

Radioprotective antioxidant gene therapy: Potential mechanisms of action

Review Article

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Abbreviations: ataxia telangiectasia gene, (ATM); plasmid/liposome (PL); manganese superoxide dismutase, (MnSOD); Photodynamic, (PDT); adenosine tri-phosphate, (ATP); epitope-tagged hemagglutinin (HA)-MnSOD; total body irradiation, (TBI)

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Summary

The plasmid/liposome (PL) delivery of the manganese superoxide dismutase (MnSOD) transgene to lung, esophagus, oral cavity, bladder, or delivery of the same transgene by viral vectors including Herpes virus, adenovirus, and retrovirus to cells in culture or to intestine have been demonstrated to confer cellular and tissue resistance to ionizing irradiation damage including apoptosis. In contrast, transgene mediated expression of other antioxidant transgenes including Cu/ZnSOD, metallothionein, glutathione peroxidase, was ineffective. Delivery of protein by liposomes containing MnSOD, lazaroid, or vitamin E was also ineffective for normal tissue radioprotection. Radioprotective MnSOD transgene protection has been shown to be specific to localization of protein to the mitochondrial membrane in that deletion of the mitochondrial localization signal from the transgene was associated with cytoplasmic overexpression and was not radioprotective. Addition of the mitochondrial localization to cytoplasmic Cu/ZnSOD transgene, localization of transgene product to the mitochondria and conferred radioprotection. The biochemical mechanism of MnSOD radioprotection has been shown to be associated with stabilization and an increase in intracellular thiol and antioxidant pool size at the level of the mitochondrial membrane suggesting that overexpression of MnSOD stabilizes cellular antioxidant reserves, and that its antioxidant reserve capacity is associated with cellular and tissue radioprotection. In three orthotopic tumor model systems (Lewis lung carcinoma at the carina, SCC-VII murine floor of the mouth tumors in C57BL/6J mice, and mediastinal sarcomas in C3H/HeJ mice), tumor radioprotection was not detected under conditions of MnSOD-PL protection of the associated normal tissue: lung, oral cavity/oropharynx, and esophagus respectively. Reduced ability of many tumors to handle increased hydrogen peroxide bioavailability, which is the catabolite of MnSOD dismutation of superoxide, has been reported to synergize with ionizing irradiation to enhance tumor killing. In this regard, radiosensitization of oral cavity and lung tumor cells in vitro has been demonstrated with MnSOD-PL transfection. The data suggest that differences between normal tissue and tumor management of oxidative stress may be exploited by MnSOD-PL gene therapy to facilitate simultaneous normal tissue radioprotection and tumor radiosensitization.

I. Introduction

Clinical radiotherapy and environmental radiation protection are different disciplines but share several common principles. Ionizing irradiation killing of cells, tissues, and organs is dependent upon total dose and volume treated, dose rate (including fraction size in protocols of multiple exposures), and overall time of exposure (Hall, 1999). There are well known phylogenetic, and ontogenic determinants of ionizing irradiation tissue

and total body damage (Hall, 1999). Genetic determinants of ionizing irradiation sensitivity have been demonstrated in mouse models (Rubin and Casarett, 1968; Franko and Sharplin 1994; Johnston et al, 1995; Dileto and Travis, 1996; Hall, 1999). These variables have been shown to relate to normal tissue damage and relative tumor killing capacity (therapeutic ratio) in clinical radiotherapy (Hall, 1999). These variables have also been shown to relate to the toxicity of environmental irradiation exposure either by external beam (high energy photons, gamma rays, particle

beam exposure) and internally ingested radionuclide exposure.

II. Mechanisms of radiation cell killing

Classical radiation biology defines principles of irradiation killing based on DNA strand breaks specifically targeted to the nucleus (Hall, 1999). Studies in bacterial and yeast systems of ionizing irradiation damage and repair, defined multiple classes of DNA repair genes (Bakkenist and Kastan 2003; Shiloh 2003), which were shown to have analogs in mammalian cells including human cells (Bakkenist and Kastan 2003; Shiloh 2003). More recently, the molecular mechanism of ionizing irradiation cell killing has been shown to follow pathways common to multiple other forms of oxidative stress including that induced by chemotherapeutic alkaline agents, ultraviolet irradiation, hyperthermia, and agents which induce oxidative stress (Oberley and Buettner 1979; Oberley, 1982; Oberley and Oberley, 1986). Furthermore, immunologic mechanisms of cell killing including immunocompetent cell killing of microorganisms and tumor cells, and humoral immune cytotoxicity mediated by cytokines and lymphokines have been shown to mediate cell killing by molecular pathways common to ionizing irradiation-induced apoptosis (Epperly et al, 2001a, 2003a). Ionizing irradiation induces radiochemical changes within cells within 10-13 seconds including the generation of singlet oxygen, superoxide, hydroxyl radicals, and other free radical moieties attributable to chemical interactions between high energy photons, electrons, and the molecular targets of oxygen and water within cells (Spitz et al, 1990; McCord, 2000). Radiochemistry techniques, which are sensitive to changes that are detected at the femtosecond level of sensitivity (Hall, 1999), are followed by detectable changes, which are measured at the sensitivity of current molecular biology techniques (Shiloh 2003; Bakkenist and Kastan 2003). Phosphorylation of the ataxia telangiectasia gene (ATM) has been shown to occur "immediately" after ionizing irradiation exposure (as rapidly as cells can be transferred from the irradiation device) (Bakkenist and Kastan 2003). In the time scale of minutes transport of molecules from the nucleus to cytoplasm and mitochondria has been demonstrated with respect to movement of p53, BAX, and stress activated protein kinases (SAP kinases) including Jnk1, Mp38 (Epperly et al, 2002c). Radiochemical changes in lipid membranes are detectable within minutes, the measurement of which is restricted in many cases to the speed with which an assay can be carried out (Nimura et al, 1988; Lynch et al, 1994; Morrow and Roberts LJ 1994; Halpern et al, 1995; Elsayed et al, 1996; Karoui et al, 1996; Ritov et al, 1996; Kagan et al, 1998; Day et al, 1999; Ashcroft et al, 2000).

