Akt and ERK1/2. Activation of caspase-3 and -9 was determined with anti-caspase-3 or -9 antibodies. PARP cleavage analysis was performed with anti-PARP antibody. Data presented is representative of three separate experiments.
inhibition of Akt dephosphorylation or degradation. The protective effects of OA and VA were maximal at 200 nM and 50 µM, respectively, and higher concentrations of both appeared to be toxic to A549 cells (data not shown).

Okadaic acid, as well as another serine/threonine phosphatase inhibitor calyculin A, have been shown to inhibit apoptosis (24-26, 47). There are also some reports emphasizing an important role of PTPase in the regulation of programmed cell death (33, 48). Hence, we investigated the effects of PSPase and PTPase inhibitors on caspase-3 activation and subsequent poly(ADP-ribose) polymerase (PARP) cleavage (Fig. 5-3B). Okadaic acid treatment prevented crocidolite-induced apoptosis while VA had little effect.

DISCUSSION

We have previously reported that exposure of relevant human lung and pleural target cells to asbestos fibers resulted in dephosphorylation and subsequent inactivation of EGFR (see Chapter 3). Also, we observed that iron-dependent p38 MAPK activation played a central role in crocidolite-induced apoptosis of human lung epithelial cells via inhibition of ERK1/2 and Akt survival pathways and concurrent activation of caspase-3 and -9 (see Chapter 3). Results presented here strongly suggest that crocidolite-induced p38 MAPK-dependent activation of serine/threonine phosphatases directs the cells to apoptosis. Most importantly, it appears that the fibers must be internalized for the PPases to be activated, and that the iron associated with fibers was directly or indirectly responsible for some of this effect.

It is apparent that cells contain simultaneous survival and apoptotic pathways that
are in part regulated by protein phosphorylation and dephosphorylation. Inhibition of both protein kinases and phosphatases activity has been shown to prevent apoptosis in a variety of cell lines (33, 49, 50). There are contradictory reports in the literature regarding the effects of VA and OA on cells. Both have been shown to inhibit apoptosis (24-26, 31-36) or potentiate growth inhibition and apoptosis (22, 23, 34, 37) in a number of cell lines. It is apparent that the balance between protein phosphorylation pathways regulates signal transduction events that control a variety of cellular processes ranging from cell growth and differentiation to apoptosis. Any disturbance of this finely tuned mechanism may direct the cells to either survival or death. Thus, it appears that the ultimate effects of VA and OA may be opposite depending on the dominance of either the survival or apoptotic pathway in a particular cell line (51).

Cytochalasins, the endocytosis inhibitors, blocked asbestos-induced injury to mesothelial and lung epithelial cells (52). It has also been observed that iron mobilized from asbestos fibers was responsible for DNA oxidation (53) and mitochondrial dysfunction (54-56). Additionally, iron mobilized from fibers inside the A549 cells was shown to initiate crocidolite-induced activation of the apoptotic pathway (see Chapter 3). Consistent with that, our results presented here showed that decreasing fiber uptake or removal of bioavailable iron with DF prevented the observed cellular protein dephosphorylation.

We have recently demonstrated that p38 MAPK played a central role in crocidolite-induced apoptosis in A549 cells (see Chapter 3). Stimuli other than asbestos, i.e. nitric oxide (57), arsenite (58), phorbol esters (59), have also been shown to induce apoptosis in a p38 MAPK-dependent manner. Our results reported herein suggest that the
upstream activation of p38 MAPK is required for an increased activity of PPases. The precise mechanism by which p38 MAPK may lead to PPase activation is unclear. Phosphorylation of the PP2A regulatory subunit has been shown to promote its \textit{in vitro} activity (60). It has been suggested that PPases may be direct targets for p38 MAPK-mediated phosphorylation. Recent studies showed that PP2A could be activated by p38 MAPK in NIH 3T3 cells (61) and neutrophils (25).

