Antioxidant defense mechanisms of human mesothelioma and lung adenocarcinoma cells

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Childrens Hospital, University of Helsinki, 00029 Helsinki; Finnish Institute of Occupational Health, 00250 Helsinki; Department of Internal Medicine, University of Oulu, 90220 Oulu Finland; and Department of Environmental Health, University of Washington, Seattle, Washington 98105-6099

J ärvinen, Kristiina, Petra Pietarinen-Runtti, Kaija Linnaimaa, Kari O. Raivio, Cecile M. Krejsa, Terrance Kavanagh, and Vuokko L. Kinnula. Antioxidant defense mechanisms of human mesothelioma and lung adenocarcinoma cells. Am J Physiol Lung Cell Mol Physiol 278: L696–L702, 2000.—The development of drug resistance of tumors is multifactorial and still poorly understood. Some cytotoxic drugs generate free radicals, and, therefore, antioxidant enzymes may contribute to drug resistance. We investigated the levels of manganese superoxide dismutase (Mn SOD), its inducibility, and its protective role against tumor necrosis factor-α and cytotoxic drugs (cisplatin, epirubicin, methotrexate, and vindesin) in human pleural mesothelioma (M14K) and pulmonary adenocarcinoma (A549) cells. We also studied other major antioxidant mechanisms in relation to oxidant and drug resistance of these cells. A549 cells were more resistant than M14K cells toward both oxidants (hydrogen peroxide and menadione) and all the cytotoxic drugs tested. M14K cells contained higher basal Mn SOD activity than A549 cells (28.3 ± 3.4 vs. 1.8 ± 0.3 U/mg protein), and Mn SOD activity was significantly induced by tumor necrosis factor-α only in A549 cells (+524%), but the induction did not offer any protection during subsequent oxidant or drug exposure. Mn SOD was not induced significantly in either of these cell lines by any of the cytotoxic drugs (0.007–2 µM, 48 h) tested when assessed by Northern blotting, Western blotting, or specific activity. A549 cells contained higher catalase activity than M14K cells (7.6 ± 1.3 vs. 3.6 ± 0.5 nmol O2−·min−1·mg protein−1). They also contained twofold higher levels of glutathione and higher immunoreactivity of the heavy subunit of γ-glutamylcysteine synthetase than M14K cells. Experiments with inhibitors of γ-glutamylcysteine synthetase and catalase supported our conclusion that mechanisms associated with glutathione contribute to the drug resistance of these cells.

oxidant; hydrogen peroxide; drug; A549 cells; superoxide dismutase; catalase; glutathione; γ-glutamylcysteine synthetase

HUMAN MALIGNANT MESOTHELIOMA is an uncommon and fatal tumor associated in most cases with exposure to asbestos fibers (33). One typical feature of mesothelioma is its high resistance to chemotherapeutic agents and to radiation. Lung tumors such as squamous cell carcinoma and adenocarcinoma are often primarily resistant to chemotherapy and/or they develop the resistance rapidly during the first courses of therapy. Because both radiation and several anticancer drugs work by generating free radicals, intracellular antioxidants may partly contribute to the drug resistance of this and other malignant cells.

Manganese superoxide dismutase (Mn SOD) is one of the most important antioxidant enzymes scavenging superoxide radicals in the mitochondria (8, 23, 46, 50). This enzyme has low activity in most cancer cells (37, 38), and it has been suggested that Mn SOD may be a tumor suppressor gene (29). Recent studies have, however, documented high levels of Mn SOD in many malignant tumors such as mesothelioma, glioma, thyroid carcinoma, and colon carcinoma (7, 19, 20, 28, 36). One study also suggested that the survival of colon cancer patients with elevated Mn SOD immunoreactivity in the tumor cells was shorter than that of patients with low Mn SOD immunoreactivity (19). In recent studies by our laboratory, Mn SOD expression was found to be higher in malignant mesothelioma than in healthy human mesothelium (20) and higher in four malignant mesothelioma cell lines than in nonmalignant mesothelial cell cultures (25). It has, however, remained unclear to what extent Mn SOD may contribute to the resistance of human mesothelioma cells against cytotoxic drugs used in the treatment of this disease.

