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Gene Therapy for Radioprotection

William H. Everett, B.S.[1] and **David T. Curiel, M.D., Ph.D.**[1]

[1] Division of Cancer Biology, Department of Radiation Oncology, Washington University School of Medicine in St. Louis, St. Louis, Missouri, USA

Abstract

Radiation therapy is a critical component of cancer treatment with over half of patients receiving radiation during their treatment. Despite advances in image guided therapy and dose fractionation, patients receiving radiation therapy are still at risk for side effects due to off-target radiation damage of normal tissues. To reduce normal tissue damage, researchers have sought radioprotectors, agents capable of protecting tissue against radiation by preventing radiation damage from occurring or by decreasing cell death in the presence of radiation damage. While much early research focused on small molecule radioprotectors, there has been a growing interest in gene therapy for radioprotection. The amenability of gene therapy vectors to targeting, as well as the flexibility of gene therapy to accomplish ablation or augmentation of biologically relevant genes, makes gene therapy an excellent strategy for radioprotection. Future improvements to vector targeting and delivery should greatly enhance radioprotection through gene therapy.

Introduction

Radiation therapy (XRT) is a commonly used and effective modality for the treatment of cancer, with over half of cancer patients receiving XRT at some point during their treatment.[1] However, the use of XRT is associated with significant off-target effects on normal tissues that limit the dosages and locations used in XRT.

The pathology of radiation damage is mediated by the creation of free radicals and reactive oxygen species (ROS) inside cells.[2] These ions and radicals damage components throughout the cell, most significantly causing double strand DNA breaks. This damage initiates a signaling pathway that either results in the arrest of the cell cycle or in apoptosis. Thus, radiation damage results in a decreased population of cells, as well as a decreased ability to repopulate. The dual nature of this pathology is most apparent and appears rapidly in tissues that require replication to maintain physiological function, such as bone marrow and intestinal epithelia. In tissues that replicate more slowly or not at all, the damage takes longer to appear and is often of an inflammatory, fibrotic form.[2] Thus, the rate of cellular division influences the timing and nature of normal tissue response to radiation damage.

Correspondence: David T. Curiel, 4511 Forest Park Avenue, St. Louis, MO 63108 Phone: (314) 747-5443 Fax: (314) 362-9790 dcuriel@radonc.wustl.edu.

Conflict of Interest

The authors declare no conflict of interests.

The responses of normal tissue to irradiation can be classified as early, intermediate, or late depending upon the time it takes for them to develop following radiation exposure.[2] The early radiation responses that occur in days to weeks following irradiation are dominated by the effects on the hematopoietic, gastrointestinal, and cerebrovascular systems. At doses up to 5Gy, hematopoietic effects are dominant, with lymphopenia, neutropenia, thrombopenia, and anemia occurring. With higher doses in this range (2.5-5Gy) death may occur within approximately two months. From 5 to 12Gy, damage to the gastrointestinal system leads to bloody diarrhea, denudation of epithelia, destruction of intestinal crypt cells, and death within nine to ten days. Doses of 12Gy and above cause catastrophic damage to the neurological and cardiovascular systems, leading to death within 48 hours. Together, these patterns of normal tissue damage are known as the Acute Radiation Syndrome.[2] Damage to the skin is also classified as an early radiation response, with erythema occurring within hours of radiation exposure and desquamation within two to three weeks, depending on the dose. Finally, damage to the testes and ovaries is classified as early, as the stem cells and oocytes, respectively, are quickly killed by radiation exposure.[2]

The intermediate effects of radiation damage occur within a few months of radiation exposure. The main form of intermediate radiation response is acute pneumonitis of the lung, which may occur two to six months after irradiation.[2]

The late effects of radiation damage occur months to years following exposure.[2] Thickening of epithelium and fibrosis occur throughout the gastrointestinal tract, from the esophagus to the intestines. Fibrosis also occurs as a late effect in the lungs, bladder, and heart, with the heart also being vulnerable to the development of pericarditis.[2] The response of the kidneys to radiation is a late-developing nephropathy that leads to arterial hypertension and anemia. Exposure of the liver to radiation can lead to a rapid loss of function several months post-exposure. Finally, the late effects of radiation on the CNS are transient demyelination, leukoencephalopathy, and radionecrosis.[2] Table 1 summarizes the effects of radiation on normal tissues.