Independent studies have provided strong evidence that asbestos triggers apoptosis in both rodent (49, 52, 62-64) and human (52, 54-56) cells. It appears that asbestos-induced apoptosis in lung and pleural target cells may account for the pathogenic effects of the fibers. However, in contrast to rodent cells, the signaling cascades involved in asbestos-induced apoptosis in human cells have not yet been identified. Kamp and coworkers (54-56) have established that asbestos induces apoptosis in human lung epithelial cells. It was proposed that apoptosis resulted from mitochondrial dysfunction and was, in part, due to iron-derived ROS. Nevertheless, the signaling cascades involved in asbestos-induced apoptosis in human cells have not been thoroughly explored. We have previously observed that exposure to crocidolite resulted in a dose- and time-dependent activation of the pro-apoptotic p38 MAPK, and subsequently initiation of apoptosis in human lung epithelial cells (see Chapter 3). The characteristic features of apoptosis provided by our studies included a dose- and time-dependent activation of caspase-3, -9, and subsequent cleavage of PARP. Our results presented here showed that OA, a potent inhibitor of PSPase PP1 and PP2A at the concentrations used, was able to prevent not only inactivation of EGFR and survival pathways (ERK1/2 and Akt), but also activation of caspases and cleavage of PARP. We have previously
observed that crocidolite treatment resulted in pro-survival Akt dephosphorylation and subsequent degradation (see Chapter 3). Akt has been shown to be degraded during apoptosis through a caspase-dependent mechanism (65). Consistent with inhibition of caspase activity by OA, this treatment was also able to prevent crocidolite-induced Akt degradation. Our results suggest that PSPases regulate not only phosphorylation of the serine/threonine residues, but also of tyrosine residues. This was supported by the observation that treatment of cells with OA, but not with VA, blocked crocidolite-induced global cellular serine/threonine as well as tyrosine dephosphorylation. Consistent with that, OA treatment also prevented crocidolite-induced dephosphorylation of the tyrosine residue of EGFR, as recently observed by others (50). It is ambiguous though how PSPases regulate, directly or indirectly, the dephosphorylation of tyrosine residues. PP2A, a member of the serine/threonine phosphatase family, is known to display a low basal phosphotyrosine phosphatase activity (66-69). Most importantly, Jackson et al. (70) showed that PP2A was able to regulate the PTPase activity in Lewis lung carcinoma tumor variants. It appears that PP2A could link serine/threonine and tyrosine signaling pathways by regulating PTPases. VA treatment was able to block crocidolite-induced EGFR and ERK dephosphorylation, but, in contrast to OA, was not able to inhibit Akt dephosphorylation and degradation, nor did it prevent apoptosis. Interestingly, VA prevented dephosphorylation of ERK1/2 on both residues, i.e. threonine 202 and tyrosine 204, which could have been due to inhibition of a DSPase that functioned as a MAPK phosphatase (71). Taken together, it appears that PSPases, PP1 and PP2A in particular, play a central role in crocidolite-induced apoptosis in A549 cells. Even though OA is a potent inhibitor of both PP1 and PP2A, one should be aware that some of the less
abundant PPases (PP4, PP5, and PP6) are inhibited by these compounds in the same nanomolar concentration range as PP1 and PP2A are inhibited (72). Since it is believed that oxidation of critical cysteine residues within PPases catalytic sites leads to their inactivation rather than activation, our findings may seem a little bit controversial. However, it appears that oxidative stress-sensitive p38 MAPK activation (73) may link asbestos-derived ROS and activation of PPases.

Stimuli other than asbestos have also been shown to induce apoptosis in a PSPase-dependent manner (74, 75). The precise mechanism by which PSPases lead to apoptosis is unclear. It has been proposed that phosphorylation and dephosphorylation are crucial in caspase signaling pathways of apoptosis (72). Many signals activate caspase-9, an initiator protease that activates caspase-3 and downstream caspases to initiate cellular destruction. Cardone et al. (76) reported that Akt induced phosphorylation of procaspase-9. This reversible phosphorylation, which led to procaspase-9 inactivation, appeared to promote cell survival. Moreover, cytochrome c-induced activation of caspases-9 and -3 required PSPase activity in chronic lymphocytic leukemia cells (77). It was suggested that the PP2A-induced dephosphorylation of proapoptotic Bcl-2 family members, such as Bad, was responsible for the observed cell death. Additionally, Allan et al. (78) observed that ERK MAPK inhibited caspase-9 through phosphorylation at threonine 125. Finally, activated phosphatases have been shown to regulate the alternative splicing of specific apoptotic factors, i.e. caspase 9 and Bcl-x (75).