In contrast, measurable changes associated with cellular apoptotic and non-apoptotic death appear to be delayed for 15-45 minutes after irradiation of cells in culture and tissues in situ, perhaps again attributable to the sensitivity of measuring techniques (Epperly et al, 1999a, 2002c). The available evidence indicates that ionizing

irradiation induces cellular changes at many levels in addition to the known induction of DNA single and double strand breaks (Bakkenist and Kastan 2003; Shiloh 2003). Furthermore, there is evidence that these initial strand breaks are repaired very rapidly (within minutes) after irradiation, and it is the steps distal to DNA strand breaks, including communication of the death inducing signals from nucleus to mitochondria which are associated with ionizing irradiation killing (Epperly et al, 1999a, 2002c). The role of mitochondria in ionizing irradiation cellular damage, as well as damage induced by multiple other classes of agents has become prominent in recent years (Epperly et al, 2002c, 2003c). Mitochondria play a central role in energy production within mammalian cells producing ATP by energy producing pathways, and reducing free radicals produced during oxidative metabolism, to water, thereby neutralizing and preventing further cellular damage (Epperly et al, 2002c, 2003c). Mitochondrial "aging" or inefficiency in neutralization of free radicals has been associated with cellular senescence, defective self-renewal of tissues within aging organ systems, and such recent knowledge has prompted a careful investigation of the potential role of antioxidants delaying the effects of cellular aging as well as oxidative stress in general (McCord, 1992).

With respect to ionizing irradiation damage, mitochondrial membrane permeability has been shown to play a potentially critical role in irradiation killing (Epperly et al, 2002c). Stabilization of the mitochondrial membrane by Bcl2 family members including Bcl-xl has been shown to confer irradiation resistance (Epperly et al, 2002c). Recent evidence has suggested that mitochondrial membrane permeability and leakage of cytochrome C into the cytoplasm mediates a critical step in the activation of caspase 3 and distal caspase death effector cascade proteins leading to poly-ADP-ribosyl polymerase activation and DNA fragmentation (Epperly et al, 2002c). These latter steps have been shown to be common to induction of apoptosis by TNF binding to its receptor, as well as other cytokine receptor mediated apoptotic mechanisms (Epperly et al, 2003a). There is increasing evidence that other events within the mitochondria, specifically the outer mitochondrial membrane may be critically relevant to ionizing irradiation induced as well as other forms of apoptosis (Epperly et al, 2002c, 2003c). Specifically, the stability of antioxidant reserves within the mitochondria including levels of thiols, glutamine, and antioxidant enzymes may be very relevant to cellular radioresistance or radiosensitivity (Greenberger et al, 2001). Agents or conditions, which elevate mitochondrial antioxidant reserves, have been shown to confer resistance to apoptosis where as depletion of mitochondrial antioxidant pools confers sensitivity to apoptosis. Both mitochondrial membrane stability, and antioxidant reserves appear important to this phenomenon in that stabilization of the mitochondrial membrane in the state of depleted antioxidant reserves confers some irradiation protection whereas lack of mitochondrial membrane protection in the presence of elevated antioxidant reserves also confers some radiation protection (Pearce et al, 2001; Epperly et al, 2002c, 2003c).

Studies of cells in culture demonstrated the importance of cell contact mechanisms in the transmission of irradiation damage between ionizing irradiation exposed, and non-exposed cells (Little and Wakeford, 2001; Sawant et al, 2002; Xue et al, 2002) using microbeam radiation techniques, irradiation of one cell and a monolayer of C3H10T1/2 cells was demonstrated to induce the overexpression of p21 (Azzam et al, 2001) (unknown irradiation response gene product) not only in the irradiated cell but in cells distant from the beam margin. Co-cultivation of non-irradiated hematopoietic cells with recently irradiated bone marrow stromal cells have been demonstrated to induce cytotoxic free radicals in non-irradiated cells (Gorbunov et al, 2000).

