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Modulation of Redox Signal Transduction Pathways in the Treatment of Cancer

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ABSTRACT

Reactive oxygen species (ROS)-mediated damage to DNA is associated with induction of stress-activated protein kinases leading to secondary and tertiary effects on the nuclear matrix, cytoplasmic transport mechanisms, and altered mitochondrial and cell membranes. The cellular defenses against ROS damage are associated with up-regulation of gene products that can significantly alter cell biology, including antiapoptotic Bax family proteins and inflammatory proteins. Altered cell integrity can occur either directly or by indirect paracrine and juxtacrine interactions within tissues. Previous approaches toward therapeutic intervention against ROS damage have included administration of radical scavenger compounds, use of novel drugs that increase cellular production of constitutive antioxidants, or pharmacologic agents that modify the intracellular transport of antioxidants. Strategies to modify the cellular effects of ROS in hyperbaric oxygen injury to the lung, reperfusion injury to transplanted organs, and cancer have led to novel approaches of gene therapy in which the transgenes for antioxidant proteins can be expressed in specific tissues. Reducing tissue-damaging effects of ROS may have relevance to cancer patients by ameliorating normal tissue damage from ionizing irradiation therapy, photodynamic therapy, and cancer chemotherapy. Antioxid. Redox Signal. 3, 347–359.

INTRODUCTION

IT IS NOW WIDELY ACCEPTED that redox reactions within cells, tissues, and organs are involved in both the mediation of pathologic effects of malignant transformation (1, 30) and the efficiency and potential side effects of therapeutic modalities used in the management of cancer (21). DNA-damaging agents widely used in cancer chemotherapy, including ionizing irradiation, alkylating agents, anthracyclines, taxotere derivatives, platinum-based chemotherapeutics, and many other front-line and investigational agents, are known to mediate cellular damaging effects by producing imbalance in redox states within cells (21, 26). Antiviral agents, protease inhibitors, and pharmacological agents used in the treatment of acquired immunodeficiency syndrome and other systemic viral infections, such as those caused by the cytomegalovirus, herpes viruses, hepatitis viruses, and the inflammatory responses to these pathogens, are known to be associated with induction of imbalances of the redox state within cells (18, 19, 21, 22). Furthermore, these viral, as well as other bacterial, microbial, and parasitic, organisms are known to induce tissue trauma by mechanisms involving alteration of the redox state (27, 30, 36, 45). As new research uncovers new influences of redox re-

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actions within cells, it follows that involvement of this pathway in many physiologic and therapeutic settings relative to the management of cancer patients will be identified.

Redox imbalance within cells has been demonstrated to play a vital role in aging (54) and in the disease-specific processes of atherosclerotic damage to blood vessels (27, 35, 53), amyloid β damage to brain tissue in Alzheimer's disease (3, 20), and necrotizing and apoptotic damage to the liver, kidney, and heart in several pathologic states (3, 6, 19, 20, 48, 49). Twenty years ago, the biological mechanisms for many of these disease states were not appreciated to include a role of redox changes within the tissue environment.

With a new awareness of the importance of antioxidant levels within cells, and the significant pathophysiologic alteration resulting from incomplete neutralization of free radicals, new therapies have been focused on ways to intervene in the redox pathways associated with diseases. New *in vitro* systems, animal models, and clinical trials utilizing agents that alter the redox state within cells have brought new promising strategies for the therapy of these disease states. These investigations have also uncovered new questions regarding the interaction of redox state alterations with other pathophysiologic mechanisms in cell and tissue biology (1, 6, 35). This article will focus on specifically those areas of application of the use of antioxidant therapies in the context of gene therapy (introduction of transgenes that allow expression of antioxidant proteins within specific cells) and pharmacologic therapies, in which antioxidant proteins are directly delivered to organs in specific disease states. New data on the effects of manganese superoxide dismutase (MnSOD) gene therapy for ionizing radiation protection will be presented. The implication of these novel approaches and future prospects for preclinical evaluation and clinical trials will be discussed.

MATERIALS AND METHODS

Spin-trap assay

Measurements of reactive oxygen species (ROS) were performed in 32D cl 3, 1F2, 2C6, or 32D-Bcl-xl cells using a spin-trap assay. At 0, 0.5, 1, 2, 3, 6, 18, or 24 h after irradiation, cells were incubated in 100 m*M* DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) for 30 min, washed in phosphate-buffered saline (PBS), resuspended in 100 μl of PBS, and analyzed by electron paramagnetic (spin) resonance (EPR) (19, 41).