Since the inception of XRT, attempts have been made to abrogate side effects by increasing the radiation resistance of normal tissues (radioprotection). The ideal radioprotection agent would affect only normal tissue and not increase the resistance of cancer cells. Such an increase in the therapeutic index would allow the use of current levels of radiation with fewer side effects or allow increased levels of radiation with an acceptable side effect profile. Strategies to accomplish radioprotection have followed three general avenues. The first is to protect normal tissue by increasing the ability of the tissue to detoxify free radicals to prevent radiation-induced damage from occurring; the second is to mitigate radiation damage by overriding the cellular signaling network with growth factors or other proteins to sustain the cell cycle and prevent apoptosis. The former strategy is true radioprotection, whereas the latter is more accurately described as radiomitigation. Finally, a third strategy is to use the expression of transgenes as therapy to restore function to tissues that have already been damaged. In all of these strategies, the goal is the maintenance of tissue function through either protection against damage, mitigation of damage, or restoration of function after damage. Therefore, all of these strategies are radioprotective in that they seek to prevent the off-target radiation from affecting the function of normal tissues. It is in this

more general sense that “radioprotection” will be used throughout the rest of this paper. In early work, many groups have focused on the use of small molecule pharmaceuticals as radioprotectors; however, despite numerous studies, only two drugs, amifostine and palifermin, have received FDA approval as radioprotection agents.[3]

In recent years, there has been a growing interest in the use of gene therapy for radioprotection. Gene therapy is an attractive strategy for radioprotection for several reasons. Gene transfer can be targeted to particular sites and tissues by either the intrinsic properties of the gene transfer vector or by the method by which the vector is administered. Further, gene therapy has extensive flexibility to carry out a variety of radioprotection strategies due to the ability to accomplish ablation or augmentation of biologically relevant genes. Therefore, gene therapy can be used in any of the general strategies of radioprotection—increasing detoxification of free radicals through the delivery of antioxidant genes, improving survival and proliferation of damaged cells through the delivery of growth factor genes, or delivering transgenes to restore tissue function following radiation damage. The ability of gene therapy to target specific sites with a variety of therapeutic genes may thus allow for the radioprotection of normal tissues without affecting the sensitivity of tumors.

The choice of vector is of critical importance in gene therapy for radioprotection. To achieve clinically significant radioprotection, a vector must be able to specifically and efficiently transduce the normal tissue desired. This is complicated in practice by the widely distributed nature of normal tissues important to radioprotection (bone marrow, gastrointestinal tract) and the *in vivo* delivery of radioprotective genes. Therefore, an ideal vector for radioprotection would be amenable to targeting to normal tissues and efficiently transduce those tissues. Further, to limit the side effects of radioprotective transgenes, many of which have effects on cellular growth and proliferation, the duration of expression should be controlled. This makes integrating and long-term expression vectors, such as retroviral and adeno-associated viral vectors, respectively, less attractive candidates for the delivery of some radioprotective transgenes. Two promising vector systems for radioprotection are plasmid liposomes and adenovirus. Plasmid liposomes incorporating polymers have been developed and have shown the potential to be targeted to specific tissues.[4] In adenoviral systems, incorporation of tissue specific promoters[5] and the incorporation of targeting ligands (including single domain antibody species) into the adenoviral capsid proteins[6] have yielded adenoviral vectors capable of targeting a variety of normal tissues.

Superoxide Dismutase

In following the antioxidant approach to radioprotective gene therapy, the proteins of the Superoxide Dismutase (SOD) family have been extensively studied. The SOD proteins are a family of three metalloproteins that catalyze the conversion of superoxide (O_2^-) into hydrogen peroxide and oxygen. As radiation damage is mediated by the creation of superoxide and other ROS in cells, SOD gene therapy was seen as a means to reduce the damage caused by XRT. One member of the family, CuZnSOD (SOD1), is localized to the cytoplasm and constitutively expressed, while MnSOD (SOD2) is localized to mitochondria and expression is induced by several factors, including radiation.[7] The third member of the

SOD family, ECSOD (SOD3), is also CuZn based but is localized to the extracellular space. Despite the similar enzymatic action of these proteins, MnSOD generally shows superior radioprotective capacity. This is thought to result from the mitochondrial localization of MnSOD, as MnSOD without the mitochondrial localization sequence shows decreased radioprotective capability.[8] The exact mechanism is unknown, but it is possible that MnSOD inhibits apoptosis by stabilizing the mitochondrial membrane.[8] This evidence of MnSOD-mediated radioprotection calls into question the model of radioprotection solely by antioxidant effects within the cytoplasm of cells. On the other hand, there is evidence that ECSOD is a potential radioprotective agent in the lungs, by virtue of its extracellular localization.[9] Thus, it is possible that the mechanism of radioprotection via SOD expression varies depending on the particular tissue with further work being necessary to clarify the mechanism of SOD radioprotection. In addition to acting as a radioprotector, there is evidence that MnSOD acts as a radiosensitizer in some cancers[10] and is dysregulated in tumor development and progression.[11]

Superoxide Dismutase gene therapy has been carried out using a variety of vectors and administration methods to control the localization of expression. Vectors used with SOD include recombinant viruses,[12,13] plasmid liposomes,[14-17] and minicircle-plasmid liposomes (a plasmid in which remaining bacterial sequences have been eliminated.)[18] To achieve more specific expression of SOD, several methods have been used to administer these vectors as well. Direct injection has been used for intratracheal[12,14,19] and intraesophageal[20,21,22] protection. Intravenous administration has also been used for whole body protection experiments.[17,23] Radioprotection experiments with SOD gene therapy have also been carried out using oral administration of plasmid liposome vectors.[24,25] Finally, plasmid liposomes have been administered in a manner making use of naturally occurring anatomical isolation such as inhalation via nebulizers[16] and intravesicular instillation.[15]

