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Review

Antioxidant Gene Therapeutic Approaches to Normal Tissue Radioprotection and Tumor Radiosensitization

JOEL S. GREENBERGER and MICHAEL W. EPPERLY

Department of Radiation Oncology, University of Pittsburgh Cancer Institute, 200 Lothrop Street, Pittsburgh, PA 15213, U.S.A.

Abstract. *Administration of manganese superoxide dismutase-plasmid liposomes (MnSOD-PL) has been demonstrated to provide local radiation protection to the lung, esophagus, oral cavity, urinary bladder and intestine. Radiation protection has been shown to be mediated in part by MnSOD stabilization of the antioxidant pool including glutathione and total thiols within cells and in normal tissues. In experiments to determine whether organ-specific radioprotection would also protect orthotopic tumors, mice with Lewis lung carcinoma orthotopically placed at the carina or in other experiments with mice with cheek pouch placed SCCVII orthotopic squamous cell tumors demonstrated paradoxical and beneficial tumor radiosensitization following intratracheal or intraoral MnSOD-PL, respectively. The mechanism of MnSOD-PL tumor radiosensitization may involve a difference in redox balance between tumors and normal tissues. Differences in handling radiation-induced oxidative stress between tumors and normal tissues can provide a fundamental basis to design new cancer therapeutic agents which can exploit differences between normal tissue and tumor mechanisms of handling the oxidative stress of ionizing irradiation damage.*

Squamous tumors are commonly hypoxic and show decreased mitochondrial cytochrome c oxidase activity (1-2). Ionizing irradiation induces superoxide and two superoxide molecules are dismutated by superoxide dismutase to produce H_2O_2. H_2O_2 neutralization following irradiation is carried out by either GSH/peroxidase oxidizing to oxidized glutathione or glutathione disulfide (GSSG) and water, or alternatively, hemoproteins are oxidized and produce cellular damage. These two common ways that H_2O_2 is metabolized in cells determine a non-toxic or toxic outcome. Furthermore, two groups of investigators have hypothesized that H_2O_2 generation in tumors overexpressing an introduced MnSOD is of potential therapeutic benefit (3-11).

Several lines of evidence suggest that a transient increase in the levels of antioxidant gene product within normal tissues can provide protection from ionizing irradiation or CRT-induced cellular damage. Experiments done in nontumor-bearing mice have demonstrated that ionizing irradiation induces a rapid increase in cytokine levels for TGF-β1-β2, IL-1 and TNF-α, and that peak cytokine elevation decreases rapidly within 24-48 hours. A second, slow increase in levels of TNF-α within the irradiated lung occurs around day 50 and continues until day 100, when a second peak of elevation of TGF-β1 is detected. Finally, a second peak of elevation of TGF-β1-β2 is coincident with the appearance of organizing alveolitis/fibrosis and pulmonary death (12). In the esophagus model, cytokine elevation is also detected rapidly after irradiation, but different patterns of cytokines are detected compared to those in the lung associated with different cell types within the tissues that differentiate these organs (13). A similar pattern of an acute peak cytokine production associated with inflammation, and a second latent period peak is associated with fibrosis in both organs. The administration of MnSOD-PL in single dose prior to single fraction irradiation or in several doses during fractionated irradiation has been shown to decrease the magnitude and duration of both the acute peak elevation in cytokine production and the late peak elevation (12, 13). MnSOD-PL administration to C57BL/6J or C3H/HeNHsd mice decreases irradiation-induced organizing alveolitis and esophageal stricture, respectively (12, 14). *In vitro* experiments utilizing MnSOD overexpressing hematopoietic progenitor cell lines, embryo fibroblast lines, and freshly explanted esophagus and lung have demonstrated decrease in irradiation-induced apoptosis, cytokine induction and cell killing (13-16).

Correspondence to: Joel S. Greenberger, MD, Professor and Chairman, Department of Radiation Oncology, University of Pittsburgh Medical Center, 200 Lothrop Street, Pittsburgh, PA 15213, U.S.A.

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MnSOD-PL Radiation Protective Gene Therapy

