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Differential Expression of Manganese Superoxide Dismutase and Catalase in Lung Cancer[1]

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ABSTRACT

Reactive oxygen species (ROS) are important in the initiation and promotion of cells to neoplastic growth. In this context, cigarette smoke exposure, the primary risk factor in lung cancer development, leads to high levels of ROS within the human airway. Although well-equipped with an integrated antioxidant defense system consisting of low-molecular weight antioxidants such as glutathione and intracellular enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, the lungs are vulnerable to increased endogenous and exogenous oxidative insults. Antioxidants increase in response to oxidative stress and minimize ROS-induced injury in experimental systems, indicating that antioxidant levels may determine whether ROS can initiate lung carcinogenesis. On this basis, we hypothesized that antioxidants would be decreased in lung carcinoma cells as compared with tumor-free adjacent lung tissues. Antioxidant expression was evaluated in 16 lung tumor and 21 tumor-free lung tissues collected between the years 1993 and 2001 from 24 individuals with surgically resectable non-small cell lung cancer, *i.e.,* adenocarcinoma and squamous cell carcinoma. Total SOD activity was increased ($P = 0.035$), catalase activity decreased ($P = 0.002$), and glutathione and glutathione peroxidase were similar in tumors compared with tumor-free lung tissues. Alterations in antioxidant activities were attributable to increased manganese SOD and decreased catalase protein and mRNA expression in tumors. Immunohistochemical localization of catalase in the lung revealed decreased or no expression in the tumor cells, although healthy adjacent airway epithelial cells were strongly positive for catalase. Parallel changes in antioxidant activities, protein, and mRNA expression were noted in A549 lung carcinoma cell lines exposed to cytokines (tumor necrosis factor-α, interleukin 1β, and IFN-γ). Thus, inflammation in the lung may contribute to high levels of manganese SOD and decreased catalase, which together may lead to increased hydrogen peroxide intracellularly and create an intracellular environment favorable to DNA damage and the promotion of cancer.

INTRODUCTION

Cellular prooxidant states promote cells to neoplastic growth, in part because of DNA damage caused by ROS,[3] *i.e.,* superoxide anion and hydroxyl radicals (1). Thus, many of the agents that generate ROS are promoters or complete carcinogens (1). For example, tobacco smoke is a major source of ROS in the lungs of smoking individuals, and cigarette smoking is the primary risk factor in the development of lung cancer. Tobacco smoke results in both an exogenous oxidant stress, with 10^{14} radicals/cigarette puff, and endogenous ROS production through the activation of phagocytes in the respiratory tract (2, 3). In direct support of the increased oxidizing environment in lungs of cigarette-smoking individuals, exhaled breath condensate from smoking individuals contains more H_2O_2 than condensate of nonsmoking individuals (4, 5).

Conversely, only a small percentage of individuals with tobacco-smoke exposure develop lung cancer (6, 7). This may be attributable in part to a well-developed antioxidant system in the lung, which includes SOD, catalase, and glutathione-dependent enzymes (8). SOD enzymes include the intracellular Mn SOD and CuZn SOD and an extracellular SOD that is present in epithelial lining fluid and blood vessels (9). SOD enzymes convert superoxide anion to H_2O_2, and catalase converts H_2O_2 to water and oxygen (10). GPxs include extracellular and intracellular enzymes which also function in the detoxification of H_2O_2 and lipid peroxide molecules in a reaction dependent on glutathione and other cofactors (11). Previous studies suggest that antioxidant activity is impaired in lung cancers (12–17). One interindividual risk factor proposed in the development of lung cancer is airway epithelial cell expression of the glutathione-related antioxidant genes (16). In fact, extracellular GPx expression is increased in airway epithelial cells and alveolar macrophages in healthy smoking individuals, *i.e.,* without lung disease (18). Interestingly, ingestion of foods high in antioxidant activity is associated with a decreased incidence of lung cancer (19). However, administration of vitamins A and E, which function as antioxidants, fail to prevent the development of lung cancer (20, 21).

If a decrease in antioxidant activity predisposes to lung cancer, we hypothesized that the activity and expression of antioxidants would be decreased in lung carcinoma tissues as compared with tumor-free lung. Here, we show that catalase activity is decreased in lung cancer because of a striking decrease of catalase protein and mRNA in the tumor cells. In contrast, Mn SOD was increased in lung cancer as compared with tumor-free tissue. Exposure of lung cancer cell lines to cytokines relevant to the lung environment in cancer leads to similar changes in Mn SOD and catalase expression, revealing one potential mechanism for these opposing changes.

SUBJECTS AND METHODS

Individuals with histologically proven non-small cell lung carcinomas undergoing surgical resections at the Cleveland Clinic Foundation (Cleveland, OH) were recruited into the study. Demographic, clinical, and follow-up data were collected prospectively. Tumor characteristics were described, including cell type, differentiation, and the revised Tumor-Node-Metastasis staging (22). Preoperative lung function indices and histological or computer tomographic evidence of emphysema were recorded. Postoperative adjuvant or rescue treatments, time to tumor progression, and survival time were also described. Lung parenchymal tissues representing the tumor-involved and nearby tumor-free areas were stored immediately after resection in liquid nitrogen until subsequent processing. The study was approved by the Cleveland Clinic Institutional Review Board.

RNA and Protein Extraction. Lung specimens (tumor or tumor-free lungs) were homogenized by the PowerGen 35 tissue homogenizer (Fischer Scientific, Brightwaters, NY). Total RNA from the tissue homogenate was extracted by the Guanidium thiocyanate [4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl, and 0.1 M β-mercaptoethanol]-CsCl gradient method (23). Tissue homogenate in lysate buffer (40 mM HEPES, 1.25

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[3] The abbreviations used are: ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; Mn SOD, manganese superoxide dismutase; CuZn SOD, copper-zinc superoxide dismutase; GPx, glutathione peroxidase; GSH, total glutathione; TNF-α, tumor necrosis factor α; IL-1β, interleukin-1 β; IFN-γ, interferon γ 1β.

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μg/ml leupeptin, 2.5 μg/ml aprotinin, 1.25 μg/ml pepstatin, 0.125 mg/ml pefabloc, 0.09 mM DTT, and 1% NP40, all from Boehringer Mannheim, Indianapolis, IN) was freeze-thawed three times for protein extraction. Total protein concentration was measured by bicinchoninic protein assay (Pierce, Rockford, IL).

