Intraoral Manganese Superoxide Dismutase-Plasmid/Liposome (MnSOD-PL) Radioprotective Gene Therapy Decreases Ionizing Irradiation-induced Murine Mucosal Cell Cycling and Apoptosis

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Abstract. Background: Single or multiple intraoral administrations of manganese superoxide dismutase-plasmid/liposomes (MnSOD-PL) to C3H/HeNHsd mice receiving single fraction or fractionated ionizing irradiation to the head and neck region have been shown to significantly decrease mucosal ulceration, weight loss and to improve survival. Materials and Methods: To elucidate the mechanism of irradiation protection by MnSOD-PL and explore possible additive or synergistic protective effects with Amifostine (WR2721), mice received a single fraction of 19, 22.5, 25 or 30 Gy, or 24 fractions of 3 Gy irradiation to the oral cavity and oropharynx. Multiple parameters of irradiation-induced toxicity were quantitated in subgroups of each irradiated group of mice treated with single or multiple administrations of intraoral MnSOD-PL and/or intravenous WR2721. Results: In 19 Gy single fraction irradiated mice, MnSOD-PL treatment the day before irradiation alone or in combination with intravenous WR2721 significantly decreased the irradiation induction of mucosal cell cycling as measured by 5-bromo-2-deoxyuridine (BuDR) uptake in oral cavity mucosal cells at 48 hours and decreased ulceration of the tongue at nine days after irradiation compared to control, irradiated or irradiated, WR2721-treated mice. Mice treated in single fractions of 22.5, 25 or 30 Gy showed MnSOD-PL protection against irradiation-induced oral mucosal apoptosis and xerostomia measured in decreased saliva output. In fractionated irradiated mice, twice weekly hemagglutinin (HA) epitope-tagged MnSOD uptake in oral cavity and tongue mucosal cells was not detectably altered by daily WR2721 intravenous administration. Mice treated with both radioprotective agents (MnSOD-PL and WR2721) demonstrated a significant decrease in irradiation-induced xerostomia (measured as reduced salivary gland output volume), mucosal ulceration and improved survival. Conclusion: Enhanced salivary gland function in WR2721-treated mice in the absence of detectable mucosal protection, coupled with relatively low uptake of HA-MnSOD in the salivary glands of intraorally-treated mice, suggests that a combination of both radioprotective agents may prove optimally effective for the prevention of the acute and late normal tissue toxicities of fractionated radiotherapy for head and neck cancer.

A major complication of chemoradiotherapy for head and neck cancer is toxicity to normal tissues (1-5). Most prominently xerostomia, resulting from irradiation of major and minor salivary glands (4) and mucosal alterations, resulting from toxicity to proliferating epithelial tissues in the oropharynx, oral cavity and tongue (2, 5), results in dose-limiting toxicities, which are exacerbated by administration of effective chemotherapeutic agents (6, 7). Intensity-modulated radiotherapy (IMRT) protocols have facilitated treatment plans, which can spare one parotid gland and significantly reduce the late complications of xerostomia (1); however, other strategies of normal tissue protection are needed. The radioprotective agent Amifostine (WR2721) has been shown to provide irradiation protection of the salivary glands (6, 8-17), perhaps attributable to an increased concentration of WR2721 in salivary gland tissue (8, 9, 17). Both IMRT and WR2721 radioprotective strategies might be further enhanced by the availability of yet another radioprotective strategy, which could focus on the protection of epithelial mucosal surfaces within the radiation therapy target volume.

We have previously demonstrated that intraoral administration of MnSOD-PL to mice bearing orthotopic floor of the mouth tumors provided significant protection for...
the oral cavity, oropharyngeal and tongue mucosa while not protecting tumors from irradiation (18). In previous experiments, comparison of MnSOD-PL intraoral administration with systemic WR2721 showed no additive or synergistic effects in normal tissue protection of single fraction, irradiated mice (18). However, the known concentration of WR2721 in salivary glands (8) and the recent reports of a significant reduction in xerostomia in patients receiving WR2721 as part of clinical protocols for chemoradiotherapy of head and neck cancer (8, 9) suggested that the two agents might prove additive or synergistic in fractionated radiation therapy models. Organ-specific irradiation protection with MnSOD-PL has been demonstrated in the murine lung (19-21), esophagus (22-24) and oral cavity/oropharynx (18). Other studies suggested organ-specific irradiation protection using MnSOD transgene targeted to intestine (25) or bladder (26). In each of these studies, a relative lack of uptake and expression of transgene in tissues outside the target organ was documented (27).

In the present studies, we sought to determine whether the relative effectiveness of WR2721 in concentrating activity and protection to salivary glands could be combined with MnSOD-PL protection of the mucosal epithelium of the oral cavity and oral mucosa as a potential therapeutic paradigm for additive or synergistic irradiation protection.