Superoxide Dismutase gene therapy has shown promise in preventing radiation damage in several tissues at both the early and late stages of radiation damage. In the lungs, MnSOD therapy has been shown to reduce histological and clinical signs of organizing alveolitis and fibrosis, late sequelae of radiation exposure.[14] This protective effect appears to be limited to normal tissues with no bystander effect on tumor models.[26] Accordingly, administration of SOD gene therapy reduces apoptosis in irradiated lungs[27] and reduces the expression of inflammatory cytokines IL-1, TNFα, and TGF-β.[12,19] At later time points, the administration of SOD gene therapy results in a decrease in the expression of VCAM-1 and ICAM-1, potentially decreasing immigration of leukocytes that contribute to pulmonary fibrosis.[28]

The esophagus is another site that has been extensively studied in the context of SOD gene therapy radioprotection. Administration of MnSOD gene therapy vectors has been shown to improve clinical markers in murine models following irradiation such as decreasing weight loss[21] and increasing overall survival.[20] Histologically, the protective effect is corroborated by findings of decreased vacuole formation,[20] increased side population stem cell survival,[29] and increased engraftment of marrow-derived progenitor cells within the damaged esophageal tissue.[30] Additionally, administration of SOD gene therapy is associated with

decreased lipid peroxidation[31] and homologous recombination in irradiated esophageal tissues.[32]

Superoxide Dismutase gene therapy also shows promise in the radioprotection of hematopoietic tissue. *In vitro* studies using the murine myeloid cell line 32Dcl 3 have shown that MnSOD gene therapy decreases the apoptosis of these cells in response to irradiation and TNFα.[33] Further, administration of MnSOD gene therapy reduces death due to the hematopoietic syndrome *in vivo.*[34]

Together, these studies demonstrate that gene delivery of members of the SOD family, particularly MnSOD, is a promising strategy for radioprotection of a variety of tissues. The radioprotection conferred by this strategy has been detected through clinical markers such decreased weight loss and increased survival, as well as through histological markers such as decreased apoptosis. However, use of SOD gene delivery is limited by the specificity of available gene delivery vectors. This constraint has led to the use of direct injection of SOD vectors or administration to anatomically compartmentalized locations such as the bladder to achieve specific expression within targeted tissues. To enable the use of SOD radioprotection in other tissues while maintaining specific gene delivery, new vectors capable of enhanced targeting need to be developed.

Catalase

Another antioxidant protein that has been used in radioprotective gene therapy is catalase. Acting downstream of the SOD proteins, catalase catalyzes the decomposition of hydrogen peroxide into water and oxygen, thus protecting the cell from the oxidative effects of hydrogen peroxide. When targeted to the mitochondria, catalase has shown some radioprotective effect,[35] offering the possibility of dual therapy with an SOD and catalase to enhance further the pathway of ROS degradation. Further, catalase gene therapy has been shown to enhance the engraftment of transplanted hematopoietic stem cells following irradiation.[36] Further work is necessary to determine the potential of catalase as a radioprotective agent, either alone or in conjunction with other radioprotectors.

Roof plate-specific spondin 1

In contrast to antioxidant radioprotective strategies which attempt to reduce radiation-mediated damage, growth-modulating radiomitigation therapies attempt to sustain the cell cycle and prevent apoptosis from occurring despite radiation damage. One key regulatory pathway in the proliferation of gastrointestinal mucosa from the oral cavity to the intestine is the Beta-catenin/Wnt signaling pathway.[37] Together, Beta-catenin and Wnt signaling control stem cells in intestinal crypts, ensuring that progenitor cells remain for regeneration.[38,39] Roof plate-specific spondin 1 (R-spondin1) is a secreted agonist of the Wnt/Beta-catenin pathway that results in intestinal hyperplasia when expressed transgenically in mice.[40-42] Additionally, administration of R-spondin1 has been shown to reduce the severity of experimentally-induced colitis in mouse models,[43] and administration of recombinant R-spondin1 showed a radioprotective effect in an oral mucosa irradiation model.[44] These results encouraged the exploration of R-spondin1 as a potential agent for radioprotective gene therapy.

Bhanja et al. used an adenoviral vector expressing R-spondin1 (AdRspo1) in radioprotection experiments in the small intestine.[45] Administration of AdRspo1 resulted in a 6-8 fold increase in R-spondin1 serum levels that persisted for a week. Treatment with AdRspo1 before administration of lethal WBI (10.4Gy) showed a radioprotective effect with the median survival time increased as compared to the control vector. However, the mice eventually succumbed to the hematopoietic syndrome. Additionally, these mice maintained their body weight and well-formed stools.[45] This was correlated with markers of normal intestinal histology such as crypt proliferation, decreased crypt apoptosis, and an increase in crypt microcolonies as compared mice receiving the control vector.[45] Based on these results and the fact that R-spondin1 does not increase the resistance of tumors to chemotherapy[42] or radiation,[45] R-spondin1 is a promising radioprotective agent.