Antioxidant Activity. The quantitation of catalase activity in the tissue homogenate was based on the reaction with H_2O_2 as described previously (24). In brief, the initial rate of disappearance of H_2O_2 (0–60 s) was recorded spectrophotometrically at a wavelength of 240 nm. One unit of catalase activity was defined as the rate constant of the first-order reaction. The catalase activity was expressed as units/mg protein in the tissue sample.

SOD activity in the tissue homogenate was determined from the rate of reduction of cytochrome *c* (9, 25), with one unit of SOD activity defined as the amount of SOD required to inhibit the rate of cytochrome *c* reduction by 50%. The final reaction volume was 3 ml and included 50 mM potassium phosphate buffer, 2 mM cytochrome *c*, 0.05 mM xanthine, and a 0.1 mM EDTA solution. Xanthine oxidase (Sigma Chemical Co., St. Louis, MO) was added at a concentration sufficient to induce a 0.020 change in absorbance per min at 550 nm. The SOD activity was expressed as units/mg protein in the tissue sample.

Total GPx activity was determined spectrophotometrically in tissue homogenate through an indirect coupled assay (26). The reactions were carried out using the Bioxytech GPx-340 assay kit (Oxis, Portland, OR). The tissue homogenate was put in the proprietary assay buffer and reagent consisting of glutathione, glutathione reductase, and reduced β-$NADP^+$ (NADPH). The reaction was initiated by the addition of 350 μl of 0.007% tert-butyl hydroperoxide. The decrease in absorbance at 340 nm over 3 min, as NADPH was converted to $NADP^+$ (NADP), was proportional to the GPx activity. One unit of activity was defined as the activity that catalyzed the oxidation of 1 nmol NADPH/min, with a molar coefficient of extinction of 6.22×10^6 M^{-1} cm^{-1} used for NADPH. The GPx activity was expressed as milliunits/mg protein in the tissue sample.

Glutathione Assay. Total or reduced glutathione concentration in the tissue homogenate was measured spectrophotometrically (27). Tissue homogenate was mixed with 10 mM 5,5′-dithio-bis(2-nitrobenzoic acid) in 100 mM potassium phosphate buffer (pH 7.5) and 17.5 μM EDTA. The reaction was started by the addition of 0.5 unit of glutathione reductase and 0.4 mM NADPH. The change in absorbance at 412 nm over 2 min was obtained, and the concentration of GSH was calculated against a standard curve. The GSH activity was expressed as nmol/mg protein in the tissue sample.

Western Analysis. The protein expressions of catalase, Mn SOD, and CuZn SOD in tissue homogenates were detected by Western analysis (23). In brief, the protein samples (50 μg/lane) were resolved by 8 and 12% SDS-PAGE for catalase and SODs, respectively, under denaturing and reducing conditions and transferred to nitrocellulose membrane. Mouse monoclonal anti-catalase antibody (Sigma Chemical Co.), sheep polyclonal anti-Mn SOD antibody (Oxis, Portland, OR), and sheep polyclonal anti-CuZn SOD antibody (Oxis) were used for immunoblotting, all at 1:1000 dilutions. The protein expression of β-actin, as a marker for protein loading, was determined by using a mouse monoclonal anti-β-actin antibody (Sigma Chemical Co.) at 1:2000 dilution. Secondary antibodies included a peroxidase-linked species-specific sheep antimouse antibody (Amersham, Arlington Heights, IL) and a donkey antisheep antibody (Sigma Chemical Co.) for monoclonal and polyclonal primary antibodies, respectively. Signal detection was accomplished by using the enhanced chemiluminescence Plus Western blotting reagents (Amersham). The images of signals were electronically digitalized by scanning, and the intensity of images were quantitated by ImageQuant version 1.2 (Molecular Dynamics, Sunnyvale, CA).

Northern Analysis. The mRNA expression of catalase and Mn SOD in tissue homogenates were detected by Northern analysis (23). In brief, the RNA samples (10 μg/lane) were resolved by 1% agarose gel electrophoresis and transferred to nylon membranes. The hybridization was done with a [^{32}P]-labeled catalase probe (CAT 1991), a [^{32}P]-labeled Mn SOD probe (pHMn-SOD4), and a control γ-actin cDNA probe (pHFγA-1) and then subjected to autoradiography. Quantitation of scanned images of catalase mRNA and Mn SOD mRNA relative to γ-actin was accomplished using the ImageQuant version 1.2 (Molecular Dynamics, Sunnyvale, CA).

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections (5 μm) were used for immunohistochemistry with mouse monoclonal anti-catalase antibody (Sigma Chemical Co.) and mouse monoclonal anti-p53 antibody (Dako, Carpinteria, CA) after 15 min of proteinase digestion. Immunohistochemical staining was performed by an automated biotin-avidin peroxidase system (Ventana-ES-320) with amino-ethyl-carbonyl (Ventana, Tucson, AZ) as a chromogen. Positive controls for catalase consisted of the normal bronchial epithelium, RBCs, and endothelium. Positive control for p53 consisted of a tissue section of a lung carcinoma. Negative control of secondary antibody alone was performed on each section of tissue studied. A specialized pulmonary pathologist, who was blinded to the other results of the study, was responsible for the interpretation of immunohistochemical staining.

Cell Culture. A549, a human adenocarcinoma cell line (American Type Culture Collection, Manassas, VA), was cultured in MEM (Life Technologies, Inc., Gaithersburg, MD) with 10% FCS, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine (28). The effects of recombinant human cytokine stimulation on expressions of antioxidants were studied by stimulating the cells at 70% confluence in 100-mm diameter culture dishes with TNF-α (Biosource, Camarillo, CA), IL-1β (Genzyme, Cambridge, MA), and/or IFN-γ (InterMune, Burlingame, CA). The corresponding concentrations for TNF-α, IL-1β, and IFN-γ used in the experiments were 10 ng/ml, 0.5 ng/ml, and 10^4 units/ml, respectively, which were shown to induce Mn SOD expression (29) and IL-8 production (30) in A549 cells. Cells were harvested at baseline, 8, 24, 48, and 72 h after stimulation for RNA and protein extractions as outlined above. Antioxidant activities, protein, and mRNA expressions were determined. All cell culture experiments were performed in triplicate.