III. Tissue and organ effects of ionizing irradiation

Bystander killing of non-irradiated cells in culture by contact or close proximity to irradiated cells is a clear phenomenon, however, there is yet another more complicated phenomenon of bystander tissue and organ injury which cannot be explained by cell contact mechanisms alone (Azzam et al, 2001; Little and Wakeford, 2001; Sawant et al, 2002; Xue et al, 2002). Irradiation damaged, dying, and dead cells are known to release cytotoxic cytokines as well as cytotoxic breakdown products of nuclear and cellular fragmentation including free nucleic acids, activated caspase, and proteinases. These produce apoptotic and non-apoptotic killing of cells not only in close physical proximity, but in those available by spread through tissues and organs via the lymphatic and blood vascular systems, and interstitial tissue fluid circulation (Dileto and Travis, 1996; Hall, 1999).

Finally, the total body irradiation (TBI) effect (sensitivity of organism to lethality by lower doses of irradiation than that similarly toxic for a hemibody, or isolated organ irradiation) has remained a mystery for decades (Hall, 1999). Current evidence suggests that TBI sensitivity is mediated in part by the loss of a critical protective mechanism for the organism, namely circulation of unirradiated immune competent cells, and multilineage stem cells, which can repopulate irradiated organs. Pioneering studies (Terry and Travis, 1989) demonstrated protection of mice from otherwise lethal whole abdominal irradiation by injection of bone marrow and more recent studies have demonstrated protection of the esophagus from lethal esophageal irradiation by bone marrow transplantation (Epperly et al, in pressa). The latter studies have demonstrated bone marrow origin of cells, which can reconstitute the esophagus. Repopulation of non-hematopoietic organs by circulating cells of bone marrow origin, suggested by early studies of partial body ionizing irradiation (Anklesaria et al, 1987; Terry and Travis, 1989) have now been confirmed by sophisticated cell sorting techniques which have demonstrated subpopulations of bone marrow stromal cells and hematopoietic stem cell candidates which can reconstitute elements of liver, intestine, lung, esophagus, smooth muscle, cardiac muscle, striated muscle, as well as central nervous system and other organs (Olmsted et al, 2003; Cao et al, 2004). Total

body irradiation may be lethal at relatively lower doses because of the removal or neutralization of this circulating cell reconstitution mechanism. Alternatively, a toxicity of TBI may relate to the "radioprotective bystander" effect, which has also been described (Hall, 1999).

Radiation protection of cells, which are intrinsically radiosensitive, by close proximity to resistant cells, has also been demonstrated. A clonal bone marrow stromal cell line with intrinsic radioresistance has been shown to protect genetically marked radiosensitive bone marrow stromal cell lines by co-cultivation (Santucci et al, 1990). Plateau phase, noncycling bone marrow stromal cells, have been shown to confer apoptosis resistance to attached tumor cell lines including multiple myeloma and leukemia, and hematopoietic stem cells. Mechanisms of bystander radioprotection appear to be complex and may involve cellular transfer through tight junctions or cell membrane apposition of signals including cytokines, and intracellular trafficking molecules, which prevent irradiation-induced cell cycling in sensitive target cells, allowing both DNA repair and induction of antioxidants within the sensitive cell population. For example, TGF β , released from irradiated recovering cells, binding to TGF β receptor on radiosensitive cells may confirm signals, which neutralize the CDC2 DNA checkpoint signal preventing irradiation-induced cell cycling (Bhowmick et al, 2003).

IV. Ionizing radiation protection

Just as multiple molecular biologic and biochemical pathways appear to be involved in ionizing irradiation killing, multiple pathways seem to be involved in irradiation protection (Greenberger et al, 2001, 2003). Since both the killing mechanism and protection mechanisms are redundant (action at one place in the path may not prevent multiple actions at other places in the pathway), there has been difficulty in demonstrating a final common pathway for either killing or protection (Greenberger et al, 2003; Shiloh 2003). Multiple pathways for organ and tissue protection have been developed which relate to neutralization of free radicals (Andreassen et al, 2003; Greenberger et al, 2001, 2003), cytokine neutralization or anti-inflammatory treatments (Anscher et al, 1998; Roesink et al, 1999; Horiot et al, 2000; Huang et al, 2000; Makkonen et al, 2000; Ertekin et al, 2004; Ozturk et al, 2004;). However, the accumulating evidence of the importance of oxidative stress mechanisms in irradiation killing has prompted and stimulated research into the role of the antioxidant pool for radiation protection.

V. Identification of targets for antioxidant mediated radiation protection

The common role of oxidative stress in both cellular agent and ionizing irradiation killing has become a focus of intense investigation. *C. Elegans* strains, which produce intrinsically high levels of antioxidants, as well as control strains grown in culture medium rich in antioxidant have been demonstrated to have a longer life span (Samlowski

et al, 2003). Small molecule analogs of the antioxidant enzyme MnSOD have been shown to confer irradiation resistance to cells in vitro (Samlowski et al, 2003). Radiation protection has been demonstrated by biochemical techniques of increasing Thiol or glutamine antioxidant reserves in cells in culture (Hall, 1999). Studies with cell lines in culture, or microorganisms in fluid phase have been difficult to reproduce with tissues and organs in higher species, however, there is accumulating evidence that an increase in antioxidant capacity provides resistance to ionizing irradiation (Greenberger et al, 2003)