Effect of inducers of apoptosis on the mitochondrial respiratory chain

Measurements of activity of the mitochondrial respiratory chain following initiation of apoptosis were performed using a Clark oxygen electrode (Model 5300, Yellow Spring Instrument Co., Yellow Spring, OH, U.S.A.) as previously described (26, 35). Mitochondria were isolated from 32D cl 3, 1F2, and 2C6 cell lines as previously described (11, 16), at various times after initiation of apoptosis. Oxygen consumption studies were performed on purified mitochondria (0.1–0.5 mg/ml) at 37°C in 0.3 *M* sucrose, 5 m*M* 3-(*N*-morpholino) propanesulfonic acid, 5 n*M* potassium phosphate, 1 m*M* EGTA, pH 7.4. A 0.8-ml aliquot of the suspension was injected into a respiratory chamber together with 1 m*M* ADP with a mitochondrial substrate (5 m*M* malate/glutamate, 5 m*M* succinate, or 1.0 m*M* ascorbate/0.2 m*M* tetramethyl-*p*-phenylenediamine) added to quantify complex I, II, III, or IV activity. Respiration was calculated as the rate of change in the O_2 concentration following the addition of substrate, assuming an initial O_2 concentration of 217 μM/ml.

Glutathione (GSH) measurement

Determination of GSH levels were performed in 32D cl 3, 1F2, 2C6, or 32D-Bcl-xl cells at 0, 1, 2, 3, 6, 18, and 24 h following irradiation as previously described (10). GSH content was estimated by an immediate fluorescence response resulting from the addition of ThioGlo-1, a maleimide reagent producing a highly fluorescent product upon reaction with thiol (SH) groups, to cell homogenates. Once the initial response had reached a plateau, total protein thiols were determined as an additional response observed after the addition of 2 m*M* sodium dodecyl sulfate to the same sample.

Cell lines and cytokines

The cell line 32D cl 3, and MnSOD-overexpressing subclones 1F2, 2C6, and Bcl-xl overexpressing line 32D-Bcl-xl, were described previously (10). The Lewis lung carcinoma cell line (3LL) expressing the bacterial β-galactosidase gene (LacZ) has been described (14).

Mice

To investigate the mechanism by which MnSOD transgene expression may protect against irradiation-induced damage to the lung, a mouse model system was used. This model has been used previously to study the development of fibrosis following irradiation of the lung, and in the setting of orthotopic tumors compared with Lewis lung carcinoma cells (10–12, 14), C57BL/6J mice were intratracheally injected with plasmid/liposome (PL) complexes containing the genes for either human MnSOD or LacZ and irradiated to doses of 1,900 or 2,000 cGy. Only the pulmonary cavity was irradiated with the rest of the mouse, including the head, shielded from the irradiation. The mice were examined closely for signs of respiratory distress, as determined by increases in breathing patterns, loss of weight, ruffling of the fur, and hunching of the back. The mice were killed by Nembutal overdose, and the lungs removed and examined for development of fibrosis or alveolitis. At various time points following irradiation, the mice were anesthetized and killed. Expression of mRNA for cytokines such as transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), and interleukin-1α (IL-1α) was measured in the bronchoalveolar macrophages compared with bronchoalveolar lung cells as indicators of acute-phase lung damage (6). Mice were killed by Nembutal overdose in a manner consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Veterinary care was provided by the Central Animal Facility of the University of Pittsburgh. The mice were not subjected to any discomfort, distress, pain, or injury other than what has been described.

Inhalation model for delivery of MnSOD-PL to mice

MnSOD-PL complex containing 500 μg of pRK5-MnSOD plasmid DNA (50 μl) and 28 μl of lipofectin was prepared as previously described (10).

RESULTS

Implications of antioxidant therapy in cancer: historical perspectives

Since the discovery of the electron cascade, description of the cytochrome system within the mitochondria, and elucidation of the biochemical processes involved in neutralization of free one- electron oxygen reduction intermediates during oxidative metabolism, there have been increasing reports of the potential role of cellular damage by ROS in a wide variety of disease states. The techniques of measuring the redox state within individual cells and tissues were first carried out using electrode sensing devices (1). These techniques facilitated measurement of alterations in an acid–base balance within the heart, kidneys, and liver of experimental animals exposed to pathogenic organisms and to perfusion and reperfusion injuries. The development of hydroxyl radical, superoxide radical, and singlet oxygen, as well as hydrogen peroxide-sensing fluorochrome dyes (26, 30), facilitated measurement of specific alterations in the redox pathway within cells under the conditions of hyperbaric oxygen injury, ionizing irradiation damage, and the toxic effects of a variety of chemotherapeutic agents, including cancer chemotherapy drugs. The techniques of isolation of the mitochondria and study of the complex I–IV pathways during alterations of the mitochondrial respiratory chain added new biochemistry tools to the strategy of studying effects of ROS in basic cellular and tissue injury (16, 18, 19, 29, 30). A summary of much of this work has recently been published in several excellent reviews (1, 3, 6, 20, 26, 42). These studies over the last 30 years led to the undeniable conclusion that free radical cellular damage is involved in the mediation of many disease processes for cellular responses to therapeutic

agents for use in the management of disease states.