Several previous studies (8, 47, 50–52) have emphasized the importance of SOD in protection against oxidants and hyperoxia. Also, intraperitoneal injection of tumor necrosis factor (TNF-α) and subsequent induction of Mn SOD have rendered adult rats more resistant to oxygen toxicity (46). Previous studies (30, 34, 48, 51) have also shown that induction or transfusion of the Mn SOD gene blocks TNF-α- and/or oxidant-mediated cytotoxicity, at least in fibroblasts, breast cancer cells, tracheal epithelial cells, and H820 lung adenocarcinoma cells. On the other hand, several studies (12, 15, 16, 24) using transfusion of the SOD gene, transgenic animals, or cells treated with TNF-α to cause Mn SOD induction have indicated that high levels of SOD con-
very minimal protection or no protection at all or even increase susceptibility to oxidant effects. Thus the role of Mn SOD in the host defense against exogenous oxidants remains inconclusive. Oxidant and/or drug resistance of tumor cells may be related to the constitutive level of Mn SOD or its inducibility, to the species and cell type investigated, or to other antioxidant mechanisms of the cells. The most important of these other mechanisms are the hydrogen peroxide (H₂O₂) scavenging enzymes catalase and glutathione peroxidase as well as other antioxidant mechanisms related to glutathione (44, 45).

To further evaluate the role of Mn SOD and other H₂O₂ scavenging mechanisms in malignant cells, we investigated oxidant and drug resistance of mesothelioma and lung adenocarcinoma cell lines with high or low Mn SOD activity. Mesothelioma cells established from primary tumors contain high constitutive levels of Mn SOD (20, 25). A549 adenocarcinoma cells were selected for comparison because they represent well-characterized malignant lung cells and, in our preliminary experiments, low Mn SOD activity. Because cytotoxic drugs may lead to drug resistance by induction of intracellular antioxidant enzymes, the induction of Mn SOD by TNF-α was compared with the effects obtained by various cytotoxic drugs. In additional studies, the levels of catalase and mechanisms related to glutathione metabolism were assessed in these cells.

**METHODS**

**Cell cultures.** Human M14K mesothelioma cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 0.03% L-glutamine (all from Life Technologies, Paisley, UK) at 37°C in a 5% CO₂ humidified atmosphere (40). Human A549 lung adenocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA) and were grown in F-12 nutrient mixture supplemented with 15% FCS (Life Technologies).

**Exposures.** Subconfluent M14K and A549 cells were treated for 24–48 h with 10 ng/ml of TNF-α (Boehringer Ingelheim) or exposed to one of four cytotoxic drugs: epirubicin (Farmorubicin), methotrexate (Trexan), vindesin (Elsidine), or cisplatin (Platinol). Anthracynines (epirubicin) inhibit RNA transcription and may cause changes in the cellular redox state; cisplatin inhibits DNA synthesis by mechanisms closely related to anthracynines; and vinca alkaloids (vindesin) inhibit microtubule formation in the mitotic spindle. Methotrexate inhibits dihydrofolate acid reductase, but a recent study (3) also showed that methotrexate inhibits γ-glutamylcysteine synthetase (γ-GCS) and glutathione reductase, at least in HeLa cells. Drug concentrations of 0.5, 0.05, and 0.005 µg/ml (0.01–1 µM epirubicin, 0.02–2 µM cisplatin, 0.01–1 µM methotrexate, and 0.007–1 µM vindesin) were selected on the basis of previous studies performed in our laboratory and preliminary viability tests conducted for this study. The exposures varied between 24 and 48 h. In the oxidant experiments, the cells were exposed to 5–100 µM menadione, which generates superoxide in a redox cycling reaction intracellularly, or to 0.01–1 mM H₂O₂. In selected experiments, the cells were pretreated with 0.2–1 mM buthionine sulfoximine (BSO) for 18 h. BSO inhibits the rate-limiting enzyme γ-GCS in glutathione synthesis and causes glutathione depletion. To inhibit catalase, the cells were pretreated with 30 mM aminotriazole (ATZ) for 60 min and were also incubated with the same concentration of ATZ for the last 24 h of exposure (31). Previous and present studies have indicated that the BSO concentration required for nearly complete glutathione depletion without toxicity is 0.2 mM for M14K cells (22) and 1 mM for A549 cells (39), the concentration of ATZ being 30 mM (22). The concentrations of inhibitors used in this study showed no detectable toxicity. Oxidant and drug resistance of A549 cells pretreated with TNF-α was investigated by first incubating the cells with TNF-α (10 ng/ml) for 48 h to induce Mn SOD and then exposing the cells either to 0.05 µg/ml of epirubicin for 48 h or to 50 µM menadione for 16 h, both exposures causing 50% loss of cell viability in control cells.