The development of WR2721 (Amifostine) serves as a case in point (Andreassen et al, 2003). This pharmacological radical scavenger compound has been demonstrated to neutralize ionizing irradiation induction of free radicals, but also to modulate several steps involved in ionizing irradiation killing including those cited above with respect to mitochondrial membrane stabilization, caspase path reactivation, and DNA fragmentation. Animal studies, and clinical trials with WR2721 have demonstrated both local and systemic effects of an increase in antioxidant capacity (Andreassen et al, 2003). One potential limitation of WR2721, and other systemically delivered antioxidants, for clinical radiotherapy, is the possibility that equivalent or even greater radioprotection of tumor cells may obviate any potential increase in therapeutic ratio (Andreassen et al, 2003). Organ specific or tissue specific radiation protection, focusing on those targets within the radiotherapy treatment volume provide a challenge for increasing the therapeutic ratio, particularly since the tumor is also within that volume (Andreassen et al, 2003). A method by which to deliver radioprotection to normal cells while preventing such protection of tumor has remained an attractive alternate approach to the other strategy for increasing the therapeutic ratio, namely tumor radiosensitization by targeting to target tumors with specific pharmacologic agents or a gene product that would be specific to tumor cells. An ideal locally administered agent would be one providing both normal tissue specific protection and tumor radiosensitization. There is accumulating evidence that MnSOD, a naturally occurring form of superoxide dismutase with localization of gene product to the mitochondrial membrane, may in fact be such an ideal agent (Epperly et al, 1998; Goltry et al, 1998; Zwacka et al, 1998; Epperly et al, 1999a,b,c; Stickle et al, 1999; Gorbunov et al, 2000; Epperly et al, 2000a,b,c; Epperly et al, 2001a,b,d; Greenberger et al, 2001; Pearce et al, 2001; Epperly et al, 2002b,c). Two unrelated areas of research have converged in recent years to inform the use of MnSOD gene therapy for clinical radiotherapy programs. One area of research is focused on the demonstration that squamous cell carcinoma as well as adenocarcinomas from a variety of sources demonstrate an intrinsic deficit in MnSOD biochemical activity (Oberley and Buettner 1979; Fernandez-Pol et al, 1982; Oberley, 1982; Oberley and Oberley, 1986; Beckman et al, 1989; Spitz et al, 1990; Bravard et al, 1992; St. Clair et al, 1992, 1997; Church et al, 1993; Li et al, 1995; Yan et al, 1996; Zhong et al, 1996, 1997; Liu et al, 1997; Li et al, 1998a,b;

Xu et al, 1999). A relative lack of MnSOD has been shown to be attributable to inactivating mutations in the MnSOD transcriptional activator site within tumor cells, decreased production of mRNA for MnSOD, increased consumption of MnSOD, or a combination of these factors (Fernandez-Pol et al, 1982; Beckman et al, 1989; Bravard et al, 1992; St. Clair et al, 1992, 1997; Church et al, 1993; Li et al, 1995; Yan et al, 1996; Zhong et al, 1996, 1997; Liu et al, 1997; Li et al, 1998a,b; Xu et al, 1999). Such a decrease in MnSOD biochemical activity prompted some investigators to study the effects of gene transfer of MnSOD into tumor cells. Several studies demonstrated potentially beneficial effects of this overexpression, with sensitization of orthotopic tumors to the chemotherapeutic drug BCNU, sensitization to ionizing irradiation, and/or induction of non-malignant phenotypic differentiation of tumor cells to a "benign" phenotype (Beckman et al, 1989; Church et al, 1993; St. Clair et al, 1997). Biochemical analysis of events associated with MnSOD overexpression in tumors, demonstrated an increased sensitivity of tumor cells to the catabolite of MnSOD, hydrogen peroxide. Neutralization of the over produced hydrogen peroxide by simultaneous overexpression of glutathione peroxidase prevented the beneficial effects of MnSOD overexpression in tumors cells (Oberley and Buettner 1979)

A second body of research has demonstrated in a variety of cells, tissues, and normal organ systems, a significant radioprotective effect of MnSOD overexpression, when transgene was delivered by plasmid liposomes, adenovirus, herpes virus, or retrovirus (Epperly et al, 1998; Goltry et al, 1998; Zwacka et al, 1998; Epperly et al, 1999a,b,c, 2000a,b,c, 2001a,b,c,d, 2002a,b,c, 2003a,b,c,d,e,f, in press a,b,c; Stickle et al, 1999; Gorbunov et al, 2000; Greenberger et al, 2001, 2003; Pearce et al, 2001; Kanai et al, 2002, in press; Guo et al, 2003a,b,c,d; Wong et al, submitted)