The role of redox reactions in basic cell biology

There are many examples of the importance of free radical generation and neutralization in normal cell physiology. Generation of free radicals in polymorphonuclear leukocytes (neutrophils) and macrophages mediates bacteriocidal killing (22, 30, 36, 38). Phagocytes, as part of the normal host defense, have been demonstrated to mediate pathogen killing effects in part by the generation of free radicals and biochemical processes involving cellular lysosomes (phagosomes) and extracellular killing mechanisms (30, 40). This work has been extended to include the natural killer cell, T-lymphocyte, and phagocytic cells in many tissues, including Kupffer of the liver and alveolar macrophages in the lung (21, 48, 53). Damage to normal tissues caused by ROS generation mechanisms that were initially activated as part of the bacterial/viral/fungal cytopathic response have been shown to mediate many of the chronic effects of disease states. A prominent example is pulmonary fibrosis in which chronic phagocyte responses to bleomycin during exposure or irradiation mediate the destructive lung pathophysiology of the disease (30, 48, 50, 52). A lack of these phagocytic cellular responses, conversely, is found in the following disease states: cyclic neutropenia, aplastic anemia, chronic granulomatous disease, catalase deficiency, and myeloperoxidase deficiency (6, 20). In the granulocytopenia resulting from total body irradiation or chemotherapeutic treatment of malignancy, tissue-damaging effects are also mediated by free radicals; in this case, produced by both the cancer therapy and the destructive organisms that are not being adequately combated due to the lack of functional phagocytic cells (21).

At the molecular biologic level, the pathways leading to induction of ROS and cellular responses in the antioxidant system, designed to neutralize ROS, have been shown to have important roles in many basic developmental and pathophysiologic states (6, 20, 35, 42, 43, 51).

Free radical mediation of apoptosis (programmed cell death) has been shown to be critical in normal development, tissue modification, and many disease states (20). The elucidation of the molecular pathways in DNA damage, transport of stress-activated protein (SAP) kinases, Bax, p53, and other proteins from the nucleus to mitochondria, and subsequent alteration in mitochondrial membrane permeability have been shown to be linked to the caspase cascade of activation of serial proteins mediating cell death (16). The studies of knockout mice in which the genes for one or more critical proteins in the caspase pathway are deleted or inactivated demonstrate the importance of structured elimination of cells within a specific population during growth and development. Animals overexpressing a mutation in antioxidant gene products such as copper/zinc superoxide dismutase (Cu/ZnSOD), as well as animals deficient in this gene product, have been shown to have abnormalities in central nervous system development leading to the condition of amyotrophic lateral sclerosis (reviewed in 10). Thus, the underexpression or overexpression of an antioxidant gene product may lead to abnormalities in growth and development. The underexpression of the critical protein involved in the process of apoptosis within specific tissues can also lead to an unstable and disabling development of a specific organ system.

Cancer prevention by antioxidant therapy

There is much evidence to suggest that continuous exposure to ROS induces genetic changes associated with accumulation of multiple mutations. Recent evidence documents the effects of carcinogens in cigarette smoke on induction of p53 mutations in bronchoepithelial cells in culture (8, 29), and recent evidence indicates that alcohol in conjunction with cigarette smoke may further increase the frequency of p53 mutations. In experimental animal model systems, continuous exposure to chemical oxidants has been shown to increase the frequency of induction of tumors in several systems; and in many of these model systems, administration of antioxidant compounds, including vitamin E, selenium, and antioxidant enzymes, has been associated with a decrease in the incidence of tumors.

In these model systems, it is not known whether continuous, low-level ROS exposure leads to direct genetic changes that are associated with malignant transformation, or whether indirect effects mediate these changes. Indirect effects include the following: lack of cell-cycle regulation (pushing cells in various organ systems out of quiescence and into cell cycle); simulation of production of matrix metalloproteases or other enzymes associated with invasion and metastasis; and initiation of angiogenesis or immunosuppression. Carcinogenesis associated with ROS exposure may be mediated by the indirect effects of inflammatory cytokines. It is known that free radical oxygen, peroxynitrite, and nitric oxide can stimulate immunocompetent as well as stromal cells of several organ systems, to produce inflammatory cytokines including TNF-α, IL-1, and TGF-β (6, 8, 42). These cytokines have been shown to induce the proliferation of cells in culture, and to mediate and amplify toxic effects of other inflammatory mediators.