Statistics. The demographic and clinical data were shown as mean ± SD. The antioxidant activities, protein, and mRNA expressions were reported as mean ± SE. Because the tissue samples were either paired (tumor-free and tumor tissues from the same individual) or unpaired (only tumor-free or tumor tissues from a single individual), the comparison of tumor-free and tumor tissues in the whole group with respect to antioxidant expressions was carried out using an ANOVA. The subgroup analyses of the paired samples in terms of antioxidant expressions were done by paired *t* test. Correlations between antioxidants and clinical parameters were performed with Pearson correlation coefficients, and *P*s were reported. The level of significance for *P* was chosen at 0.05. The effects of antioxidants on time to tumor progression and survival time were tested by the score test from a proportional hazards regression model. The data from cell culture experiments were reported in mean ± SD. The comparisons between stimulated and unstimulated groups were done by paired *t* test. The Statistical Analysis System (SAS Institute Inc., Cary, NC) was used to perform the above mentioned statistical tests.

RESULTS

Demographic and Clinical Data. There were 37 lung samples collected from 24 individuals from 1993 to 2001. Twenty-six samples were taken as paired samples of tumor and tumor-free lung tissues from 13 individuals. Eleven individuals had lung resections which allowed for samples of only tumor ($n = 3$) or only tumor-free lung ($n = 8$). Because samples were limiting for some experiments, not all analyses could be performed on all samples. The number of samples used in each experiment is stated in the text. The demography, tumor characteristics, preoperative lung function indices, and postoperative treatments are summarized in Table 1. Two-thirds of individuals with lung cancer were males, with a mean age of 67 ± 8 years (47–86 years). All except two were cigarette smokers with 14 ± 9 pack-years. The main tumor cell types were adenocarcinoma (54.2%) and squamous cell carcinoma (37.5%), predominantly well differentiated (66.7%). There were >90% of individuals with stage I or II diseases. The preoperative lung functions were preserved with FEV_1 2.44 ± 0.74 L (83.1 ± 24.6% predicted). Most individuals received pneumonectomy (83.3%), and postoperative treatments included radiotherapy (29.2%) and chemotherapy (12.5%). There was no statistically significant difference ($P > 0.05$) in demographic and clinical parameters between the individuals with tumor-free samples and those with tumor samples collected.

Antioxidant Activities. The total protein concentration for tumor-free lung (L) and tumor tissues (T) were similar [total protein (mg/

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Table 1 *Demographic and clinical data*[a]

Clinical parameters	
Gender	8 females, 16 males
Age (yr)	66.9 ± 8.5
Smoking status	
Current smoker	6 [25]
Ex-smoker	16 [67]
Nonsmoker	2 [8]
Smoking—pack-yr	14.4 ± 8.6
Histology	
Squamous cell	9 [37.5]
Adenocarcinoma	13 [54.2]
Large cell	2 [8.3]
Grade	
Well	16 [66.7]
Poor	8 [33.3]
Stage	
IA	8 [33.3]
IB	10 [41.7]
IIB	4 [16.7]
IIIA	1 [4.2]
IIIB	1 [4.2]
Presence of emphysema	7 [29.2]
FEV_1 (L)[b]	2.44 ± 0.74
FEV_1 (% predicted)	83.1 ± 24.6
FVC (L)	3.73 ± 0.94
FVC (% predicted)	93.6 ± 14.6
Types of lung resections	
Pneumonectomy	20 [83.3]
Lobectomy	4 [16.7]
Postoperative treatment	
Radiotherapy	7 [29.2]
Chemotherapy	3 [12.5]

[a] All data are mean ± SD; values in brackets are the percentage of total population studied.
[b] FEV, forced expiratory volume in 1 second; FVC, forced vital capacity.

ml): L, 12.98 ± 0.51; T, 11.93 ± 0.58; $P > 0.05$]. SOD activity was significantly higher in tumor than in tumor-free lung [SOD (units/mg): L, 6.84 ± 1.98; T, 13.94 ± 2.32; $P = 0.035$] whereas catalase activity was less in tumor than in tumor-free lung [catalase (units/mg): L, 148 ± 16; T, 81 ± 18; $P = 0.002$; Fig. 1*A*]. GPx and GSH activities were similar between the groups [GPx (milliunits/mg): L, 47.6 ± 8.3; T, 55.9 ± 10.0; $P > 0.05$; GSH (nmol/mg): L, 0.485 ± 0.112; T, 0.533 ± 0.128; $P > 0.05$; Fig. 1*A*]. Among the subgroup with paired tumor-free lung and tumor tissues obtained from the same individual ($n = 13$), there was a trend of higher SOD activity in tumor than in tumor-free lung [SOD (units/mg): L, 6.05 ± 0.67; T, 12.09 ± 3.31; $P > 0.05$], whereas catalase activity was significantly lower in tumor than tumor-free lung [catalase (units/mg): L, 145 ± 21; T, 78 ± 13; $P = 0.003$]. The GPx and GSH activities were similar in paired samples of tumor and tumor-free lung [GPx (milliunits/mg): L, 49.4 ± 8.5; T, 44.2 ± 12.6; $P > 0.05$; GSH (nmol/mg): L, 0.314 ± 0.045; T, 0.381 ± 0.109; $P > 0.05$]. Catalase was directly correlated to SOD activity in the tumor-free tissues ($r = 0.64$; $P = 0.001$; Fig. 1*B*), but catalase was not related to SOD activity in the tumor tissues ($r = 0.30$; $P > 0.05$).

Antioxidant Protein Expression. To investigate the mechanisms leading to higher SOD activity and lower catalase activity in tumor than in tumor-free lung, Mn SOD, catalase, and CuZn SOD protein expression were evaluated by Western analysis ($n = 32$). Tumors had higher levels of Mn SOD protein as compared with tumor-free lung [Mn SOD/β-actin: L, 0.61 ± 0.16; T, 1.35 ± 0.19; $P = 0.009$], whereas there was less catalase protein in tumor than in tumor-free lung [catalase/β-actin: L, 0.62 ± 0.06; T, 0.26 ± 0.07; $P = 0.001$; Fig. 2, *A* and *B*]. There was no significant difference in CuZn SOD levels between tumor and tumor-free lung [CuZn SOD/β-actin: L, 0.67 ± 0.08; T, 0.70 ± 0.09; $P > 0.05$]. Similarly in the subgroup with paired tumor-free lung and tumor tissues obtained from the same individual, there were higher Mn SOD protein levels in tumor than in tumor-free lung [Mn SOD/β-actin: L, 0.56 ± 0.09; T, 1.24 ± 0.35; $P = 0.047$], whereas catalase protein was lower in tumor than in tumor-free lung [catalase/β-actin: L, 0.64 ± 0.10; T, 0.26 ± 0.06; $P = 0.003$]. The CuZn SOD protein level was similar in paired tumor and tumor-free lung [CuZn SOD/β-actin: L, 0.73 ± 0.08; T, 0.82 ± 0.15; $P > 0.05$]. Overall, the catalase activity was positively correlated with the catalase protein expression ($r = 0.514$; $P = 0.003$; Fig. 2*C*). However, CuZn SOD protein levels were not correlated to total SOD activity ($P > 0.05$), although Mn SOD trended to correlate with total SOD activity ($P = 0.06$).