VI. Applications to clinical radiotherapy

There are two general categories of difficulty in achieving local control of epithelial tumors with ionizing irradiation: volume related limitations of dose and dose escalation to produce local control of radioresistant tumors (Bhatnagar et al, 2002). In the first category of volume effects, it is now well established that multifield conformal radiotherapy approaches toward treating lung and esophagus cancer, which require transit of radiotherapy beams through large volumes of lung will produce radiation pneumonitis and dose limiting toxicity despite the relatively low dose to any one segment of lung. Several clinical studies have demonstrated that the volume of lung, which receives doses between 20 and 30 Gy, is directly related to acute toxicity (Bhatnagar et al, 2002). This phenomenon is independent of the well known resistance of smaller volumes of lung to doses as high as 5500 – 6000 cGy in situations of either large tumor target volume or treatment plans which produce beam transit through multiple areas of ipsilateral and contralateral lung, volume effects limited effect of radiotherapy treatment courses. In the protocols which rely upon concomitant

chemotherapy with drugs including Taxol, Carboplatin, Etoposide, and Gemcitabine (all radiosensitizing agents, as well as effective tumoricidal drugs), volume dependent pulmonary toxicity is even greater. The second category of dose limiting toxicity applies to those tumors, which are intrinsically non-radioresponsive. A second group of patients with lung and esophagus cancer demonstrate significant clinically demonstrable resistance of tumor shrinkage to chemoradiotherapy utilizing small volume irradiation fields, which do not transit significant proportions of normal lung, and in protocols where doses reach 7000 cGy (97). This data has led several experts in lung radiotherapy to conclude the doses of 8500 – 9000 cGy may be required to produce local control of this sub-population of lung cancer and esophagus cancer patients (Bhatnagar et al, 2002). Oral administration of radioprotective agents including WR2721 has been attempted in these categories of patients and some encouraging results have been achieved (Andreassen et al, 2003). However, the distribution of radioprotector compounds delivered intravenously suggests that both tumor volumes and normal tissue receive significant exposure to the radioprotective agent (Andreassen et al, 2003) Techniques by which to selectively protect normal tissue in the transit volume are attractive.

In additional studies, MnSOD-PL administration by intratracheal injection to mice with orthotopic tumors demonstrated no radiation protection of the tumor, and in several instances suggested radiosensitization. The encouraging results prompted focus on methods by which to protect normal tissue in other organs utilizing this gene therapy approach.

Administration of MnSOD-PL by intratracheal injection provided protection against fractionated irradiation of whole lung in the C57BL/6J mouse model (Epperly et al, 1998). The requirement for neck dissection and intratracheal cut-down, and associated trauma to the normal tissues prevented multiple fraction administration of MnSOD-PL in the initial studies, therefore, inhalation techniques were developed. Such inhalation technology using a nebulizer system (**Figure 1**) was felt necessary to allow eventual translation of this technique to clinical radiotherapy where 28 – 38 fractions of radiotherapy are

delivered over 7 – 8 weeks. Inhalation studies in the C57BL/6J mouse model demonstrated persistence of transgene product for 48 to 72 hours following an inhalation administration (Epperly et al, 1998, 2003d). These data were comparable to those for intratracheal injection. Furthermore, multi-fraction administration of epitope-tagged hemagglutinin (HA)-MnSOD demonstrated persistent transgene delivery to the pulmonary distal alveolar structures, and confirmed studies with intra-tracheal injection, that a single administration of HA-MnSOD does not prevent uptake of subsequent fractions and full administration of HA-MnSOD was also demonstrated with oral cavity and oropharynx, and in a second system with multiple intraesophageal administration (Epperly et al, 2002a, 2003e; Guo et al, 2003c). Thus, in three systems (lung, oral cavity/oropharynx, and esophagus) multiple administrations of HA-MnSOD proved feasible to provide a continuous level of production of transgene-MnSOD product during a protocol of multiweek fractionated radiotherapy. Inhalation of HA-MnSOD by nebulizer to C57BL/6J mice provided radiation protection against late fibrosis (Guo et al, 2003b; Epperly et al, 2003b). The mechanism of lung protection by MnSOD-gene therapy is not yet known, however, there is a clear decrease in initial acute irradiation-induced elevation of TGF β , TNF α , IL-1, and an increase in irradiation-induced upregulation of pulmonary endothelial VCAM-1 and ICAM-1, but not P-Selectin, E-Selectin, or L-Selectin (Epperly et al, 2002b, in press b). At the time of initiation of late irradiation fibrosis/organizing alveolitis, HA-MnSOD-PL administration prior to irradiation significantly prevented late cytokine elevation at around 100 – 150 days in the mouse model, and also significantly reduced the migration of bone marrow myoepithelial progenitor cells to the lung as a major component of the fibrotic lesion (Epperly et al, 2003d,f). Available evidence indicates that multiple biochemical and cell biologic processes associated with late irradiation fibrosis are inhibited by administration of MnSOD-PL gene therapy prior to irradiation exposure even though this exposure has occurred 100 to 150 days earlier in the mouse model (Epperly et al, 2003d,f).



Figure 1. Nebulizer system for inhalation gene therapy

In recent studies, administration of HA-MnSOD-PL after irradiation was also associated with decrease in late fibrosis

while the particular effect was less significant than that observed with administration of gene therapy prior to

irradiation. Several components of late irradiation lesions have been evaluated with respect to the protective effect of MnSOD-PL gene therapy. Inflammation macrophages and macrophage progenitors migrate into irradiated tissues, while the number of fibroblast progenitors is reduced (Epperly et al, 2003d).