With much experimental evidence for a direct ROS effect on inducing genetic change, including p53 mutations, and a significant body of evidence suggesting that the indirect effects of ROS can influence tumorigenesis (including elaboration of cytokines and mediation of angiogenesis and invasion), it has followed that therapeutic trials of antioxidant therapy have been designed based on these observations. Such trials were designed to attempt to prevent tumor formation or tumor recurrence. Several clinical trials of "chemoprevention" have included administration of antioxidant compounds to patients at high risk for second tumor formation or tumor recurrence. A very large trial carried out by the Veterans Administration Hospital System included administration of antioxidants to patients who had a head and neck cancer or lung cancer. Initial studies suggested a decreased incidence of formation of second primary tumors in the setting of continuous antioxidant therapy (1). The study was not confirmed in other clinical trials. Difficulties with the study were noted to include individual variation and processing of antioxidant compounds, varying levels of total extracellular and intracellular antioxidants between patients, simultaneous exposure of patients to varying levels of both oxidant and antioxidant compounds, and their diets and environments. Other studies suggest that administration of antioxidant compounds as daily dietary supplements may provide some protection from carcinogenesis with respect to tumors of the aerodigestive tract, bladder cancer, and breast cancer (1). No convincing randomized, prospective clinical trials have identified a particular category of antioxidants or dose scheduling that reproducibly affords protection.

A separate area of protection from oxidant injury, which has been the subject of discussion, has been the possibility of delivering significant levels of antioxidant agents around the time of an acute, intense exposure to oxidant compounds (52). In the setting of ionizing radiation therapy or chemotherapy for cancer, in which the therapeutic agents are known to induce free radical damage as part of the mechanism of tumor killing, normal tissue injury represents a major side effect of these therapeutic modalities (2, 4, 10–12, 14). Methods that provide selective protection of normal tissues without altering tumor sensitivity to the toxic effects of oxidants have been a subject of intense investigation.

Pharmaceutical or chemical antioxidant therapies have been tested in many model systems over the past 20 years. Acetylcysteine (Mucomyst), a ROS scavenger compound used in inhalation therapy of lung toxicity from infection or inflammation, was tested in both animal models and clinical trials for the prevention of radiation pneumonitis (37, 52). Some encouraging results were achieved in experimental animals, but difficulties in aerosol delivery of this compound posed a problem. Amifostine is a new antioxidant compound currently undergoing clinical trials in many areas of clinical radiotherapy, including protection of the salivary glands and oral mucosa during radiotherapy of head and neck cancer (5), and protection of the esophagus, lung, intestine, and bladder from the side effects of radiation therapy in targeted treatment of tumors in these anatomic locations. One possible explanation for the successful use of antioxidant compounds in protecting normal tissues without significant simultaneous protection of tumor may be explained by the differing baseline

levels of expression of antioxidants within tumors (34).

MnSOD deficiency has been observed in a wide variety of human cancers (34). The mechanism of decreased expression of MnSOD appears to be nonuniform with some tumor cells clearly demonstrating alterations in the promoter region of the MnSOD gene (34); in other cases, decreased production may be attributable to altered RNA processing or protein stability. Total antioxidant levels within tumors may also differ from that of surrounding tissues. In recent experiments, introduction of the transgene for MnSOD or overexpression of other antioxidant genes within tumor cell lines was demonstrated to either induce differentiation or provide synergy with other tumoricidal drugs (14, 28).

Systemic delivery of antioxidant compounds for cancer therapy has been associated with systemic side effects, including hypotension, and may not be possible in all patients, particularly those with compromised renal function (5, 37). Other methods of increase in antioxidant levels within normal tissues are needed to provide protection from irradiation chemotherapy.

Therapeutic intervention with the use of antioxidants

Therapy with γ-glutamyltranspeptidase, MnSOD (at the University of Alabama studies in hyperbaric oxygen trials), amiphostine, radical scavenger compounds, Mucomyst, vitamin E, selenium, and vitamin C has been tried (reviewed in 5).

Other gene therapy approaches to the use of antioxidants MnSOD and Cu/ZnSOD have been reported. A trial of hyperbaric oxygen damage in infants, gene therapy trials in cancer management, radiation damage, and photodynamic therapy have been carried out by Biotechnology General Corp., Tel Aviv, Israel (presented at the International SOD Conference, Institut Pasteur, Paris, France, May 18–20, 2000).

Protection of organs and organ systems from irradiation or chemotherapy damage by MnSOD-PL

Damage to organs by accumulation of ROS has been the subject of intense investigation in the setting of reperfusion injury following cardiac surgery, as well as reperfusion injury following organ transplantation. The use of antioxidant gene therapy in these settings has been a subject of investigation, and several encouraging reports suggest that overexpression of genes for MnSOD, Cu/ZnSOD, catalase, glutathione peroxidase, and other antioxidant proteins may provide some level of protection (10–17, 19).

The localized delivery of MnSOD-PL for protection of the lung from ionizing radiation (10, 12, 14, 15), esophagus (17, 47), and bladder has recently been reported. In the C57BL/6J mouse model, the delivery of 1,900–2,000 cGy to both lungs has been associated with a highly reproducible incidence of fatal organizing alveolitis/fibrosis at 100–120 days after radiation (10). Delivery of MnSOD-PL to mice 24 h prior to irradiation was associated with a detectable increase in biochemical activity of MnSOD in explanted lung 24 h after injection (10). Furthermore, human MnSOD transgene mRNA was detected by nested reverse transcription–polymerase chain reaction (RT-PCR) in explanted, purified alveolar type II cells of the distal area, as well as in tracheobronchial cells of the more proximal airway (10, 12, 14). Overexpression of human MnSOD in mouse lung was associated with a significant decrease in irradiation-induced acute and chronic injury (10).