**Cell viability.** After the exposures, the cells were collected by trypsinization and counted with a microscope. Cell survival was also assessed by the XTT method with a commercial kit according to the instructions of the manufacturer (Boehringer Mannheim, Mannheim, Germany) with a spectrophotometer capable of reading microtiter plates (1420 Victor multititer counter, Wallac, Turku, Finland).

**Northern blot analysis.** The cells were scraped into 4 M guanidine thiocyanate buffer and immediately frozen at −70°C. Total RNA was isolated with the acid guanidium method (6). Denatured RNA samples were electrophoresed on 1% agarose gels containing 0.36 M formaldehyde. After ethidium bromide staining and ultraviolet examination to confirm loading homogeneity, the RNA was transferred onto Hybond-N nylon filters (Amersham) and cross-linked to the filters by ultraviolet illumination. The filters were prehybridized at 58.5°C in a buffer containing 50% deionized formamide, 5x saline-sodium citrate, 50 mM sodium phosphate (pH 6.5), 5x Denhardt’s reagent, and 100 µg/ml of herring sperm DNA. The full-length cDNA of Mn SOD, kindly provided by Dr. Y.-S. Ho (Wayne State University, Detroit, MI), was cloned into pSP65 vector and transcribed into a 32P-labeled riboprobe. Purified probe was added to the prehybridization solution and hybridized overnight at 58.5°C with shaking. The filters were then washed in 2x saline-sodium citrate and exposed to Kodak (Rochester, NY) X-OMAT AR photographic film at −80°C. After autoradiography, the same filters were hybridized with a β-actin control probe transcribed from the p-TRI-β-actin plasmid (Ambion, Austin, TX).

**Western blot analysis.** The cells were mixed with the electrophoresis sample buffer and boiled; 50 µg of cell protein (5) were applied to 12% sodium dodecyl sulfate-polyacrylamide gels (27). The gels were electrophoresed for 1.5 h (90 V) at room temperature and transferred onto Hybond ECL (Amersham, Arlington Heights, IL) nitrocellulose membranes in a Mini-PROTEAN II cell (Bio-Rad). The blotted membranes were incubated with rabbit antibody to recombinant human Mn SOD (1:10,000; a gift from Dr. J. D. Crapo, Department of Medicine, National Jewish Medical and Research Center, Denver, CO) or with rabbit anti-actin antibody (1:2,500) followed by sheep anti-mouse antibody conjugated to horseradish peroxidase (Amersham). The reactivity was detected by an enhanced chemiluminescence system (Amersham). β-Actin expression of the cells was detected with a monoclonal anti-actin antibody (1:2,500) followed by sheep anti-mouse antibody conjugated to horseradish peroxidase (1:3,000; Amersham).

**ImageQuant software version 3.0 Fast Scan (Molecular Dynamics, Sunnyvale, CA).**

Cell protein was measured with the method of Bradford (5) (Bio-Rad, Hercules, CA).
Mn SOD activity. Total SOD was measured spectrophotometrically with the method of McCord and Fridovich (32). Mn SOD activity was distinguished from Cu/Zn SOD by its resistance to 1 mM potassium cyanide. The activity is expressed as units per milligram of protein.

Catalase activity. Catalase was assayed by measuring oxygen production in cells exposed to H₂O₂ with a Clark-type oxygen electrode (22). Enzyme activity is expressed as nanomoles of oxygen produced per minute per milligram of protein.

Glutathione S-transferase activity. The activity was measured spectrophotometrically with 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM glutathione (13). Enzyme activity is expressed as units per milligram of protein.

Glutathione. Total glutathione content was determined spectrophotometrically after the reduction of 5,5'-dithiobis-(2-nitrobenzoic) acid by NADPH in the presence of glutathione reductase (4). Glutathione content is expressed as nanomoles per milligram of protein.

Statistical analysis. Results are means ± SE; two groups were compared with two-tailed Student's t-test. P < 0.05 was considered significant.

RESULTS

The basal level of Mn SOD was higher in M14K cells (28.3 ± 5.8 U/mg protein) than in A549 cells (1.8 ± 0.6 U/mg). TNF-α treatment for 48 h enhanced the protein levels of Mn SOD in both cell types (Fig. 1), whereas none of the cytotoxic drugs at two concentrations had any effect (data not shown). The specific activity of Mn SOD increased significantly with TNF-α only in A549 cells (∗P < 0.05 compared with cont) (Fig. 2), suggesting that at least part of the immunoreactive protein in M14K cells may be enzymatically inactive. The specific activity of Mn SOD was not changed significantly by any of the drugs (Table 1). All the cytotoxic drugs with the exception of methotrexate (0.05 and 0.005 µg/ml) caused a modest increase in the mRNA level of the 4-kb transcript of Mn SOD in A549 cells (177% for cisplatin, 183% for epirubicin, and 164% for vindesin); this change was, however, small compared with the effect of TNF-α, which was the only agent causing an increase also in the 1-kb transcript (Fig. 3).