Antioxidant mRNA Expression. To determine the mechanisms for higher Mn SOD and lower catalase protein levels in tumor compared with tumor-free lung, Mn SOD and catalase mRNA expression were evaluated by Northern analysis ($n = 27$; Fig. 3*A*). Mn SOD mRNA expression was higher in tumor than in tumor-free lung [Mn SOD/γ-actin: L, 0.61 ± 0.14; T, 1.2 ± 0.18; $P = 0.049$], whereas the

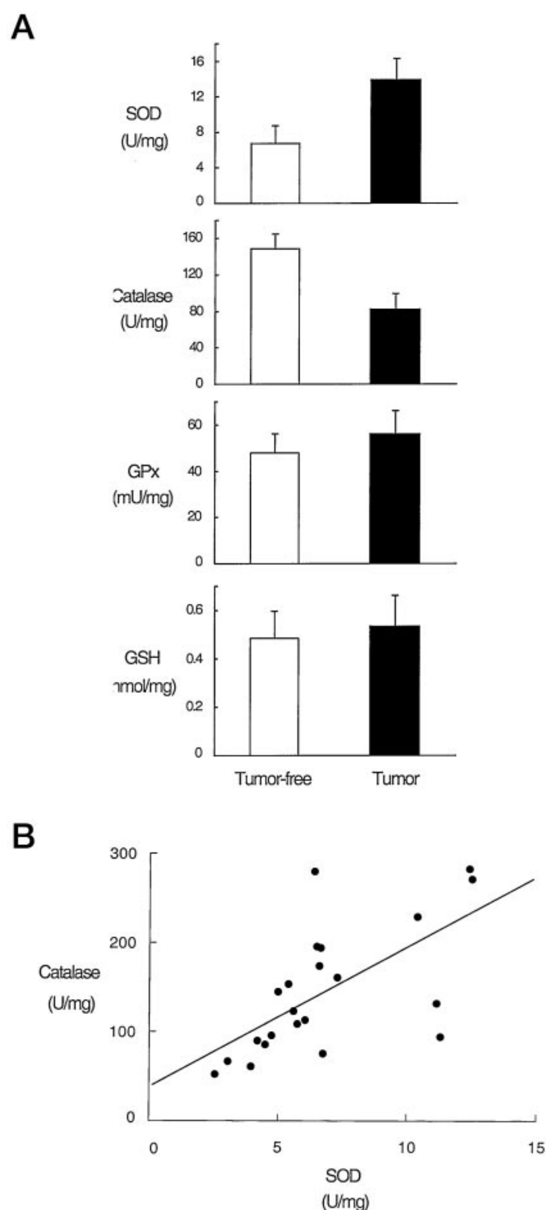

Fig. 1. Antioxidant activities in lung tumor and adjacent tumor-free lung. *A,* SOD activity is increased ($P = 0.035$) and catalase activity decreased ($P = 0.002$) in tumor compared with tumor-free lung from individuals with lung cancer. GPx activity and GSH levels are similar in tumor-free lung and tumor ($P > 0.05$). Mean and SE are shown. *B,* catalase activity is directly related to SOD activity in tumor-free lungs from individuals with lung cancer ($r = 0.64$; $P = 0.001$) but not in tumor.

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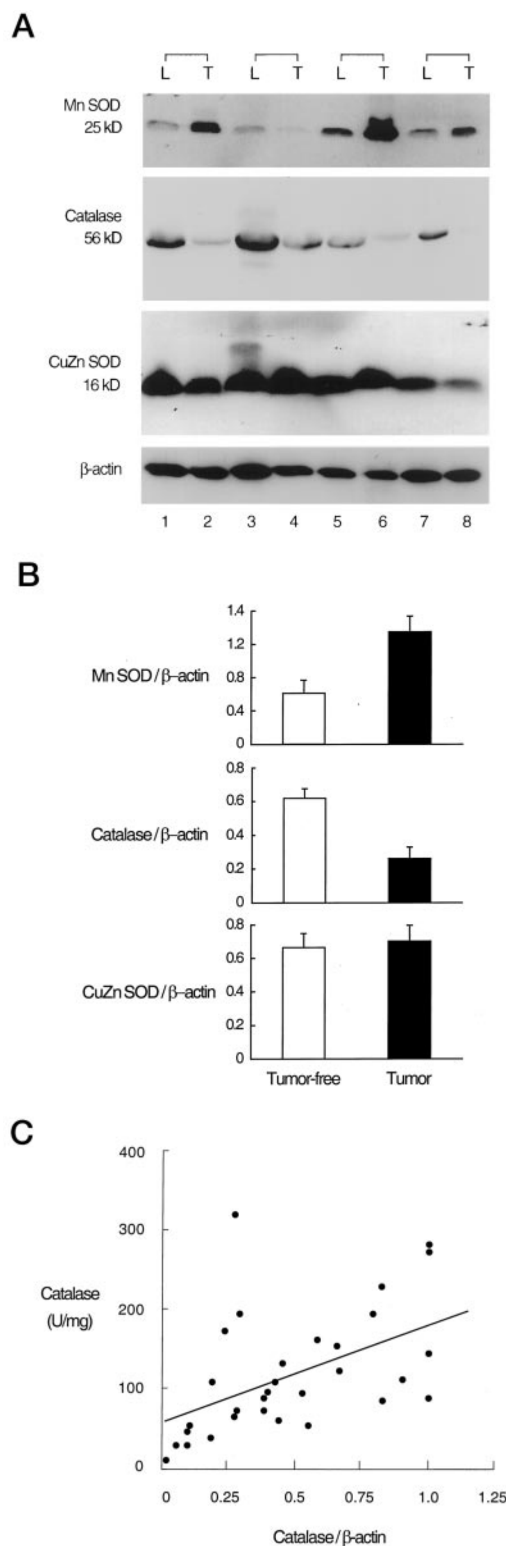