Acute injury to the lung mediated by irradiation is known to be associated with up-regulation of mRNA levels for TNF-α, IL-1, and TGF-β (12). Mice pretreated with MnSOD-PL, but not with a control transgene for LacZ, demonstrated a significant reduction in irradiation-induced cytokine mRNA levels. Furthermore, the increase in endogenous murine MnSOD, seen as a reaction to irradiation injury at 2–4 days after irradiation, was significantly blunted by prior overexpression of human MnSOD in mouse lung (10).

Cellular damage after irradiation is known to follow a biphasic process, with initial DNA strand breaks that are usually repaired within 1 h after irradiation, and then a second pathway of mediation of cellular injury that includes a component of mitochondrial destabilization. In recent studies, SAP kinases Jnk1

and p38 were shown to be translocated from the nucleus to the mitochondria following irradiation of cells in culture (16). This translocation was not altered by overexpression of MnSOD in the cells. However, steps distal to mitochondrial concentration of these SAP kinases were clearly modulated by MnSOD overexpression. Cells unprotected by MnSOD transgenic overexpression prior to irradiation showed a significant increase in mitochondrial membrane permeability, leakage of cytochrome c into the cytoplasm, and resultant activation of caspase-3 and poly(adenosine diphosphate-ribose) polymerase (PARP) (16). This pathway was followed by significant DNA fragmentation in a pattern consistent with apoptosis. Stabilization of the mitochondrial membrane with MnSOD overexpression decreased these elements of the pathway.

As a control for these experiments, the Bcl-xl mitochondrial stabilization product was overexpressed in the same cell line, and radiation protection was also observed. In this latter situation, antioxidant levels prior to irradiation were not detectably increased in the 32D-Bcl-xl cell line compared with two subclones of the parent line 32D cl 3, which overexpressed human MnSOD (lines 1F2 and 2C6) (16). Following irradiation, transport of SAP kinases Jnk1 and p38 from nucleus to mitochondria was the same in all four cell lines, and in both the Bcl-2-overexpressing and MnSOD-overexpressing clones, mitochondrial membrane permeabilization induced by irradiation was reduced (16). However, there was a difference in the mechanism by which the mitochondria were protected, as shown by levels of mitochondrial complex I/IV ratios and GSH levels within the mitochondria.

Recently generated data demonstrated that the 32D-Bcl-xl cell line showed alterations in spin trap DMPO-detected ROS hydroxyl radical adducts induced by ionizing irradiation and in complex I/IV similar to that of the 32D cl 3 cell line, suggesting accumulation of ROS within the mitochondria; moreover, GSH levels were depleted following irradiation in 32D-Bcl-xl, as well as 32D cl 3 (Table 1; Figs. 1 and 2). In contrast, MnSOD-overexpressing lines 1F2 and 2C6 showed decreased irradiation-induced changes in spin trap-detected ROS and complex I/IV ratios, and significantly less GSH depletion (Table 1; Figs. 1 and 2). These results indicate an increased antioxidant level in the mitochondria within the MnSOD-overexpressing clones of 32D cl 3 cells (16). Whether this mechanism is also active in cells of the bronchoalveolar system, bronchoendothelium, and

Table 1. Changes in Mitochondrial Respiratory Complex Activity in 32D cl 3 or 2C6 Cells Following Irradiation, *S*-Nitrosoglutathione (SNOG) Induction of Nitric Oxide, or Both

		Activity of complex I, II, or III normalized to complex IV activity			
Cell line	*Treatment*	*I/IV*	*II/IV*	*III/IV*	*ATP/ADP*
32D cl 3	Control	8.1*	0.064	0.62	1.9
	SNOG	8.4	0.077	0.70	
	5,000 cGy	9.3	0.059	0.79	
	5,000 cGy + SNOG	6.3†	0.052	0.31†	1.3
2C6	Control	14.7*	0.160*	1.40*	1.7
	SNOG	14.8	0.170	1.20	
	5,000 cGy	15.6	0.150	1.30	
	5,000 cGy + SNOG	16.4	0.190	1.60	1.7

Cells from 32D cl 3 and subclone 2C6 expressing the human MnSOD transgene were irradiated to 1,000 cGy in the presence or absence of the nitric oxide donor SNOG. Mitochondria were isolated from the cells by washing three times in PBS, freezing at −80°C, thawing, homogenizing, and centrifuging at 8,000 *g*. The activities for complexes I–IV were determined as described in Materials and Methods, with the results expressed as ratios to the measured complex IV activity of the same sample. Cells from 32D cl 3, but not 2C6, had decreased activity for complexes I and III following irradiation in the presence of SNOG. Decreased complex I and III activity in the 32D cl 3 cells was accompanied by decreased adenosine triphosphate/adenosine diphosphate (ATP/ADP) levels.