In conclusion, our results indicate that the initial event that triggers apoptosis by crocidolite in human lung epithelial cells is the p38 MAPK-dependent increase in the activity of PSPases followed by dephosphorylation of EGFR and EGFR-associated
survival pathways (ERK1/2 and Akt) and concomitant activation of caspases. We speculate that asbestos-induced intracellular imbalance between protein phosphorylation and dephosphorylation is a critical determinant of both cell survival and death. Despite significant research advances, the exact mechanism of asbestos-induced carcinogenicity is not well understood. Increased understanding of the signaling pathways that govern the ability of cells to survive or undergo apoptosis is of critical importance to identify predictive factors controlling development of malignant tumors in humans.

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CHAPTER 6

SUMMARY

Signaling pathways initiated at the cell surface or from within the cell regulate transactivation of transcription factors and gene expression that are related to a number of critical cellular outcomes ranging from proliferation to apoptosis. While several groups have independently established that asbestos induces apoptosis in the lung cells, the components of the regulatory network controlling apoptosis, the mechanisms of action and patterns of interaction of these factors remain elusive. Since some differences were reported in the way rodent and human cells responded to asbestos fibers, the goal of this research was to examine and compare the asbestos-induced signaling events in relevant human lung and pleural target cells, and to determine if iron associated with fibers, as well as the process of fiber endocytosis, played any role in these events. A significant finding of this dissertation was that treatment of human lung and pleural cells, i.e. epithelial (A549 and SAEC) and mesothelial (MET5A) cells with asbestos fibers resulted in dephosphorylation and subsequent inactivation of the ErbB family of receptor tyrosine kinases. The results presented here support other studies in the literature showing differences between rodent and human tissue in terms of resistance to genotoxicity and/or cytotoxicity by asbestos. Another significant finding of this dissertation was that p38 MAPK, through a serine/threonine phosphatase-mediated mechanism, regulates asbestos-induced apoptosis in A549 cells. Of particular importance was that iron mobilized from fibers inside the cells initiates the crocidolite-induced signaling events preventing survival, but activating apoptotic pathways. The findings of this dissertation are
summarized in more details in the following sections.

Treatment of A549, MET5A, and SAEC cells with asbestos caused a significant reduction of EGFR tyrosine phosphorylation. Our results strongly suggest that the responses observed in A549 cells were representative of transformed and normal human cells. We have identified a potentially important mechanism by which relevant human lung and pleural target cells respond to asbestos fibers. Treatment with crocidolite for 24 h also diminished the phosphorylation levels of HER2. In addition, exposure of A549 cells to crocidolite for 3 to 24 h resulted in no apparent degradation of the EGFR. Most importantly, inhibition of fiber endocytosis and removal of iron from asbestos by desferrioxamine B or phytic acid prevented asbestos-induced decreases in EGFR phosphorylation. We also observed that the amount of EGFR dephosphorylation in A549 cells by two types of asbestos, crocidolite and amosite, was significantly greater than that by the third type, chrysotile, either short or intermediate length fibers. The effects of crocidolite, amosite and chrysotile on the EGFR phosphorylation state appeared to be related to the amount of iron mobilized from these fibers. Thus, there appeared to be a correlation between iron mobilization from asbestos and the degree of EGFR dephosphorylation. These results strongly suggest that iron plays an important role in asbestos-induced inactivation of EGFR.