Fig. 2. Antioxidant protein levels in lung tumor and adjacent tumor-free lung. *A,* increased Mn SOD and decreased catalase protein in tumor compared with tumor-free lung from individuals with lung cancer. Representative Western analysis of tissue lysates (50 μg/lane) from pairs of tumor-free lung (*L*) and tumor (*T*) is shown. Each of the paired samples (*L* and *T*) was obtained from the same individual. Mn SOD protein is increased in most *T* compared with *L* (*top*). Catalase protein is uniformly decreased in *T* compared with *L*, whereas CuZn SOD protein is similar in *T* and *L*. β-actin is shown as control for protein integrity and loading (*bottom*). *B,* Mn SOD protein/β-actin is quantitatively increased ($P = 0.009$) and catalase protein/β-actin decreased ($P = 0.001$) in tumor compared with tumor-free lung. CuZn SOD protein expression is similar in samples ($P = 0.74$). Relative units of protein expression are shown as means ± SE. *C,* catalase activity is directly related to the level of catalase protein present in all samples (tumor and tumor-free lung; $r = 0.514$; $P = 0.003$).

mRNA expression for catalase was less in tumor than in tumor-free lung [catalase/γ-actin: L, 0.58 ± 0.06; T, 0.26 ± 0.07; $P = 0.002$; Fig. 3*B*]. Thus, the changes in Mn SOD and catalase activity and protein expression in tumor compared with tumor-free lung were related to alterations in mRNA expression levels. This may have been caused by changes in gene expression, RNA stability, or half-life.

Immunohistochemistry. Lung samples contain respiratory epithelium, blood vessels, connective tissues, and inflammatory cells. Because of the heterogeneous nature of lung tissue and inflammatory changes that may occur in lung cancer tissue, there may be under- or overrepresentation of specific cell types in tumor and tumor-free lung samples. To address this, immunohistochemical staining was performed to localize the specific cell expression for catalase in tumor and tumor-free lung. Four adenocarcinomas and two well-differentiated squamous cell carcinomas underwent immunohistochemical staining. Although consistent results for Mn SOD localization could not be obtained with commercially available antibodies (Oxis), catalase immunostaining was specific and gave consistent results. As positive controls, the normal bronchial epithelium stained strongly in cytoplasm for catalase, whereas a bronchoalveolar cell lung carcinoma expressed strong nuclear staining for p53. Semiquantitative evaluation revealed that the lung carcinoma cells had undetectable or low-positive cytoplasmic immunoreactivity for catalase compared with the surrounding normal bronchial epithelium (Fig. 4). Absence of immunoreactivity for catalase in tumor cells was noted in three adenocarcinomas, despite strong immunoreactivity in airway epithelial cells in these tissue sections. The carcinoma cells tended to have strong nuclear staining for p53 protein (Fig. 4). One of four adenocarcinomas and all squamous cell carcinomas stained positive for p53.

Antioxidant Expressions *in Vitro*. Induction of Mn SOD gene expression by cytokines including TNF-α and IFN-γ has been demonstrated previously in carcinoma cell lines (31, 32). To investigate further the potential role of cytokines in regulating Mn SOD and catalase expressions in lung tumor tissues, *in vitro* stimulation of A549 lung carcinoma cells with specific cytokines (TNF-α/IL-1β/IFN-γ) was performed. The SOD and catalase activities were determined in A549 cells at baseline, 8, 24, 48, and 72 h after stimulation with cytokines, either

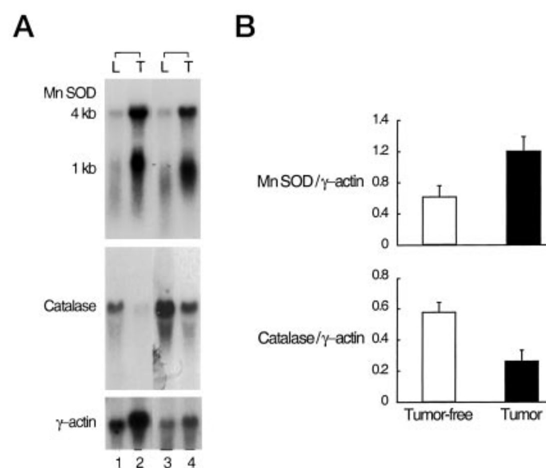

Fig. 3. Antioxidant gene expression in lung tumor and adjacent tumor-free lung. *A,* Northern analysis of total RNA (10 μg/lane) using ^{32}P-labeled cDNA in paired tumor-free lung (*L*) and tumor (*T*) is shown. Each of the paired samples (*L* and *T*) was obtained from the same individual. *Top,* the typical Mn SOD mRNAs (4 kb and 1 kb), which are increased in *T* as compared with *L*. Catalase mRNA is decreased in *T* compared with *L* (*middle*). γ-Actin is shown as control for RNA integrity and loading (*bottom*). *B,* increased Mn SOD mRNA/γ-actin and decreased catalase mRNA/γ-actin is present in tumor compared with tumor-free lung from individuals with lung cancer ($P = 0.049$ and $P = 0.002$, respectively). Relative values shown are means ± SE.