†Significant difference from control at that time point.

*Significant difference for control for the 500 cGy + SNOG time point.

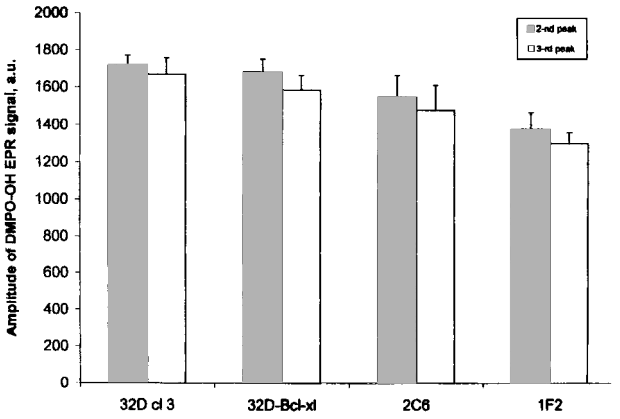

FIG. 1. Spin-trap analysis of ROS production following 1,000 cGy irradiation. Cells from 32D, 1F2, 2C6, or 32D-Bcl-xl were incubated for 15 min in 100 m*M* DMPO. The cells were washed in PBS, irradiated to 1,000 cGy, and immediately frozen in liquid nitrogen. EPR analysis demonstrated that 32D cl 3 and 32D- Bcl-xl generated a similar magnitude of ROS, whereas 1F2 and 2C6 had significantly decreased levels of ROS compared with 32D cl 3 ($p = 0.001$ and 0.021, respectively. n = 3.

influences bronchoalveolar macrophage response to ionizing irradiation in the setting of MnSOD-PL gene therapy is not yet known. Studies with bronchopulmonary macrophages from gene therapy-treated animals, obtained by bronchopulmonary lavage, and explant of both proximal and distal airway cells from MnSOD-PL-treated, irradiated mice will be required to answer these questions.

Other antioxidant gene therapy constructs used in the same experiments have not proven as useful as MnSOD transgene therapy. In a separate study in Nu/Nu mice that received intratracheal injection of adenovirus Cu/ZnSOD or adenovirus MnSOD, only those mice receiving MnSOD showed significant protection from irradiation-induced organizing alveolitis (13). Studies using this strain were chosen to minimize the inflammatory effects of E1A inactivated adenovirus, which is known to produce inflammatory responses in the lung (13). In other studies, use of metallothionein transgene therapy by PL administration prior to irradiation did not provide irradiation protection (12). Overexpression of other antioxidant gene products, including catalase, glutathione peroxidase, and other enzymes associated with an increasing concentration of intracellular antioxidant proteins (such as γ-glutamyltranspeptidase), have not shown the encouraging results seen with MnSOD-PL or MnSOD adenoviral gene therapy (10). Success with MnSOD-mediated protection from ionizing irradiation, and a comparable effect with mitochondrial membrane stabilizing Bcl-xl, have suggested that irradiation-induced alterations in mitochondrial membrane permeability may mediate irradiation damage in several systems, thus explaining the success of MnSOD transgene therapy. Experiments are currently in progress with mitochondrial nonlocalizing forms of MnSOD, and mitochondrial localizing forms of Cu/ZnSOD to test this hypothesis.

The potential for the use of multiple antioxidant gene products in gene therapy for prevention of irradiation and chemotherapy damage has been suggested. The use of MnSOD-PL in conjunction with catalase PL, compared with MnSOD-PL alone, is an experiment that should provide some interesting results. The use of nitric oxide synthase inhibitors, in conjunction with MnSOD-PL, is another experiment to be carried out.

Considerations in transient overexpression of transgenes for antioxidant enzymes

Model systems of transgenic mice in which overexpression of MnSOD or other antioxidant molecules have led to developmental abnormalities suggest concern for long-term overexpression of an antioxidant transgene. In this regard, clearance of mRNA for human MnSOD

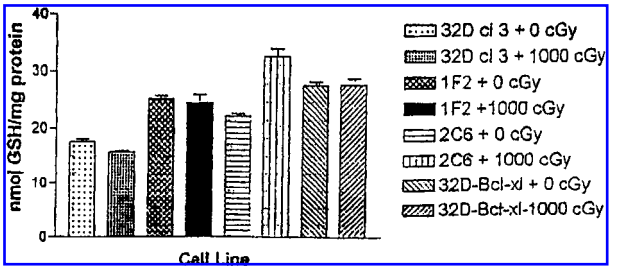

FIG. 2. GSH levels in 32D cl 3, 32D-Bcl-xl, 1F2, or 2C6 1 h after 1,000 cGy. Cells (2×10^6) from each line were irradiated to either 0 or 1,000 cGy and frozen in liquid nitrogen 1 h later. There was a significant decrease in GSH in 32D cl 3 cells 1 h after irradiation with no decrease in the other lines. Cell lines 32D-Bcl-xl, 1F2, and 2C6 had significantly higher baseline levels of GSH compared with 32D cl 3. $n = 3$.