Several independent research groups have provided evidence for the involvement of some signaling cascades in apoptosis of rodent cells following asbestos exposure. However, the effects of asbestos on the control and coordination of the signal transduction pathways in human lung cells have not been investigated. We observed that exposure of A549 and SAEC cells to crocidolite led to inactivation of the main EGFR
signaling pathways, including Akt and ERK1/2 pathways. Crocidolite also initiated apoptosis via pathways involving activation of p38 MAPK, caspase -3 and -9, and cleavage PARP. Prevention of these effects with an iron chelator or endocytosis inhibitors strongly suggests that iron mobilized from fibers inside the cells initiates the observed events. Inhibition of p38 MAPK with SB203580 prevented inactivation of EGFR, inactivation of EGFR-associated survival pathways, and initiation of apoptosis. Taken together, it appears that iron-dependent p38 MAPK activation plays a central role in crocidolite-induced apoptosis of human lung epithelial cells via inhibition of ERK1/2 and Akt survival pathways and concurrent activation of caspase-3 and -9. Our results reveal not only important species differences but also the complexity and diversity of regulatory mechanisms in asbestos-induced apoptosis. We speculate that apoptosis may be an important response of relevant human lung target cells and that asbestos pathogenesis may result from altered apoptotic mechanisms.

Phosphorylation and dephosphorylation of proteins mediate signal transduction events that control a multitude of cellular processes including cell survival and death. Recent studies strongly suggest that PTPases as well as PSPases are essential components critically involved in the control of apoptosis. We therefore investigated the role of PPases in asbestos-induced apoptosis in A549 cells, using OA and VA, specific inhibitors of PSPases and PTPases, respectively. Treatment with OA, an inhibitor of PP2A and PP1 at the concentrations used, but not with VA, protected the cells from asbestos-induced apoptosis. Importantly, inhibition of p38 MAPK with SB203580, selective decreases in fiber uptake and removal of the redox active iron from the fibers resulted in significant attenuation of asbestos-induced dephosphorylation of cellular proteins. Our results
demonstrate that p38 MAPK, through a serine/threonine phosphatase-mediated mechanism, regulates asbestos-induced apoptosis in human lung epithelial cells. We speculate that the intracellular imbalance between protein phosphorylation and dephosphorylation caused by asbestos is a critical determinant of both cell survival and death.

Taken together, we propose a model of crocidolite-induced injury in human lung epithelial cells involving p38 MAPK control of cell survival/apoptosis (Fig. 6-1). The signaling events appear to be initiated by iron mobilized from crocidolite fibers inside the cells. Additionally, p38 MAPK appears to control the molecular survival/death switch that directs the cells away from survival/proliferation to apoptosis. This involves p38 MAPK-mediated activation of protein phosphatases and subsequently dephosphorylation of EGFR and EGFR-associated survival pathways (Akt and ERK1/2) as well as activation of apoptotic pathways (caspase-3, -9, PARP). Importantly, inhibition of p38 MAPK with SB203580, blockage of PSPases with okadaic acid, decreasing fiber uptake (cytochalasins) and removal of bioavailable iron with iron chelator desferrioxamine prevented the observed cellular events and apoptosis. Increased understanding of the signaling pathways that govern the ability of cells to survive or undergo apoptosis is of critical importance to identify predictive factors controlling development of malignant tumors in humans.

A human lung epithelial cell line, A549, with characteristics of alveolar epithelial type II cells, was used for the majority of these studies. A549 cells are one of the three most common cultured human lung epithelial cell lines being used as a tissue culture model of lung epithelial cells in research. The epithelial type II cells are chosen for these
studies because not only did they appear at crossroads of inflammation, fibrogenesis and carcinogenesis, but also this type of cells is most likely to come into contact with external agents, such as particles or asbestos fibers. This cell line was also selected because of its unlimited proliferation capacity and reproducibility, which is hard to achieve with primary cells. Since A549 cells were originally obtained from an alveolar carcinoma,
there was concern that they may not be a faithful representation of normal cells.

Importantly, in some experiments presented here, A549 cells responded in the same way
to crocidolite as normal human small airway epithelial cells (SAEC). Nevertheless, some
of the studies that we have done with only A549 cells should also be carried out in the
primary human lung cells in an attempt to reproduce these results, e.g. p38 MAPK, PPase
and apoptotic pathways activation.