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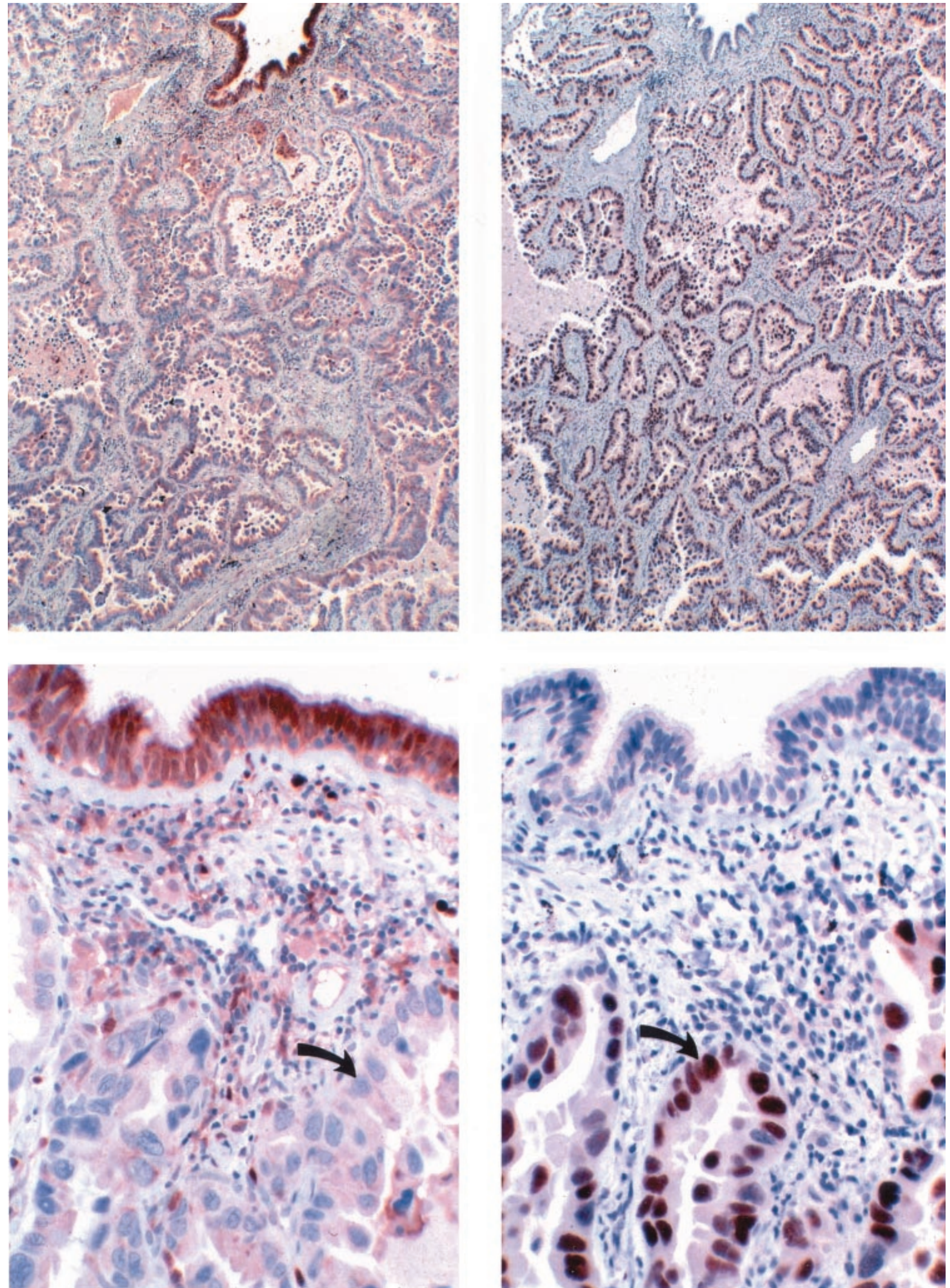

Fig. 4. Catalase and p53 immunoreactivity in lung tumor and tumor-free adjacent lung. Well-differentiated adenocarcinoma (*upper right panel*) with strong p53 immunostaining; adjacent benign bronchiole shows no immunoreactivity to p53. In contrast, the benign bronchiole (*upper left panel*) has strong catalase immunostaining, whereas surrounding adenocarcinoma shows no immunoreactivity to catalase (×20; hematoxylin). Higher magnification shows malignant tumor glands with strong p53 nuclear staining (*arrow, lower right panel*), whereas overlying bronchiole mucosa is negative. Overlying bronchiole mucosa has strong immunoreactivity to catalase (*lower left panel*), whereas malignant tumor glands (*arrow*) show no immunoreactivity to catalase (×200; hematoxylin).

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alone or in combination (Fig. 5*A*). At the time that cells were harvested, cell confluence, total protein, and total RNA from A549 cells exposed to cytokines were similar to unstimulated cells (all $P > 0.05$). There was higher SOD activity in cells stimulated with TNF-α, IL-1 β, or the combination of all three cytokines starting at 8 h ($P = 0.01$ cytokine-stimulated *versus* unstimulated cells at 8 h). Comparing with unstimulated A549 cells, the catalase activity failed to increase in the combined cytokine stimulation starting at 24 h ($P = 0.04$ at 24 h). There was also a trend of less catalase activity after 72 h in cells stimulated with IFN-γ ($P = 0.07$). Northern analysis showed a marked increase in Mn SOD mRNA expression after combined cytokine stimulation (S) compared with unstimulated (US) cells starting at 8 h [Mn SOD/γ-actin at 8 h: S, 2.09 ± 0.21; US, 0.02 ± 0.01; $P = 0.004$; Fig. 5*B*]. However, the catalase mRNA expression was less in combined cytokine stimulated cells compared with unstimulated cells starting at 8 h [catalase mRNA/γ-actin at 8 h: S, 0.405 ± 0.118; US, 0.809 ± 0.061; $P = 0.007$; Fig. 5*B*]. Western analysis confirmed an increase in Mn SOD protein expression starting from 8 h after stimulation by cytokines in combination compared with unstimulated cells (Fig. 5*C*). The Mn SOD protein level was strikingly increased at 72 h with combined cytokine stimulation compared with unstimulated cells [Mn SOD/β-actin at 72 h: S, 66.3 ± 27.0; US, 0.84 ± 0.50; $P = 0.05$; Fig. 5*D*]. On the other hand, there was a lack of increase in catalase protein expression in combined cytokine stimulation compared with the rise in unstimulated cells over time [catalase/β-actin at 72 h: S, 0.83 ± 0.43; US, 1.36 ± 0.46; $P = 0.01$; Fig. 5, *C* and *D*]. However, there was no change in protein expressions for CuZn SOD after stimulation ($P > 0.05$ at all time points). Overall, catalase activity was correlated to catalase protein levels in cells ($r = 0.913$; $P = 0.001$) and catalase protein levels were related to mRNA expression ($r = 0.675$; $P = 0.04$). Similarly SOD activity was related to Mn SOD protein levels ($r = 0.962$; $P < 0.001$), and Mn SOD protein levels were related to mRNA expression ($r = 0.908$; $P = 0.001$).