VII. Radiation protective gene therapy: considerations relevant to radiation pathology and radiation pathophysiology

Organ specific radiation protection necessitates evaluation of the known radiation pathologic and pathophysiologic changes in specific tissues. Ionizing irradiation pathology has been well described in several textbooks and clinical review articles (Dileto and Travis, 1996; Anscher et al, 1998; Hall, 1999) (**Table 1**). Common factors to most organs and organ systems include initial endothelial cell swelling, inflammatory cell infiltration, and late migration into irradiated fields of fibroblast progenitor cells, and proliferation of surviving fibroblasts to produce the late lesion of fibrosis. However, there are organ and organ system specific acute and chronic pathologic changes induced by ionizing irradiation, which relate to an application of organ specific gene therapy. In particular, measurement of acute and late ionizing radiation toxicity is organ specific. For example, acute radiation changes of oral cavity, oropharynx, cervical, and thoracic esophagus are manifest as apoptotic bodies within the submucosal layer, micro-ulceration, and the clinical symptomatology of difficulty swallowing, weight loss, and dehydration (Rosenzweig et al, 2000; Bhatnagar et al, 2002). In contrast, acute irradiation damage to the urinary bladder is associated with transitional epithelial cell swelling, microvascular

hemorrhaging, and the acute symptomatology of urinary frequency and burning (Kanai et al, in press). Both organ systems demonstrate late irradiation fibrosis such that radiation pathologic changes common to the late effect in both organ systems would not be as significant as the pathophysiologic differences between irradiation-induced injury as measured in the acute interval (Cooper, 2000; Kang et al, 2003; Martin et al, 2000; Robnett et al, 2000; Sunyach et al, 2000; Theuws et al, 2000).

VIII. Radioprotective lung gene therapy

Intratracheal or inhalation delivery of plasmid/liposomes containing the MnSOD transgene has been demonstrated to decrease both the acute and chronic side effects of ionizing irradiation damage in a mouse lung (Epperly et al, 1998). Murine acute irradiation lung damage as well as chronic damage is similar to the pathology and pathophysiology of lung damage in higher primates. Acute inflammatory injuries are associated with endothelial swelling, cellular transudates in the alveolar spaces, and the clinical symptomatology of increased respiratory rate, decreased exercise tolerance with laboratory correlates of decreased effusion capacity for oxygen which is associated with fluid accumulation in the alveolar spaces. Biochemical correlates include elevation of TNF , TGF , and IL-1 (Epperly et al, 1999c). MnSOD-PL delivery to animals receiving single fraction or fractionated irradiation, with single fraction or fractionated delivery of MnSOD was associated with a decrease in pathologic and biochemical correlates of early irradiation injury (Epperly et al, 1998, 1999c).

Late irradiation fibrosis in the lung model has been demonstrated to significantly involve migration of

Table 1. Various tested strategies for tissue or organ specific radiation protection

Agent	Mechanism of Action	Organ Specific	Reference
GMCSF	Immune Cell Stimulator/anti-infection	Oral Cavity	Makkonen et al, 2000
MCSF	Immune Cell Stimulator/anti-infection	Oral Cavity	Makkonen et al, 2000
Pilocarpine	Salivary Gland Stimulus	Oral Cavity	Roesink et al, 1999
WR2721 (Amifostine)	Thiol Radical Scavenger	Esophagus, Lung, Rectum	Andreassen et al, 2003
Antifungal Agent	Redox Purification	Oral Cavity	Ertekin et al, 2004
Glutamine	Redox Balance	Oral Cavity	Huang et al, 2000
Pentoxifilline	Redox Balance	Lung	Rube et al, 2002; Ozturk et al, 2004
MnSOD Gene Therapy	Antioxidant	Oral Cavity, Lung, Esophagus, Intestine, Bladder	Epperly et al, 1998; Stickle et al, 1999; Guo et al, 2003d; Guo et al, 2003a; Kanai et al, in press

myofibroblast precursors from the bone marrow to the

lung (Epperly et al, 2003f). MnSOD-PL intratracheal gene therapy was associated with decreased migration of

fibroblasts to the lung. Steps between the acute and chronic irradiation lung damage have been shown to involve accumulation of bronchoalveolar macrophages in the lungs (Epperly et al, 2003f). Migration of macrophages from the bone marrow in the irradiation-induced late damage model has also been shown with mouse lung. The mechanism of correlation of alveolar macrophage and fibroblast migration from bone marrow to lung is not yet known. One hypothesis is that the late spike of TGF, β 1 and TGF, β 2 production by the lung at 100 to 150 days after irradiation of the mouse model is associated with recruitment of fibroblast progenitors from the bone marrow to form the fibrotic lesions (Epperly et al, 2003f). Experiments are in progress to test this hypothesis. The mechanism by which MnSOD-PL treatment decreases irradiation fibrosis is not yet known. Whether MnSOD-PL effects are related to decreased free radical bioavailability is not known. While MnSOD scavenges superoxide and dismutates it to hydrogen peroxide, other enzymes including glutathione peroxidase metabolize hydrogen peroxide to water. Both direct ionizing irradiation induced free radicals, and those induced by cytokines in inflammatory cells may be in part neutralized by MnSOD overexpression. In fractionated irradiation protocols, delivery of MnSOD-PL daily or every third day by a nebulizer has provided a model system by which to test exhaustion of antioxidant reserves in the lung and how MnSOD-PL gene therapy may preserve antioxidant pools (Epperly et al, 2003b; Guo et al, 2003b). These experiments are in progress. A summary of tissue radioprotectants or tumor radiosensitizers is shown in **Table 2**.