from the lung or esophagus of mice intraesophageally or intratracheally injected with MnSOD-PL within 72–96 h after administration provides a level of confidence that transgene expression will not be persistent beyond the time of immediate therapy (6). Whereas long-term overexpression of intrapulmonary or intracavitary transgene expression is a sought-after goal in many other gene therapy protocols, in which long-term overexpression of a deficient or aberrant gene product is to be corrected, and where transient expression of a transgene product is felt to be an incomplete result, our system of MnSOD-PL irradiation protection of specific organs and organ systems by transient overexpression of MnSOD-PL is felt to be a highly successful outcome. Serial administration of PL three times weekly to mice receiving fractionated lung or esophageal irradiation (47) provided for continuous expression of antioxidant gene product in the normal cells of each organ system, allowing protection from fractionated irradiation. These studies are in progress.

Effects of radioprotective intratracheal MnSOD-PL administration on orthotopic lung carcinoma

A major concern for the use of radioprotective gene therapy in cancer patients would be the potential bystander protection of tumor by agents designed to protect the normal organ. In experiments recently carried out, C57BL/6J mice with orthotopic Lewis lung carcinomas at the carina received intratracheal MnSOD-PL administration (14). The results showed detectable expression of human MnSOD mRNA by nested RT-PCR within the upper and lower airways of normal lung within these animals, but no detectable transgene messenger in excised orthotopic tumor. Furthermore, 1,900 cGy single fraction lung irradiation of orthotopic tumor-bearing mice that received intratracheal MnSOD-PL showed a more effective tumor kill and longer survival than in other groups of mice, including those receiving no PL gene therapy, LacZ-PL gene therapy, or no irradiation. Death from tumor regrowth was clearly slowed in the animals that had normal tissue protection by MnSOD-PL administration (14). The mechanism of apparent tumor radiosensitization by normal tissue protection mediated by intratracheal MnSOD-PL administration is not known, but several recent experiments suggest that a decrease in irradiation-induced cytokines within the normal lung by MnSOD-PL gene therapy may have decreased tumor repair capacity.

In vitro culture in a colony-forming assay of Lewis lung carcinoma cells, used in the orthotopic tumors experiments, in the presence of TNF-α, IL-1, and TGF-β, demonstrated improved irradiation survival of tumor cells (Fig. 3). In new data, mice implanted with Alzet pumps dispensing TNF-α, IL-1, and TGF-β following MnSOD-PL gene therapy in the setting of orthotopic tumors and 1,900 cGy irradiation demonstrated improved tumor repair and more rapid death than did mice without Alzet

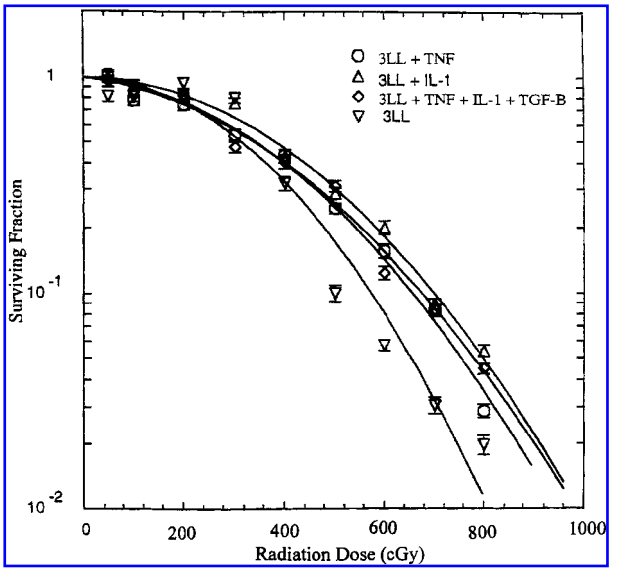

FIG. 3. Effect of cytokine levels on 3LL-LacZ tumor cell line irradiation survival *in vitro* and *in vivo*. The effect of increased cytokine levels on survival of 3LL-LacZ cells was investigated both *in vitro* and *in vivo*. 3LL- LacZ cells were grown in the presence of 20 ng of TNF-α or 10 ng of IL-1α, or in the presence of 20 ng of TNF-α, 5 ng of IL-1α, and 2 ng of TGF-β for 24 h. Untreated cells or cells grown in the presence of cytokines were irradiated to doses ranging from 0 to 8 Gy, plated in four-well tissue culture plates, and stained with crystal violet, and $\geq$50 cell colonies were counted. The data were analyzed by linear quadratic and single-hit, multitarget models. The untreated 3LL cells were more sensitive to irradiation than cells grown in TNF-α, IL-1α, and TGF-β ($p < 0.01$). $n = 3$.

pumps that restored plasma cytokine levels (Fig. 4).