Heat Shock Protein 25

Heat Shock Proteins (HSPs) are a group of proteins upregulated in response to noxious stimuli including temperature and ischemia.[46] Their capability to protect cells against apoptosis has led to exploration of several HSPs, especially HSP25 as protective agents against the insults of chemotherapy and XRT.[47,48] The mechanism by which HSPs reduces apoptosis is unknown, but putative mechanisms include binding cytochrome c,[49] degradation of unfolded proteins via the ubiquitin pathway,[50] binding Daxx,[51] delaying cell growth,[52] inducing MnSOD expression,[53] downregulating ERK1/2 expressing,[54] and inhibiting PKCδ-mediated production of reactive oxygen species.[55]

Lee et al. explored the use of HSPs as a radioprotective agent of two tissues, the salivary glands and bone marrow. In work using HSP25 as a salivary gland radioprotector, Lee et al. directly injected an adenovirus expressing HSP25 or HSP70i (AdHSP25 and AdHSP70i) into the submandibular glands of mice. The mice were then subjected to 17.5Gy of radiation directed to the submandibular glands. Both vectors were found to transfect salivary gland cells and induce the production of their respective transgene. The submandibular glands were then examined 40 and 90 days post irradiation.[47]

As radioprotective agents, AdHSP25 prevented the decrease of the mass of the submandibular glands without having an effect on overall weight loss. AdHSP70i did not show any effect on gland weight. Further, both AdHSP25 and AdHSP70i improved salivary flow rate and chemical constituency following irradiation, as compared to controls. The improvements in these markers were associated with a reduction of apoptosis in acinar cells and fibrosis within the glands.[47]

In work using AdHSP25 as a radioprotector of bone marrow, Lee et al. injected the vector into the tail veins of mice one hour prior to irradiation. AdHSP25 was found to transfect a large portion of bone marrow cells. Following irradiation, all of the mice developed thrombocytopenia, erythocytopenia, and leukopenia, with the mice that received AdHSP25 recovering more quickly. AdHSP25 was shown to enhance the recovery of bone marrow by reducing apoptosis as demonstrated by decreases in caspase activation.[48] Further, it was shown that many of the cells protected by HSP25 were c-kit-positive, a marker for stemness. This suggested that HSP25 mediated radioprotection by preventing apoptosis of

hematopoietic stem cells (HSCs). HSP25 radioprotection was also associated with the expression of Tie2, a receptor tyrosine kinase expressed on endothelial cells and HSCs that protects stem cell compartments.[56] Knockdown of Tie2 by RNA interference blocked the radioprotection mediated by HSP25.[48]

Multidrug Resistance 1

MDR1 (multidrug resistance 1) is the gene that encodes the protein P-glycoprotein (P-gp), which is widely expressed in human cancers and provides resistance against many chemotherapy agents.[57] This resistance capability has lead to the study of MDR1 in gene transfer to protect normal tissues during intense chemotherapy regimens.[58] Further study revealed that MDR1 protects cells not only through removal of toxic agents, but also by inhibiting apoptosis and that deficiency of P-gp can lead to increased apoptosis following exposure to radiation.[59] Though this is known to be achieved through suppression of caspase activity, the exact mechanism is not known.

The ability of MDR1 to suppress apoptosis has led to interest in the gene as a radioprotective agent. Maier et al. examined the effect of MDR1 as a radioprotector *in vitro*, using the human B-cell lymphoblastoid line TK6.[60] Using a retroviral vector expressing MDR1, the TK6 cells were infected and changes in gene expression were measured. The pro-apoptotic genes CASP1, CASP4, and NALP7 were all found to be down-regulated while the potentially anti-apoptotic gene AKT3 was upregulated. These changes in apoptosis-related gene expression remained after the cells were irradiated. The physiological relevance of the changes in apoptosis-related gene expression was corroborated by evidence of radioprotection. Cells transfected with MDR1 showed reduced apoptosis and increased survival after irradiation with 1-4Gy. Though the mechanism for this radioprotection clearly involves transcriptional regulation of apoptotic factors, the addition of an inhibitor of the P-gp efflux pump slightly reduced the protective effect of MDR1 transfection. Thus it is not possible to rule out the contribution of the efflux action of P-gp on the observed radioprotection.[60]

MDR1 radioprotection has also been examined in $CD34^+$ hematopoietic stem cells, a group of cells not only important for hematopoiesis but also extremely sensitive to radiation.[61] In this study, $CD34^+$ hematopoietic stem cells were transfected with a lentiviral self-inactivating (SIN) vector and two days later were irradiated at doses ranging from 0-8Gy. After twelve days, the surviving cells were analyzed for MDR1. As the dose of radiation increased, the proportion of surviving cells positive for MDR1 also increased, showing the radioprotective effect. Additionally, MDR1 was shown to confer radioprotection to hematopoietic cells differentiated from the $CD34^+$ stem cells.[61]

The further use of MDR1 as a clinical radioprotective agent depends on improvements in gene therapy targeting. MDR1 is a gene commonly upregulated in cancers that provides the cancers with resistance to common chemotherapeutics. Thus it is critical that the delivery vector used for MDR1 be able to target the population of normal tissue for protection, without off target expression within tumors.