IX. MnSOD-PL protection of the esophagus from irradiation damage

Intraoral administration of MnSOD-PL to non-anesthetized mice results in the swallowing of the material and coating of the esophageal (Epperly et al, 1998, 2001a,b). These results were derived using an epitope-hemagglutinin labeled MnSOD construct. HA-MnSOD-PL delivery, reduced irradiation mediated esophagus damage as measured by reduction in apoptotic bodies, decreased DNA strand breaks in esophageal mucosal cells, decreased ulceration, and stabilization of food and water intake and stabilized weight. Late irradiation fibrosis was also decreased in surviving animals with decreased esophageal stricture detected in those animals that received MnSOD-PL gene therapy.

Photodynamic (PDT) induced a stricture in a porcine model has also been shown to be beneficially influenced by MnSOD-PL gene therapy (Wong et al, submitted). Pigs given photofrin 24 hours before phototherapy of segments of esophagus demonstrated esophageal strictures within three months. Those animals given MnSOD-PL administered either by esophagoscope directly at the site of PDT treatment, or diffusely throughout the whole esophagus showed decreased esophageal stricture. PDT induced singlet oxygen, and mitochondrial membrane

damage associated with other free radicals has been shown to be involved in the mechanism of PDT tissue damage. MnSOD-PL gene therapy was associated with a reduction in PDT induced esophageal damage. These data provide strong evidence that oxidative stress mechanisms common to both PDT and ionizing irradiation are influenced by overexpression of one antioxidant, MnSOD, and further suggests that mitochondrial specific localization of this antioxidant is critical for protection. In mouse esophagitis models, irradiation of mice that have received CU/ZnSOD or MnSOD with the mitochondrial localization signal deleted did not demonstrate significant radiation protection.

X. Gene therapy irradiation protection of the oral cavity and oropharynx

While the cervical esophagus is similar to the thoracic esophagus with respect to cellular self-renewal systems in proposed mechanisms of radiation killing, and MnSOD-PL protection, the oral cavity and oropharynx is complicated in that late irradiation effects are significantly influenced by the integrity of the salivary glands (Guo et al, 2003c). Major salivary glands including the parotid, and submandibular glands as well as multiple minor salivary glands contribute to the prevention of irradiation-induced xerostomia and also mediate release of IgA and enzymes critical for protection of the oral cavity from bacterial and fungal organisms. Multiple strategies of reduction of acute mucositis have been attempted focusing on inhibiting steps in the infection pathway including techniques to deliver pharmacologic or therapeutic agents which might overcompensate for decreased salivary gland protection of micro-ulceration, by providing GM-CSF, antibiotics, anti-fungal agents, or in several experiments providing agents that stimulate radiation damaged salivary glands to produce more saliva. Intra-oral administration of MnSOD-PL have been shown to deliver gene product effectively to the tongue, floor of the mouth, and buccal mucosa. However, salivary gland delivery is minimal. This is not surprising since the availability in the salivary gland ducts of intra-oral administered MnSOD-PL would be minimal. In contrast, WR2721, Amifostine, which is delivered intravenously, has been shown to accumulate significantly in the salivary glands (Anscher et al, 1998). This radioprotective thiol compound is relatively ineffective in preventing irradiation-induced mucosal ulceration, but appears to preserve salivary gland function (Anscher et al, 1998; Guo et al, 2003a). Thus, one strategy suggested by our recent work is that both MnSOD-PL gene therapy and systemic WR2721 could be combined to provide oral cavity radiation protection. Oral cavity fibrosis has not been quantitated with respect to MnSOD-PL protective effects. These studies are in progress. Other measures of late irradiation toxicity include osteoradionecrosis, particularly of the mandible and subcutaneous fibrosis. Experiments to test the effects of MnSOD-PL gene therapy on these systems are in progress.

Table 2. Gene therapy and related gene product delivery strategies of normal tissue radiation protection compared to strategies for tumor radiosensitization