A comparative examination of human and rodent cells showed that patterns of
asbestos-induced signaling events were different. Several explanations for these
dissimilar responses are possible. First of all, species-specific effects may be due to
unusual resistance to cytotoxicity of asbestos by one of the cell lines. Another possibility
might be unique patterns and efficiency of DNA repair and/or protective responses to
oxidative stress in different cell types. Although direct comparison of human and rodent
cells are not fully justified, further work needs to be done to determine and understand
the differences between rodent and human tissue in terms of resistance to cytotoxicity by
asbestos.

The concept of redox regulation is emerging as an understanding of the novel
mechanisms in the pathogenesis of several disorders, including viral infections,
immunodeficiency, malignant transformation, and degenerative diseases. Generation of
ROS by iron-catalyzed Haber-Weiss reactions or by cellular response to this cytotoxic
agent may be in excess compared with the levels of thiol buffer, which leads to activation
of the stress kinase cascade and activation of apoptotic effector molecules. Many studies
have shown that ROS lead to activation of p38 MAPK. Several mechanisms have been
proposed for its activation including ROS-dependent dissociation of a molecule that
maintains the pathway in an inactive state. While strong evidence exists that ROS and changes in the redox potential of the cells can affect the activation of p38 MAPK, further studies are required to determine direct targets in this pathway. It is of great importance to identify new oxidant-sensitive players that are upstream of the p38 MAPK. Also, further work will be required to understand if and/or how p38 MAPK activation differs with cell type and between species. As the p38 MAP kinase is involved in the regulation of transcription, it will be also important to better understand how p38 MAPK activation regulates gene expression that may be linked sequentially to the development of early cell injury (apoptosis) and to subsequent compensatory proliferation. Knowledge of the signaling pathways and physiological responses to cytotoxic agents is essential to understanding the mechanism of asbestos-related pulmonary toxicity.

Today, the importance of the reversible phosphorylation of proteins in the control of apoptosis as well as the link between the phosphatases (PP2/PP2A) and apoptosis fields is clearly established. Our results reported herein suggest that asbestos stimulates PPases and that the upstream activation of p38 MAPK is required for the observed increases in PPase activity. The precise mechanism by which p38 MAPK may lead to PPase activation is unclear. It has been suggested that PPases may be direct targets for p38 MAPK-mediated phosphorylation. Nevertheless, additional work needs to be done to determine the exact mechanism by which p38 MAPK, directly or indirectly, activates PPases in A549 cells.

PPases appear to have a positive regulatory role in apoptosis by activating pro-apoptotic and inhibiting anti-apoptotic proteins by dephosphorylation. Even though
several players regulated by PPases have been determined, further work is required to identify the specific targets of asbestos-induced PPase-mediated apoptosis.

It has been proposed that apoptosis may represent an important mechanism of eliminating cells that have asbestos-induced DNA alterations during the process of carcinogenesis. Even though apoptosis is responsible for normal tissue homeostasis and elimination of defective or potentially dangerous cells, it has also been observed that excessive death of cells leads to persistent inflammation. Defects in the control of apoptotic pathways may contribute to a variety of diseases including autoimmune and neurodegenerative conditions and AIDS. In addition, some reports suggest that a primary increase in apoptosis can stimulate compensatory proliferation. Increased cell proliferation without a compensatory increase in apoptosis may be associated with tumor promotion and progression. Our understanding of the complex processes of asbestos-induced apoptosis has advanced over the past decade yet many unanswered questions remain. While there is considerable information regarding asbestos-induced apoptotic signaling pathways that involve mitochondria, much less is known about pathways that involve cell surface receptors, stress-activated protein kinases and most importantly protein phosphatases. A deeper understanding of the physiological importance of numerous molecules involved in apoptotic signaling pathways requires further investigation. In addition, much remains to be learnt about how asbestos-induced DNA damage initiates and regulates the execution of programmed cell death.
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