The mechanism of MnSOD-PL radiation protection is in part at the level of the mitochondria (16). Substitution of cytoplasm localized Cu/ZnSOD in either plasmid/liposomes or adenovirus, introduced comparable biochemical levels of enzyme, but did not induce radiation protection (1, 17, 18). A specific mitochondrial leader sequence on the MnSOD protein product has been associated with its mitochondrial concentration and radiation protective capacity (19). Overexpression of other mitochondrial localized transgene products Bcl-xl or Bcl-2 in the same cell lines *in vitro* also confers radiation protection (16, 20). The mechanism of MnSOD action in the mitochondria is not yet known, but recent experiments have demonstrated in MnSOD transgene overexpressing cells in culture that initial irradiation-induced DNA strand breaks (repaired within 15 – 20 mins. after irradiation) and translocation of stress-activated protein kinases (SAP) p38 and Jnk1, as well as Bax from nucleus to mitochondria, are unchanged by overexpression of the MnSOD transgene product (16). In striking contrast, cells overexpressing MnSOD transgene did not demonstrate the significant distal steps associated with apoptosis that follow p38, Jnk1, and Bax translocation to the mitochondria; namely, mitochondrial membrane depolarization, cytochrome-C leakage into the cytoplasm, and activation of caspase-3, PARP and DNA fragmentation (16). The mitochondrial localization of the MnSOD transgene has been demonstrated both *in vitro* and *in vivo* in the esophagus to be associated with decreased irradiation-induced apoptosis (14, 16, 19)

MnSOD-PL Tumor Radiosensitization

A potential antitumor effect of MnSOD-PL gene therapy has been reported (18, 21, 22). Cerutti *et al.* (6-11) and Oberley *et al.* (23-30) have shown that toxicity of H_2O_2 generated by dismutation of SOD in tumors by MnSOD can be therapeutically advantageous. Treatment of squamous cell carcinomas of head and neck or lung remains a therapeutic challenge (31-33). SCC cell lines of the head and neck region were established from a large number of patients and each tested for intrinsic levels of production of antioxidant proteins. The majority of tumor cell lines demonstrated stably decreased levels of MnSOD production, the mechanism of which included transcriptional shut-off, mutation in the promoter region of the MnSOD gene, and other redox changes within tumor cells both *in vitro* and *in vivo* (34-35). Introduction of the MnSOD transgene to these SCC head and neck tumor cell lines *in vitro* has demonstrated sensitization of the tumors to ionizing irradiation and BCNU chemotherapy (18). It has been hypothesized that the redox balance within tumor cells has adapted to a decreased MnSOD bioavailability, rendering cells sensitive to H_2O_2 toxicity (5). Hydrogen peroxide (H_2O_2), the natural catabolic product of MnSOD biochemical action when made abundant by introduction by the MnSOD transgene, renders the cells susceptible to peroxide-induced death. The available evidence suggests that normal tissue protection by MnSOD-PL gene therapy should not adversely affect irradiation killing of SCC tumors *in vivo* and may actually be an antitumor agent.

Mechanism of MnSOD-PL Action

Clinical doses of irradiation activate mitogen-activated protein (MAP) kinase and stress-activated protein (SAP) kinase pathways (36-37). MAP kinase increases the irradiation induction of strand breaks and DNA damage. The MAP kinase pathway is also cytoprotective such that its inhibition may be radiosensitizing (38). EGF binding to EGFR and tyrosine phosphorylation leads to MAPK activation. Blocking this pathway could also block irradiation induction of this cytoprotective pathway and thereby increase tumor kill. What role the increased H_2O_2 production generated by MnSOD expression in tumor cells has in the process is not known. Questions include the following: 1) does MnSOD-stimulated H_2O_2 production enhance the EGFR-TKI effect, 2) does MnSOD-mediated H_2O_2 production enhance or inhibit MAPK induction by irradiation, 3) will MnSOD overexpression reverse or enhance the synergistic irradiation and anti-EGFR interaction, and 4) does MnSOD disrupt the potentially beneficial effect of anti-EGFR, preventing irradiation-induction of the survival/repopulation response by tumors after irradiation?

MnSOD overexpressing cells have been shown to downregulate cytoprotective gene products VEGF1, TNF-α, and IL-β, while GADD53 (involved in repair of DNA double strand breaks) is increased 3.3 fold (30). Increasing MnSOD levels in tumor cells can modulate other downstream effecter genes. Furthermore, free radicals have been shown to mediate upregulation of gene expression by TNF-α (24). The available evidence suggests that overexpression of MnSOD in tumors could facilitate altered gene regulation through increased H_2O_2 production, which could further enhance the antitumor effects of combining irradiation with anti-EGFR (39-42). Since MnSOD overexpression decreases TNF-α production (12, 19), this result may also represent an antitumor effect, which could be further enhanced by anti-EGFR.

There is published evidence to suggest that H_2O_2 stimulates the EGF signal transduction pathway in neuroblastoma cell lines (39) and mucin producing epithelial cells (41-42). Thus, MnSOD-mediated generation of H_2O_2 in tumors may beneficially modulate the EGF signaling pathway to increase its susceptibility to an anti-EGFR agent.