Snail Family Zinc Finger 2

Snail Family Zinc Finger 2 (SNAI2 or Slug) is a member of the Slug/Snail transcription factor family that is known to suppress radiation-induced apoptosis.[62] This is accomplished by blocking the p53-induced expression of PUMA and thus blocking apoptosis through the mitochondrial pathway. Therefore, SNAI2 has potential use in radioprotective gene therapy.

Maier et al. developed a lentiviral bicistronic SIN vector to test the ability of SNAI2 to protect TK6 cells *in vitro*.[63] In their experiments, SNAI2 was shown to improve cell survival following irradiation by reducing apoptosis and decreasing expression of PUMA following irradiation.

SNAI2 shows promise as a radioprotective gene therapy agent as suppression of PUMA-mediated apoptosis could lead to protection in a variety of tissues. Further studies should be undertaken however, as SNAI2 could potentially enhance tumor invasion.[64]

Interleukin 3

IL-3 is a cytokine that plays an important role in regulating the development and proliferation of multiple hematopoietic cell lineages.[65] However, this increased proliferation is not lineage specific, and can lead to stimulation of basophils and mast cells.[66,67] Proliferation of these cells can lead to negative effects due to increased inflammation. Additionally, it appears that IL-3 plays a role in regulating the vascular system, in particular vasculogenesis.[68] The ability of IL-3 to stimulate hematopoietic cells led to exploration of IL-3 as a potential chemo- and radioprotective agent.[69,70]

Chapel et al. explored the potential of an antibody-based targeting system for the delivery of IL-3 *in vivo* following success with the strategy *in vitro*.[70] In this system, a plasmid encoding the IL-3 protein was covalently linked to IgG mAbs specific for CD117 (c-kit). The animal models were then intravenously injected with this construct and the expression of IL-3 in various tissues assayed.

Following injection of the construct into the animal model, evidence of transfection was found in the bone marrow and spleen by PCR. This expression was transient, and was only present until day 10 post injection.

Though IL-3 itself is not an ideal candidate for radioprotection due to its non-specific stimulation of hematopoietic cell lineages, the antibody-conjugation method used by Chapel shows some promise due to the specificity with which the transfection occurs. Further work should be done to evaluate whether physiologically relevant quantities of radioprotective agents can be produced via cells transduced in this manner.

Hepatocyte Growth Factor

Hepatocyte Growth Factor (HGF), so named because of its ability to induce mitosis of hepatocytes,[71] is known to have potent mitogenic and anti-apoptotic effects in a variety of tissues.[72] The activities are most pronounced in epithelial and endothelial cells, and HGF

has been shown to have angiogenic activity.[73] Because of these capabilities, HGF has been tested as a radioprotector of tissues that express C-Met, the receptor of HGF.

Hu et al. utilized an adenoviral vector to express HGF in a model of radiation-induced heart disease.[74] In their experiments, rats received 20Gy of radiation locally to the heart. As a radiation mitigation intervention, two weeks later, some of the rats were treated with adenovirus expressing HGF (AdHGF), while others received an empty adenovirus vector (AdNull). These adenoviral vectors were administered by performing a thoracotomy and directly injecting the vectors into five locations in the myocardium of the left ventricular wall.

The administered AdHGF successfully transduced the myocardium with elevated levels of HGF 3 and 7 days post administration. Using myocardial contrast echocardiography 120 days post irradiation, the hearts of rats receiving AdHGF were shown to have increased local perfusion. Further cardiac function studies at 180 days post irradiation showed preservation of left ventricular contractile function in rats that received AdHGF. On histology, there was less fibrosis in hearts that received AdHGF.[74] Cardiac dysfunction in radiation-induced heart disease is thought to be induced by damage to the microvasculature leading to decreased perfusion. Also, increases in fibrosis of the myocardium are thought to play a role. Thus AdHGF administration opposed these developments through its angiogenic and anti-fibrotic mechanisms.[74]

The hematopoietic potential of HGF makes bone marrow an attractive target for HGF-mediated radioprotection. Li et al. tested whether an adenovirus expressing HGF (AdHGF) could protect the bone marrow of mice following whole body irradiation.[75] In these experiments, AdHGF was administered to mice via tail vein injection 48 hours before the mice received radiation. From 7 to 28 days after administration of AdHGF, levels of HGF in the blood of the mice were found to be elevated. The mice receiving AdHGF were found to have higher RBC and WBC counts. AdHGF also affected levels of other cytokines following irradiation, with pro-hematopoietic erythropoietin and IL-6 levels increased and anti-hematopoietic IFN-γ levels decreased. On histology, administration of AdHGF preserved the cellularity of the bone marrow and prevented thymic atrophy. Finally, the mice received AdHGF before irradiation showed increased survival.[75]

HGF has great potential as a radioprotective agent for some of the tissues most often affected by radiation therapy. However, there is a key caveat to the use of HGF radioprotection. It has been shown that HGF promotes the growth and metastasis of cancer cells.[76] Thus giving HGF radioprotection therapy to patients who have a tumor is not advisable. Further developments in targeting and localization of gene therapy could circumvent this problem in the future.