A549 cells were more resistant than M14K cells against menadione and to epirubicin as shown in Fig. 4. Cisplatin at the highest concentration (2 µM) for 48 h reduced the viability in A549 cells by 16%, whereas the estimated concentration causing 50% loss of M14K cells was 1.7 µM. The concentrations of methotrexate causing 50% cytotoxicity in 48 h were 1 µM in A549 cells and 0.01 µM in M14K cells, and those of vindesin were 0.07 and 0.007 µM, respectively. To investigate the hypothesis that Mn SOD induction may increase oxidant and drug resistance, TNF-α-pretreated A549 cells were exposed either to menadione for 16 h or to epirubicin for 48 h at concentrations, leading to ~50% loss of control cells. Mn SOD induction did not protect these cells against either exposure (Fig. 5).

A549 cells were also more resistant than M14K cells against exogenous H₂O₂ (Fig. 6). This greater oxidant resistance is in line with the finding that both catalase

<table>
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<tr>
<th>Mn SOD Activity, U/mg protein</th>
<th>A549 cells</th>
<th>M14K cells</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.8 ± 0.3</td>
<td>28.3 ± 3.4</td>
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<tr>
<td>TNF-α</td>
<td>11.1 ± 0.5</td>
<td>36.2 ± 4.0</td>
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<tr>
<td>Cisplatin (0.005 µg/ml)</td>
<td>2.7 ± 0.3</td>
<td>29.1 ± 2.1</td>
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<tr>
<td>Epirubicin (0.005 µg/ml)</td>
<td>1.5 ± 0.2</td>
<td>29.2 ± 3.7</td>
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<tr>
<td>Vindesin (0.005 µg/ml)</td>
<td>1.9 ± 0.3</td>
<td>38.3 ± 4.2</td>
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Values are means ± SE from 4–6 separate experiments. Mn SOD, manganese superoxide dismutase; TNF-α, tumor necrosis factor-α.
and glutathione concentrations were higher in A549 cells than in M14K cells. Catalase activity was $7.6 \pm 1.3$ nmol O$_2$·min$^{-1}$·mg protein$^{-1}$ in A549 cells and $3.6 \pm 0.5$ nmol O$_2$·min$^{-1}$·mg protein$^{-1}$ in M14K cells ($P < 0.05$; $n = 4$ experiments). Total glutathione concentrations are shown in Fig. 7A. The heavy subunit of $\gamma$-GCS, the rate-limiting enzyme in glutathione synthesis, was prominently expressed in A549 cells but not in M14K cells (Fig. 7B), supporting the importance of $\gamma$-GCS in regulating cellular glutathione levels. Total glutathione S-transferase (GST) activity was $56 \pm 4.8$ U/mg protein in A549 cells and $74 \pm 6.3$ U/mg in M14K cells when analyzed in three separate experiments in duplicate. Thus the level of total GST seems not to be responsible for the higher resistance of A549 cells.

Kinnula et al. (22) previously found that total depletion of glutathione with BSO increased the toxicity and decreased the high-energy nucleotide pool in human mesothelioma M14K cells during exposure to H$_2$O$_2$ and epirubicin, whereas catalase inhibition by ATZ enhanced only the toxicity caused by H$_2$O$_2$. The present study confirmed this finding with A549 cells because both BSO and ATZ pretreatments made A549 cells more vulnerable to the oxidant injury caused by H$_2$O$_2$, and ATZ pretreatment had no effect on the toxicity caused by epirubicin (data not shown). These additional results suggest that glutathione-related mechanisms may be more important than catalase in the drug resistance of malignant lung cells.

**DISCUSSION**

The present findings with human mesothelioma cells and adenocarcinoma A549 cells suggest that glutathione-associated mechanisms play an important role in the resistance of these cells to exogenous oxidants and cytotoxic drugs in vitro. Mn SOD was induced by TNF-$\alpha$ but not significantly by cytotoxic drugs, but neither the basal level of Mn SOD nor its inducibility explained the resistance of these cells to exogenous oxidant or drug exposures.