Table I. *Antioxidant transgenes as possible cancer therapeutic agents (radioprotection of normal tissue and/or tumor radiosensitization).*

Agent	Model system	Reference
MnSOD-PL	Mouse lung	12, 22
MnSOD-PL	Mouse esophagus	13
MnSOD-PL	Mouse oral cavity	43
MnSOD-PL	Rat bladder	72
MnSOD-PL	Mouse intestine	73

MnSOD-PL Gene Therapy Protocols

Gene therapy strategies for radiation protection (unique to our laboratory) (12, 13) have recently been applied to the oral cavity and oropharynx (43) and other organ-specific radioprotection (12, 15, 17, 44-46, 47). (Table I) Intraesophageal administration of MnSOD-PL has been demonstrated to protect the mouse esophagus from CRT-induced esophagitis from both single fraction and fractionated irradiation-induced esophageal damage (21, 47, 48). Multiple administrations of MnSOD-PL were effective in maintaining elevated levels of messenger RNA and transgene product in the mouse model (21, 47, 48). Cervical esophagus specimens removed from mice at serial time-points after irradiation demonstrated a protective effect of this gene therapy approach (21, 47, 48). Administration of the MnSOD transgene in plasmid/liposomes or adenovirus reduced both the acute and chronic toxicity of total lung irradiation in the mouse model (12, 17, 44, 49, 50). In contrast, orthotopic Lewis lung carcinoma tumors in the mediastinum were not protected by intratracheal injection of MnSOD-PL (21). This result was attributed to the ability of PL to penetrate only the local organ at the site of contact. It was also discovered that mice receiving intraoral or intratracheal MnSOD-PL gene therapy demonstrated greater radiation tumor killing and longer survival (21, 22, 43). In the oral cavity and oropharynx, radiotherapy treatment complications are known to be of a more complex nature involving both salivary gland and mucosal targets for organ specific radioprotection.

The Clinical Problem and Strategy

A major problem with combined modality therapy, principally CRT of head and neck cancer, is mucositis. Patients receiving 180 or 200 cGy per day of fractionated irradiation alone typically develop significant oral cavity and oropharyngeal mucositis by the 3rd or 4th week of a 7-week RT treatment course (51-54). In the setting of combination chemotherapy with weekly platinum-based or taxane-based chemotherapeutic agents, mucositis is usually detectable by the end of the second week. Over 50% of patients require a treatment break to allow healing of mucositis and nearly all patients require oral cavity and oropharyngeal administration of antifungal agents. Other complications include decreased saliva production and late osteoradionecrosis in high radiation dose volumes. Improved RT delivery techniques, including conformal and Intensity Modulated Radiotherapy (IMRT) (53, 55, 56, 71), and the use of high-dose-rate (HDR) brachytherapy (51-52), have facilitated improvements in dose distribution and dose escalation. The availability of new chemotherapeutic drugs, including cisplatin, carboplatin, docetaxel, pacitaxel, gemcitibine, etoposide (51-52), and others, have facilitated sophisticated programs of combined modality chemotherapy and radiotherapy to both radiosensitize tumors in the head and neck region and to prevent or decrease distant metastasis. Improved techniques of hyperalimentation and supportive care have provided increased resources in managing the toxicity of high intensity CRT of SCC of the head and neck region (51-52). Combined modality programs have also included new techniques of neo-adjuvant CRT prior to surgical resection (52-53). Despite advances in the technical delivery of RT, radiation therapy treatment planning (RTP), and the use of new radiosensitizing drugs, local control of T_3N_1-T_3N_2-T_3N_3 carcinoma of the head and neck remains unacceptably low and recurrence of cancer both locally and distantly after CRT protocols remains at a suboptimal level (51-53). A major problem upon which most cancer treatment centers are focusing remains the toxicity of CRT (54-57). Toxicity both limits the ability to deliver full doses of CRT and prevents dose escalation.

Numerous approaches have been taken to attempt to decrease oral cavity and oropharyngeal toxicity of CRT. These have included the institution of hyperfractionation (52) or hypofractionation (53) RT regimens, split-course RT techniques, improvements in treatment planning with reduced field size modification, and the recent usage of radioprotective pharmacological compounds designed to protect normal tissue. Amifostine (s-2 (aminopropylamino)-ethyl phosphorothioic acid, WR2721, Ethyol) is a well-characterized radio- and chemoprotective agent (56, 58) and has been shown to decrease RT-induced sialadenitis, although a decrease in mucositis and oropharyngeal toxicity has been less consistently observed (58). Other pharmacological approaches toward decreasing oropharyngitis have included administration of pilocarpine (57, 59, 60), antibiotics to reduce oral cavity flora (55, 61), pentoxyfilline (59, 62, 63) adrenergic compounds, glutamine (64) cytokines including GM-CSF, G-CSF, M-CSF (56, 65, 70), use of a complex glucan (betafectin), kGF-palifermin (66-67), or erythropoietin (52). Preclinical and clinical trials using these materials have met with incomplete success at reducing treatment-related toxicity and morbidity from irradiation of tumors of the oral cavity and Oropharynx (68-69). Our studies with MnSOD-PL suggest it may be an effective radioprotector.

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