Fibroblast Growth Factor 2 and Vascular Endothelial Growth Factor

Fibroblast Growth Factor 2 (FGF2) and Vascular Endothelial Growth Factor are two growth factors known to play roles in angiogenesis. The binding of FGF2 to its receptors (FGFR-1 and FGFR-2) induces angiogenesis and proliferation of endothelial cells.[77] VEGF is known to induce angiogenesis and lymphangiognesis, as well as acting as a survival factor for

endothelial cells. VEGF can also induce vascular permeability.[78] As radiation-induced damage to endothelium is known to be a key factor in the development of further pathology,[79] FGF2 and VEGF have been used to protect the microvasculature and thus other tissues.

Cotrim et al. sought to use the angiogenic capabilities of FGF2 and VEGF to protect salivary glands in a model of xerostomia, a common side effect of radiation therapy to the head and neck.[80] In this work, adenoviral vectors expressing FGF2 and VEGF were constructed (AdFGF2 and AdVEGF), and mice were pretreated with these vectors 48 hours before irradiation. These vectors were administered by retrograde ductal delivery to the submandibular glands. After 48 hours, FGF2 and VEGF were detected in aqueous extracts from the salivary glands with none detected in the serum. Additionally, mice that received AdFGF2 or AdVEGF had increased preservation of microvessel density within the submandibular glands, as compared to the vector control. At 8 weeks post irradiation, the salivary flow of irradiated mice was measured. Mice receiving AdFGF2 and AdVEGF had markedly increased salivary flow as compared to irradiated mice receiving the control vector.[80]

Administration of angiogenic growth factors such as FGF2 and VEGF has potential as a therapy for the prevention of salivary gland damage and xerostomia. Caution has to be taken in the administration of growth factors that could also potentially act in a pro-tumorigenic fashion. The availability of retroductal delivery of vectors and the absence of expressed growth factors in the serum following salivary gland transduction are both encouraging features of this therapy. However, further studies will have to undertaken to confirm the localization of the expression.

Keratinocyte Growth Factor

Keratinocyte Growth Factor (KGF or FGF7) is a member of the fibroblast growth factor family that signals through the FGFR2B receptor.[81] Involved in paracrine mesenchymal-epithelial signaling, KGF powerfully stimulates mitogenesis, migration, and differentiation of epithelial cells. Recombinant KGF has been studied in animal and human models of acute lung injury[82] and has received FDA approval for the treatment of oral mucositis induced by chemotherapy.[83]

Zheng et al. used adenoviral vectors with retroviral elements[84] expressing KGF (AdKGF), administered to the salivary glands by retrograde ductal instillation in models of radiation-induced oral mucositis[85] and salivary hypofunction.[86] Treatment with AdKGF one day before irradiation reduced the severity of tongue ulceration in both single and fractionated irradiation schemes, with histology showing preservation of tongue epithelial thickness.[85] Additionally, the AdKGF groups showed improved weight gain, as compared to the vector control group. Administration of AdKGF improved salivary flow in single dose and fractionated irradiation schemes.[86] An important caveat to the use of growth factors for radioprotection is the potential for growth factors to enhance tumor growth.[87] To address this concern, Zheng et al. used AdKGF in an squamous cell carcinoma VII tumor model to determine whether AdKGF affected treatment of the tumor. Despite the presence of the

receptor for KGF in the tumor, treatment with AdKGF showed no effect on squamous cell carcinoma VII tumor growth.[86]

AdKGF has shown promising results in treatment of two common oral side effects of XRT. Also, the evidence that treatment with AdKGF does not affect the growth of solid tumors makes this strategy even more attractive for radioprotection in a cancer context.

Erythropoietin

Erythropoietin (Epo) is a glycoprotein produced in the renal cortex in response to decreases in oxygen within the tissue. This oxygen regulation is accomplished by the hypoxia-inducible transcription factors (HIFs).[88] The canonical function of Epo is to enhance erythropoiesis by acting on erythrocytic precursors, though the presence of Epo receptors in other tissues has stimulated interest in exploring other potential function of Epo. Studies have examined Epo as a protective agent in ischemia-reperfusion injuries of the heart and kidney[89] and in acute lung injury models.[90]

Rocha et al. developed an adenoviral vector with retroviral elements[84] (to extend transgene expression) expressing Epo (AdEpo) to test in a mouse model of radiation-induced dry eye syndrome (DES).[91] The vector was administered via submandibular gland duct cannulation one day before the animals were irradiated. Mice who received AdEpo before irradiation, in contrast to controls, showed increased tear production, as well as preservation of the epithelial layers of the cornea. Inflammatory (IL1β, TNF-α, and ICAM-1) and oxidative stress markers (glutathione peroxidase-3) did not show altered expression in the lacrimal gland between the groups; however, mice receiving AdEpo showed higher levels of VEGF receptors, indicating a possible mechanism of AdEpo mediated protection.[91] Further work would need to carried out to determine the exact mechanism of Epo mediated protection. Additionally, studies should be performed to examine the effect of AdEpo in the context of a tumor.