Recent findings (12, 15, 16) with transgenic animals containing high Mn SOD activities, with cells carrying Mn SOD-transfected cDNA, and with cells pretreated with TNF-$\alpha$ to cause Mn SOD induction have shown controversial results. Transfection of one single gene...
may result in a disturbance of the antioxidant-oxidant balance of the cell, with consequent changes, e.g., the levels of superoxide and/or H$_2$O$_2$ (12). The effects of TNF-$\alpha$ are complex, and besides Mn SOD, other enzymes are simultaneously induced, including $\gamma$-GCS (41). On the other hand, enzymes also associated with the initiation of the apoptotic cascade may be activated by TNF-$\alpha$ (53). The induction of Mn SOD may also be mediated by factors other than TNF-$\alpha$ (10, 49), and these mechanisms may be even more complicated in cancer cells. It has been suggested that the level of Mn SOD is consistently low in tumor cells and also that Mn SOD is not induced in malignant cells (38, 51). However, Warner et al. (48) showed that H820 human pulmonary adenocarcinoma cells treated with TNF-$\alpha$ (50 ng/ml, 48 h) had elevated Mn SOD activities and were more resistant to paraquat than nontreated cells that contained low Mn SOD activity. Another study (17) with insulinoma cells showed that transfection of Mn SOD prevented cytokine-induced toxicity. In addition, myeloid leukemic cells, which are sensitive to the cytotoxic effects of TNF-$\alpha$, showed low Mn SOD activities and significant upregulation of Mn SOD with TNF-$\alpha$, whereas resistant leukemic cells had higher levels of Mn SOD and no induction by TNF-$\alpha$ (26). The present study suggests that Mn SOD can be induced in malignant lung cells and also that this change is more
prominent in those cells that originally showed low Mn SOD activity. The basal or induced level of Mn SOD did not predict the development of oxidant or drug resistance in these cells, and, if anything, TNF-α pretreatment potentiated oxidant and drug sensitivity.

Few studies have investigated Mn SOD induction by cytotoxic drugs. Akashi et al. (1) have shown that the anticancer drug OK-432 leads to elevation of Mn SOD in human granulocytes. Das and White (II) showed that anticancer drugs such as paclitaxel, vinca alkaloids (vinblastine and vincristine), and anthracyclines (daunomycin and doxorubicin) may cause activation of nuclear factor-κB, and recently, Das et al. (9) also reported that mRNA of Mn SOD was upregulated by cytotoxic drugs through a protein kinase-dependent mechanism. In their study, the doses of the drugs were higher and the exposure times shorter (4–8 h) than in the present study, which may explain the more marked increase in the mRNA levels of Mn SOD in their experiments. They did not measure immunoreactive protein or Mn SOD activity or effects of Mn SOD induction on the oxidant or drug resistance. The results of our and other recent studies (1, 9, 11) cannot rule out the possibility that Mn SOD activity may also be induced by cytotoxic drugs in vivo.

The role of Mn SOD in relation to other antioxidant enzymes and oxidant resistance of cancer cells has not been earlier investigated with the exception of recent studies by our laboratory (20, 22) in which human M38K mesothelioma cells were found to contain higher specific activities of Mn SOD, catalase, and GST and a higher glutathione content than M14K mesothelioma cells. However, these findings did not support the importance of Mn SOD in the oxidant resistance of these cells.

Very little is known about catalase in malignant cells or about its role in drug resistance, but a recent study by our laboratory (22) suggests that catalase is not important in the drug resistance of human mesothelioma cells. The present study indicated that even though catalase was higher in A549 cells than in M14K cells, inhibition of catalase with ATZ did not enhance drug-related toxicity. ATZ does not lead to complete inhibition of catalase (22, 31), and, therefore, the final role of catalase in malignant cells remains unclear.

Previous studies (18, 35, 42, 44, 45) have suggested that the resistance to cytotoxic drugs is dependent on glutathione, GST, and/or glutathione-dependent multi-drug resistance-associated glycoprotein in numerous malignant cells. The present study showed that A549 adenocarcinoma cells contained a higher level of glutathione and γ-GCS and a lower level of Mn SOD than M14K mesothelioma cells. Furthermore, inhibition of γ-GCS with BSO enhanced the oxidant sensitivity of these cells significantly. A recent report (43) has shown that the γ-GCS light subunit, which exerts regulatory control over γ-GCS enzyme activity, maps within a critically deleted region of human malignant mesothelioma. On the basis of our laboratory’s (22) and other studies (2, 14, 21), we conclude that glutathione metabolism is important in the resistance of lung adenocarci-

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