Radiation Protection of Normal Tissue	Agent	Mechanism of Action	Organ Specific	Reference
	MnSOD	Antioxidant	Multiple Organ	Epperly et al, 1998; Stickle et al, 1999; Guo et al, 2003d; Guo et al, 2003a; Kanai et al, in press
	EC-SOD	Antioxidant	Total Body	Kang et al, 2003
	ML CU/ZnSOD	Antioxidant	Esophagus/Oral Cavity	Epperly et al, 2003c
	CU/ZnSOD	Antioxidant	Lung	Delanian et al, 1994
	Heme oxygenase (DNA-adenovirus)	Antioxidant anti-fibrosis	Lung	Tsuburai et al, 2002
Tumor Radiosensitization				
	p53			Kawabe et al, 2002; Fedorov et al, 2003
	Antisense C-Met			Danilkovitch-Miagkova and Zbar, 2002; Birchmeier et al, 2003; Christensen et al, 2003; Jankowski et al, 2003; Sattler et al, 2003
	Antisense Veg f.			Dings et al, 2003
	Marker Genes	Protein Delivery Anti-tumor	Mesothelioma	Mae and Crystal, 2002
	HSU-Thymidine Kinase	Tumoricidal	Brain	Nanda et al, 2001; Rosenberg et al, 2002
	Adenovirus-mda7	Lung Cancer delivery of p53	Lung	Kawabe et al, 2002
	Polyethylameimine plasmid	Tumoricidal proteins	Lung	Orson et al, 2002; Kircheis et al, 2002
	HSV-radiation inducible producer	Antitumor activity	Liver	Chung et al, 2002
	Lentiviral Lung Specific Promoter	Anticancer genes	Lung	Tarantal et al, 2001
	Adeno Associated Virus – Angiostatin	Antitumor Vascular	Brain	Ma et al, 2002
	Cationic Vector Endostatin	Antitumor Vascular	Intravenous	Nakashima et al, 2003
	Cytomyelovirus and radiation-inducible promoter INOS	Tumor Suicide Gene Delivery	Direct to Tumor	Worthington et al, 2002

XI. Gene therapy radiation protection of the urinary bladder

A common side effect of bladder irradiation which is

found in patients receiving pelvic irradiation for gynecologic cancer, and in men receiving prostate radiotherapy for prostate cancer is a symptom complex associated with inflammation of the transitional cell epithelium and sub-epithelial layers (Kanai et al, 2002). This is manifest as spreading of umbrella cells, as seen by electron microscopy, and is associated with increased urea and water permeability of the bladder mucosal surface. Ionizing irradiation induced changes are dose and fraction size dependent. Keeping a portion of the bladder out of the radiation field decreases side effects as with other organs and organ systems studied. Intra-vesicle administration of MnSOD-PL to rats receiving single fraction or fractionated radiotherapy to the urinary bladder demonstrated significant reduction in water and urea permeability (Kanai et al, 2002). Decreased fibrosis of the urinary bladder was also detected demonstrating a decrease in late side effects by MnSOD-PL gene therapy. Another potential antioxidant of value in radioprotective gene therapy is the use of inhibitors of nitric oxide synthase. Nitric oxide has been demonstrated to be involved in induction of steps critical to increasing cellular mitochondrial membrane permeability. Mitochondrial membrane permeability has been associated with the ionizing radiation-induced damage described above. Nitric oxide synthase naturally mediates production of nitric oxide and upregulation of NOS by irradiation has been demonstrated. Inhibitors of NOS have been shown to be effective in preventing irradiation damage to the bladder, measured by the acute parameters of water and re-permeability (Kanai et al, in press). Thus, with the bladder irradiation model system, potential exists for utilization of two different antioxidant molecules in gene therapy. Studies comparing MnSOD, and iNOS inhibitors are in progress (Kanai et al, in press).

XII. Gene therapy radiation protection of the intestine

While plasmid liposome delivery of MnSOD to the intestine has been carried out, effective absorption of transgene into mucosal epithelium has been limited by the extensive production of mucin by intestinal crypt cells. Irradiation damage to the intestine has been well studied in the assay of Withers (gut crypt colony assay), which has been a valuable method for quantitation of radiation damage (Guo et al, 2003d). Crypt depth, and crypt volume per square millimeter of intestine have also been measures of intestinal damage. Irradiation is known to damage differentiating epithelial cells causing sloughing and flattening of the intestinal mucosa. Damage is volume, irradiation dose, and fraction size dependent as with other organs. Because MnSOD-PL uptake was limited, initial studies were carried out with a Herpes simplex virus containing the MnSOD transgene (Guo et al, 2003d). Mice receiving intra-intestinal loop injection of Herpes virus MnSOD, but not control LACZ demonstrated the protection from irradiation-induced damage to intestinal crypts. These initial studies suggest antioxidant delivery to the intestine will also be radioprotective. Studies with

plasmid liposomes in an intestinal loop model are in progress (Guo et al, 2003d).

XIII. Conclusions

Radioprotective gene therapy offers an attractive strategy for organ specific and tissue specific delivery of antioxidant genes. The transient overexpression afforded by plasmid liposome administration allows temporal management of antioxidant levels. Antioxidant pool size differences and hydrogen peroxide metabolic differences between many squamous carcinomas and adenocarcinomas compared to the normal tissues and the suggested ability of MnSOD overexpression to selectively increase hydrogen peroxide levels in tumors afford the methods by which to use the biochemistry of oxidative stress management to facilitate improved tumor local control. Synergy of ionizing irradiation and antioxidant gene therapy with respect to tumor radiosensitization appears to be another avenue for development. Alternative strategies to antioxidant gene therapy are also being explored. The delivery of small molecule MnSOD mimics offers a potential attractive advantage in that large molecule transgene delivery would not be required. However, selective uptake of small molecules in specific tissues has not been demonstrated. Systemic delivery of antioxidants would only be appropriate in Clinical Radiotherapy applications if selective normal tissue protection compared to tumor killing were still demonstrable. In contrast, environmental radiation protection may be a better avenue for pursuit of small molecule SOD mimics for radiation protection (Samlowski et al, 2003). In the absence of tumor, all tissues might be equally protected.

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