Aquaporin-1

Aquaporin-1 (AQP1) is a member of a large membrane channel family that allows water to pass through the lipid bilayer of cell membranes. AQP1 is highly specific for water, excluding even hydronium ions (H_3O^+), and permits the rapid movement of water at a rate similar to diffusion.[92] Movement through the AQP1 channel is bidirectional, with the direction of movement governed by the osmotic gradient. These features lead Baum et al. to explore the use of vectors expressing AQP1 to treat xerostomia, a condition of decreased saliva production that commonly occurs following irradiation of the head and neck. In their model, AQP1 expression in ductal cells of salivary glands would permit the movement of water across these cells into the lumen of the salivary gland, by taking advantage of pre-existing osmotic gradients.[93] This increased fluid movement would alleviate many of the symptoms of xerostomia.

To accomplish gene transfer to the ductal cells of the salivary gland, Baum et al. used an adenovirus expressing AQP1 (AdAQP1) and administered the virus using retroductal cannulation. After establishment of the strategy *in vitro*,[94] work in small and large animal

models yielded promising results, with marked increased in salivary gland output following irradiation in rat and miniature pig models.[94,95] In regard to safety, administration of AdAQP1 produced few systemic effects in animal models. Effects noted were an increase in white cell count in miniature pigs[95] and reductions in food consumption, weight gain, and persistent inflammation, all in female rats.[96]

In light of these pre-clinical results, a phase I clinical trial evaluating AdAQP1 in patients with xerostomia from head and neck irradiation was carried out.[97] Through day 42 of the study (the time period covered in the report), there were few adverse events attributed to the treatment and no severe adverse events. Of the eleven patients treated, six showed an improvement in saliva flow (60-540%), and five of these six reported subjective improvement in their symptoms. Saliva flow rates in the remaining five patients showed no improvement.

Though both pre-clinical and clinical trials show promise of efficacy of this strategy to treat xerostomia, an adenovirus vector such as that used in these studies is not ideal for the treatment of xerostomia, as the expression mediated by this vector is short term. In light of this, Baum et al. are developing an adeno-associated virus vector expressing AQP1.[98] This AAV vector could provide the long term expression of AQP1 necessary to treat radiation-induced xerostomia.

Future Directions

Radioprotection by gene therapy requires effective gene delivery, that is, efficient and specific gene delivery. Also, radioprotection requires *in vivo* gene delivery, which markedly increases the difficulty of effective gene delivery. Current vectors have not been fully capable of effective gene delivery *in vivo.* Of the vectors that have been used, viruses have shown the most utility *in vivo,* and improvements in viral vector targeting could lead to improvements in the effectiveness of gene delivery.

Several strategies are currently being used to improve the specificity of delivery by viral vectors. Screening virus serotypes to identify unique tissue tropisms,[99] as well as the rationally-guided modification of viral capsids are yielding advances in transductional targeting.[100] These capsid modifications have included the use of proteins from various serotypes to create chimeric viruses,[100] the insertion of phage-biopanning derived peptides,[101] and the genetic incorporation of single domain antibodies (also known as camelid antibodies) into the virus capsid.[6] Transcriptional targeting places the therapeutic gene under the control of a promoter upregulated within the target cell. This strategy has been validated in work targeting vascular endothelium[5] and colon cancer.[102] Finally targeting utilizing RNAi can be used to control expression of the therapeutic gene after delivery.[103] In this strategy, the therapeutic gene is tagged with microRNA response elements for miRNAs that are expressed at a low level in the target tissue but at higher levels in other tissues. Thus, the gene will only be expressed within the target tissue.

The context of radioprotection opens up new avenues for the utilization of these targeting strategies. Changes to protein expression and localization,[104] transcription,[105] and miRNA

levels[106] are known to occur following exposure to ionizing radiation. Thus, these changes could be used to further target vectors to the tissue which has undergone irradiation.

In addition to improving current technologies, new approaches could be used for radioprotection gene therapy. As an alternative biological delivery strategy, gene therapy could be combined with cellular therapy by using cellular therapeutic agents as vectors. This would allow a dual approach to radioprotection through both cell-mediated and transgene-mediated mechanisms. Nanoparticles are a potential non-biological vector that could be used in radioprotection studies.[107]

Gene therapy is a promising therapeutic strategy for radioprotection that should improve the cure rates and reduce side effects of XRT. To reach this goal will require continued development of advanced delivery vectors to enable specific targeting of desired tissues.