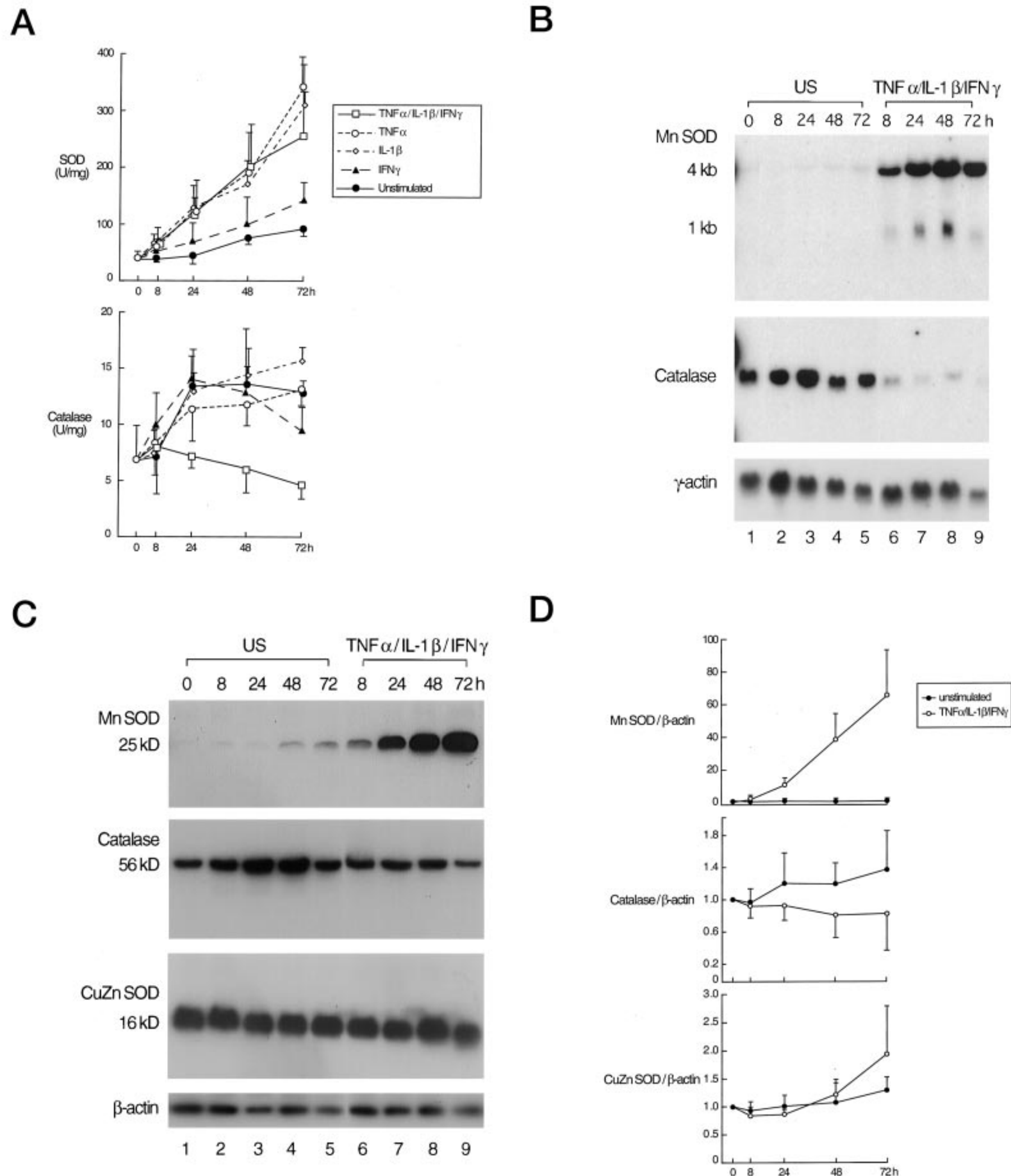

Fig. 5. Antioxidant activities in lung cancer cell line A549. *A,* SOD activity increased in cells stimulated with TNF-α, or IL-1 β, or the combination of all three cytokines as early as 8 h after exposure to cytokines (all $P < 0.01$). Catalase activity in cells increased over 72 h culture but failed to increase with exposure of cells to the combined cytokines ($P = 0.04$ at 24 h). Values are means and SD of three experiments. *B,* representative Northern analysis of total RNA (10 μg/lane) from A549 cells unstimulated (*US*) or stimulated with TNF-α (10 ng/ml), IL-1 β (0.5 ng/ml), and IFN-γ (10^4 units/ml) over 72 h time. Mn SOD mRNA (1 kb and 4 kb) increased with cytokine-stimulation (*Lanes 6–9*) compared with unstimulated cells (*Lanes 1–5*). Catalase mRNA expression was lower in cytokine-stimulated cells (*Lanes 6–9*) compared with unstimulated cells (*Lanes 2–5*). γ-Actin is shown as control for RNA integrity and loading (*Lanes 1–9*). *C,* representative Western analysis of cell lysates from A549 cells unstimulated (*US*) or stimulated with cytokines as above. Mn SOD protein increased with cytokine-stimulation (*Lanes 6–9*) compared with unstimulated cells (*Lanes 2–5*). Catalase protein was less in cytokine-stimulated cells (*Lanes 6–9*) compared with unstimulated cells (*Lanes 2–5*). CuZn SOD protein was similar in cytokine-stimulated (*Lanes 6–9*) and unstimulated cells (*Lanes 2–5*). *Lane 1* shows Mn SOD, catalase, and CuZn SOD protein at baseline. β-actin is shown as control for protein integrity and loading (*Lanes 1–9*). *D,* quantitation of antioxidant protein expression shows that Mn SOD protein/β-actin increased in A549 cells with cytokine-stimulation compared with unstimulated ($P = 0.05$ at 72 h). Catalase protein/β-actin was less in cytokine-stimulated as compared with unstimulated cells ($P = 0.01$ at 72 h), whereas CuZn SOD protein/β-actin was similar in both groups ($P > 0.05$ at all time points).

DISCUSSION

Divergent changes in Mn SOD and catalase activities are present in lung tumor tissues as compared with adjacent tumor-free lung obtained at surgical resection. The reduced catalase and increased SOD activities in tumor tissues are paralleled by corresponding changes in protein and mRNA expressions. Immunohistochemical staining confirms that decreased catalase in tumor samples are caused by reduced expression in the tumor cells. Exposure of the human lung carcinoma cell line A549 to cytokines, *i.e.,* TNF-α, IL-1β, and IFN-γ, reproduces a similar pattern of alterations in Mn SOD and catalase, which supports a role for the host inflammatory response in the mechanism leading to the changes in antioxidant expression. For the whole group, the median follow-up time was 54.6 months, and the median survival time predicted by regression modeling was 83.6 months. There were no overall significant correlations between antioxidant activities and the clinical characteristics and tumor progression or survival times ($P > 0.05$).