DISCUSSION

Summary and conclusions

Redox signal transduction events within cells, tissues, and organ systems comprise a complex set of interactions involved in normal function, as well as response to oxidant stresses, including cancer chemotherapy and radiotherapy.

Redox signal transduction pathways are also involved in many aspects of environmental carcinogenesis, particularly those associated with cigarette smoke, exposure to toxic chemicals, and low-level ionizing irradiation. Of potential importance is the observed disparity between the levels of antioxidant molecules within cancer cells, the depletion of mitochondrial protective MnSOD levels within many tumors, and the paradoxical resistance of many cancer cell lines to therapies that mediate toxic effects through induction of ROS.

Experimental strategies of preventing ROS-mediated carcinogenesis by continuous administration of antioxidants must take into account the shifting levels of antioxidant molecules within the cellular targets for transformation, and that increasing levels of one antioxidant moiety may result in down-regulation of others to provide for a balance of total antioxidant reserves that achieves a baseline similar to that prior to initiation of protocols of chemoprevention. A relatively new area of application of antioxidant therapies is that of MnSOD-PL radioprotective gene therapy, which has been shown to decrease ionizing irradiation-induced early and late side effects in the mouse lung and esophagus. Because of the transient nature of overexpression of MnSOD in these systems and the target organ-specific expression of transgene, strategies using this therapeutic modality to prevent ionizing irradiation damage may be useful. Irradiation damage to normal tissues within the radiation transit volume (principally, the esophagus and lung) during conformal multifield approaches to treatment of thoracic tumors may be decreased by radioprotective gene therapy and have potential for a clinical trial. Because of the ease of administration of swallowed PL compared with aerosolized freeze-dried PL, a clinical trial of esophageal irradiation protection in patients receiving carboplatin, Taxol, and radiotherapy for non-small cell lung carcinoma, a condition in which severe esophagitis is often encountered, appears to be a first sensible translation of this technology in the management of clinical cancer patients.

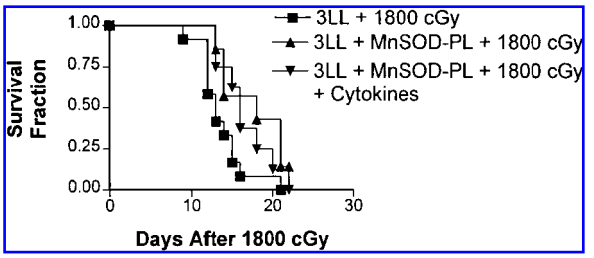

FIG. 4. Effect of Alzet pump administration of TNF-α, IL-1, and TGF-β on 3LL-LacZ orthotopic tumor-bearing C57BL/6J mice treated with MnSOD-PL 1 day prior to 1,800 cGy whole lung irradiation. C57BL/6J mice were intratracheally injected with 1×10^5 3LL-LacZ (3LL) cells. One group was intratracheally injected with MnSOD-PL (500 μg of plasmid/DNA) 24 h later, and all the mice were irradiated to 1,800 cGy at 48 h. Following irradiation, half of the mice injected with MnSOD-PL were implanted subcutaneously with Alzet osmotic pumps delivering 4 μg of TNF-α, 0.4 μg of TGF-β, and 2 μg of IL-1α over 7 days. Mice injected with MnSOD-PL had significantly increased survival ($p = 0.036$) compared with control irradiated mice. There was no increase in survival of MnSOD-PL-treated mice implanted with the Alzet pumps compared with irradiated tumor-bearing mice. Our prior data show that orthotopic 3LL-LacZ tumors are sensitized to irradiation killing by intratracheal injection of MnSOD-PL 1 day prior to 1,800 cGy whole lung irradiation [14]. The MnSOD-PL treatment of normal lung in these mice decreased irradiation induction of TNF-α, IL-1, and TGF-β (12). In new preliminary experiments, mice treated with MnSOD-PL, but also with Alzet pump injections that restored levels of cytokines, showed lack of radiosensitization, tumor recovery, and more rapid death from tumor progression (this figure). The results show that both *in vitro* (Fig. 3) and *in vivo* (this figure) the radiosensitization of lung tumor cells in vitro or in tumors by intratracheal injection of MnSOD-PL is lost by adding back cytokines that are decreased in normal lung by the MnSOD-PL treatment.

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ABBREVIATIONS

Cu/ZnSOD, copper/zinc superoxide dismutase; EPR, electron paramagnetic (spin) resonance; GSH, glutathione; IL-1, interleukin-1; LacZ, bacterial β-galactosidase gene; 3LL, Lewis lung carcinoma; MnSOD, manganese superoxide dismutase; PBS, phosphate-buffered saline; PL, plasmid/liposome; ROS, radical oxygen species; RT-PCR, reverse transcriptase–polymerase chain reaction; SAP, stress-activated protein; SNOG, *S*-nitrosoglutathione; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

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