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Table 1

Effects of Radiation on Normal Tissue

Temporal Classification	Tissue	Effects
Early (hours to weeks)	Hematopoietic	Lymphopenia, neutropenia, thrombopenia, anemia, death (2.5-5Gy)
	Gastrointestinal	Bloody diarrhea, denudation of epithelia, destruction of intestinal crypt cells, death (5-12Gy)
	Cerebrovascular	Rapid cardiovascular and neurologic breakdown, death (12Gy+)
	Skin	Erythema, desquamation
	Testes	Death of stem cells, sterilization
	Ovaries	Death of oocytes, sterilization
Intermediate (weeks to months)	Lung	Acute pneumonitis
Late (months to years)	Gastrointestinal	Epithelial thickening, fibrosis
	Lungs	Fibrosis
	Bladder	Fibrosis
	Heart	Fibrosis, pericarditis
	Kidneys	Nephropathy, arterial hypertension, anemia
	Liver	Hepatitis, rapid loss of function
	CNS	Transient demyelination, leukoencephalopathy, radionecrosis.

Table 2

Summary of gene therapy strategies for radioprotection.

Gene, Mechanism of Action	Vector	Administration	Effects	Ref.
Superoxide Dismutase (SOD2, MnSOD), radioprotector	Adenovirus	Intratracheal Injection	Decreased alveolitis and expression of IL-1, TNF-α, and TGF-β	12,14
	Herpes Simplex Virus 1	Intestinal Injection	Preservation of villi area	13
	Plasmid/ Liposome	Intratracheal Injection	Decreased expression of IL-1, TNF-α, TGF-β, VCAM-I and ICAM-I, increased survival, no protection of tumors	19,26, 28
		Inhalation	Increased survival	16
		Intravesicular Instillation	Enhanced recovery of barrier function, improved voiding	15
		Intraesophageal Injection	Increased survival, decreased weight loss, tongue ulceration, xerostomia, lipid peroxidation, and recombination	20,21, 24,31
		Intravenous Injection	Increased survival	34
	Minicircle Plasmid	Intraesophageal Injection	Increased survival	18
		Intravenous Injection	Increased survival	18
Catalase, radioprotector	Plasmid/ Liposome	Intratracheal Injection	Increased survival, decreased alveolitis	35
	Retrovirus	Ex-*vivo* infection	Improved engraftment of hematopoietic stem cells	36
Roof-plate specific spondin 1 (Rspondin1), radiomitigator	Adenovirus	Intravenous Injection	Increased survival, decreased weight loss, decreased crypt apoptosis	45
Heat Shock Protein 25 (HSP25), unclear (possible radioprotector or radiomitigator)	Adenovirus	Salivary Gland Injection	Increased salivary gland mass, increased salivary flow rate, reduction of acinar cell apoptosis, reduction of fibrosis	47
		Intravenous Injection	Enhanced bone marrow recovery, protection of hematopoietic stem cells	48
Multidrug Resistance 1 (MDR1), radiomitigator	Retrovirus	*In vitro* Infection	Decreased expression of pro-apoptotic genes, increased cell survival	60
	Lentiviral self-inactivating vector	*In vitro* Infection	Increased cell survival	61
Snail Family Zinc Finger 2 (SNAI2), radiomitigator	Lentiviral self-inactivating vector	*In vitro* Infection	Increased cell survival, apoptosis	63
Interleukin 3, radiomitigator	Plasmid Conjugated with Antibody	Intravenous Injection	Expression of IL-3 in bone marrow and spleen	67
Hepatocyte Growth Factor (HGF), radiomitigator	Adenovirus	Myocardial Injection	Improved local perfusion, decreased fibrosis, improved ventricular function	71
	Adenovirus	Intravenous Injection	Increased RBC and WBC, increased cellularity of bone marrow, increased	72

Gene, Mechanism of Action	Vector	Administration	Effects	Ref.
			survival	
Fibroblast Growth Factor 2 (FGF2) and Vascular Endothelial Growth Factor (VEGF), radiomitgator	Adenovirus	Retrograde Ductal Delivery to Salivary Glands	Increased microvessel density, increased salivary flow	77
Keratinocyte Growth Factor (KGF), radiomitigator	Adenovirus with retroviral elements (ref. 81)	Retrograde Ductal Delivery to Salivary Glands	Reduced oral ulceration, improved weight gain, improved salivary flow, no effect on tumor growth	85, 86
Erythropoietin (Epo), radiomitigator	Adenovirus with retroviral elements (ref. 81)	Retrograde Ductal Delivery to Salivary Glands	Increased tear production, corneal epithelium preservation, increased VEGF receptor expression	91
Aquaporin-1 (AQP1), restoration of tissue function	Adenovirus	Retrograde Ductal Delivery to Salivary Glands	Increased salivary gland output in animal and human trials	94-97
	Adeno-Associated Virus	Retrograde Ductal Delivery to Salivary Glands	Increased salivary gland output	98