Although some studies have shown increased antioxidant activities in tumor cells or the lung epithelial lining fluid of individuals with lung cancer (15, 17), others have reported reduced antioxidant activities in lung tumors (12–14). Impairment of the antioxidant system renders cells vulnerable to the damaging effects of ROS, including

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DNA damage and protein modifications which contribute to carcinogenesis. In support of this concept, lower GPx activities are associated with higher levels of DNA base modifications in human lung cancer than in tumor-free lung (12). Similarly, reduced gene expression of glutathione transferases and GPx in normal bronchial epithelial cells has been implicated as a risk factor for the development of lung cancer in cigarette-smoking individuals (16). Here, limited stage lung cancers obtained from lung resections were evaluated. Previous studies in other types of cancers show that the antioxidant expression in metastatic tumor may differ from primary tumor. For example, higher Mn SOD expression has been found in metastatic lesions than in primary prostate cancer (33). Thus, the antioxidant status in lung tumors may also vary depending on the extent of the disease.

Mn SOD plays an essential role in the conversion of superoxide anion into H_2O_2 in the mitochondrial matrix (8) and is a key survival factor in cells (32, 34, 35). For example, Mn SOD is a determinant of cell resistance to prooxidant cytokines, *e.g.,* TNF-α and IL-1β, and contributes to the survival of cells against ionizing radiation and tumoricidal chemotherapeutic drugs (34, 35). Notably, inhibition of SOD has recently been identified as the primary mechanism for selective killing of human leukemia cells by estrogen derivatives (36). Inhibition of SOD caused superoxide-mediated mitochondrial damage with release of cytochrome *c* resulting in apoptosis of cells. Increased SOD also produces higher levels of H_2O_2, which is normally detoxified by catalase or GPx (11). Catalase is a primary antioxidant defense that converts H_2O_2 to water (10). Decreased catalase activity in tumor cells leads to the accumulation of H_2O_2, which causes DNA damage and/or cell death (37–39). Thus, the combination of increased Mn SOD and decreased catalase in lung tumors undoubtedly results in increased production and decreased detoxification of H_2O_2, a condition which may regulate tumor growth and development. Accumulation of H_2O_2 or other hydroperoxides also promotes DNA damage (40). Taken together, the high levels of Mn SOD with decreased catalase may create an antiapoptotic intracellular environment which is especially susceptible to increased frequency of mutations, a situation likely to lead to cell transformation and cancer. Analysis of Mn SOD expression in early carcinomas or carcinoma *in situ* would provide support for an *in vivo* mechanism specifically related to carcinogenesis. Interestingly, healthy smoking individuals with no lung dysfunction have less SOD in lung epithelial lining fluid as compared with healthy nonsmokers (15, 25), suggesting that the increase in Mn SOD is associated with progression to lung dysfunction and/or cancer.

In this study, we show that Mn SOD gene expression is regulated by cytokines *in vitro*. This is consistent with previous studies which have similarly shown that IFN-γ and TNF-α act synergistically in the induction of Mn SOD expression in different cell lines, including human A549 lung carcinoma cells (31). There have been divergent reports on the effect of IFN-γ in the induction of Mn SOD in A549 cells, *i.e.,* in one report IFN-γ alone caused dose-dependent induction of Mn SOD expression (31), whereas IFN-γ had little inducing effect on Mn SOD in the absence of TNF-α and IL-1 in another study (41). Although not statistically significant, SOD activity tends to be higher in A549 after IFN-γ. However, IL-1β and/or TNF-α clearly have the greatest effect on SOD. Similarly, TNF-α increases Mn SOD activity and mRNA in a dose- and time-dependent manner in the human NIH H820 lung adenocarcinoma cell line (32). The generation of ROS by cytokines is essential in the induction mechanism of Mn SOD (42–44). TNF-α and IFN-γ stimulation also increase Mn SOD in the transformed human bronchial epithelial cell line BEAS-2B (45). BEAS-2B antioxidant responses are similar to primary human cells (46), which suggest both may behave similarly to the A549. The A549 cell line has higher SOD activity than tumor samples, which likely is caused by the homogeneous population of A549 cells as opposed to tumor samples, which include other stromal tissues besides the carcinoma cells.

In general, overexpression of cytokines, including TNF-α, IL-1β, or IFN-γ, is observed in tumors, *e.g.,* renal cell carcinoma (47, 48), gastrointestinal tumors (49), and lymphoma (50). The expression of various cytokines in individuals with lung cancer is clearly different from those without lung cancer. Specific cytokines like TNF-α and IL-1β are elevated in serum (51) and monocytes (52) obtained from individuals with lung cancer compared with controls. *In situ* hybridization provides direct evidence of cytokine production in lung carcinomas. More IL-1 β- and IFN-γ-positive lymphomononuclear infiltrates are present within squamous cell carcinoma than within tumor-free lung regions on histological sections of lung carcinoma (53). In particular, IFN-γ-positive cells are located in the stroma around the neoplastic nests and in contact with the neoplastic cells.

In addition to cytokines, the tumor suppressor protein p53 also regulates Mn SOD expression in cells. For example, transformed mouse fibroblasts lacking p53 have higher Mn SOD expression than wild-type controls (54). Furthermore, transient transfection of wild-type p53 in cervical adenocarcinoma cells suppresses Mn SOD mRNA and activity, indicating that p53 exerts a negative control on *Mn SOD* gene expression (54). Nonfunctional mutant p53 occurs in ~90% of lung cancer, and it was noted in lung tumors in this study. Thus, the presence of nonfunctional p53 and proinflammatory antitumor cytokines may cooperatively contribute to the higher levels of Mn SOD expression in lung tumors.

In contrast to Mn SOD, the human *catalase* gene is a "housekeeping" gene, *i.e.,* with no TATA box, a high GC content, multiple CCAAT boxes and transcription start sites, and low levels of constitutive promoter activity (55). The *catalase* gene expression in human bronchial epithelial cells is insensitive to oxidant stress (55). In fact, treatment of human bronchial epithelial cell lines (BEAS-2B) with TNF-α in combination with IFN-γ decreases catalase activity (45). Here, catalase expression is noted to increase over the time of the cell culture. The lower levels of catalase in response to cytokines is related to a lack of increase over time of cell growth, suggesting that expression may be related to cell cycle mechanisms.

Taken together with other studies, our data suggest that alterations in antioxidants contribute to the development of lung cancers. Loss of antioxidant activity may be a contributory event, leading to increased ROS and subsequent DNA damage and cell death. In response to subsequent local inflammation and/or the occurrence of p53 mutations, Mn SOD expression may increase and function as an antiapoptotic factor allowing selective accumulation of cells that have the potential for increased mutation frequency and immortality.

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