Materials and Methods

Mice. Female C3H/HeNHsd mice (Sprague-Harley, Indianapolis, IN, USA) were housed 5 per cage and maintained by the Division of Laboratory Animal Resources of the University of Pittsburgh, USA. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh.

Mouse irradiation. Hemagglutinin-epitope manganese superoxide dismutase (HA-MnSOD) contains the human MnSOD transgene epitope-tagged with a hemagglutinin transgene under the control of a CMV promoter (28). HA-MnSOD plasmid/liposome complexes were prepared by mixing 200 μg of plasmid (20 μl) DNA with 11.2 μl of lipofectant (Invitrogen, Inc., Carlsbad, CA, USA), incubating for 30 minutes at room temperature, adding 69.8 μl of water and storing on ice until the time of injection. Mice were injected i.p. with Pilocarpine (25 mg/kg) to stimulate saliva output and the saliva was collected following irradiation, mice were injected 24 hours before irradiation with 100 μl of water into the oral cavity, saliva output was determined (18). At various times after irradiation, mice were injected i.p. with Pilocarpine (25 mg/kg) to stimulate saliva output and the saliva was collected over 5 minutes. Since the density of saliva is 1 mg/ml, the volume of saliva was calculated by weighing the saliva and recording the results in μl. The results are reported as volume of saliva (μl) per minute.

Results

HA-MnSOD-PL intraoral administration decreases irradiation-induced mucosal cell cycling. Groups of C3H/HeNHsd mice first received 19 Gy irradiation to the oral cavity/oropharynx according to the Materials and Methods section. The first

Histology. Following sacrifice, the tongue was removed, frozen in OCT and sectioned. The sections were stained with hematoxylin and eosin (H&E) and examined for ulceration.

BuDR labeling. Forty-eight hours after 19 Gy, the mice were injected (100 μl) i.p. with 50 mg/kg of BuDR. The mice were sacrificed 1 hour later, the tongue removed, frozen in OCT and sectioned. The sections were fixed in chilled ethanol, washed in PBS and stained for BuDR incorporation using a "5-bromo-2-Deoxyuridine Labeling and Detection Kit II" (Roche Diagnostic Corporation, Indianapolis, IN, USA) by incubating the slides in a 1:10 dilution of an anti-BuDR antibody at 37°C for 2 hours. Sections were then covered in a 1:10 dilution of alkaline phosphatase conjugated anti-mouse IgG and incubated for 30 minutes at 37°C. The sections were washed in PBS, immersed in freshly prepared color substrate solution (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate) and incubated at room temperature for 15-30 minutes. The slides were coverslipped and examined microscopically and the percent of cells incorporating BuDR calculated. The score represented the number of cells in the DNA synthesis phase of proliferation.

Measurement of irradiation-induced apoptosis in the oral cavity. C3H/HeNHsd female mice had been injected 24 hours earlier with either MnSOD-PL or LacZ-PL, or control mice were irradiated to 22.5 Gy and sacrificed 24 hours later. From these mice the tongue was removed, frozen in OCT and sectioned. Apoptotic cells were determined by staining slides using a DeadEnd Fluorometric Tunel Kit (Promega, Inc., Madison, WI, USA). Sections were fixed in 4% formaldehyde, incubated in a 1:10 dilution of an anti-BuDR antibody at 37°C for 2 hours. Sections were then covered in a 1:10 dilution of alkaline phosphatase conjugated anti-mouse IgG and incubated for 30 minutes at room temperature and the equilibration buffer removed. The sections were covered in equilibration buffer containing TdT enzyme, ATP, CTP, GTP and FITC-conjugated UTP, and incubated in the dark for 1 hour at 37°C. The reaction was stopped by the addition of 2 X SSC for 15 minutes at room temperature. The sections were washed in PBS, coveredslipped with anti-fade and examined microscopically for cells expressing FITC, which is indicative of apoptotic cells. The percent of apoptotic cells was determined.

Saliva output. As an indicator of irradiation-induced damage to the oral cavity, saliva output was measured (18). At various times following irradiation, mice were injected i.p. with Pilocarpine (25 mg/kg) to stimulate saliva output and the saliva was collected over 5 minutes. Since the density of saliva is 1 mg/ml, the volume of saliva was calculated by weighing the saliva and recording the results in μl. The results are reported as volume of saliva (μl) per minute.
experiments were carried out with a single 19 Gy fraction. Subgroups of mice received intraoral HA-MnSOD-PL therapy 24 hours prior to irradiation alone, or with WR2721 intravenous administration on the day of irradiation. The method of intraoral MnSOD-PL administration is shown in Figure 1. Control groups included WR2721 alone, irradiation alone, or no treatment. The mice were evaluated for percent of cells in the oral cavity and oral mucosa going through the DNA synthesis (S) phase by the technique of BuDR labeling. Mice were sacrificed at representative time points including 24, 48, 72 hours and 9 days after irradiation. Histopathological sections from the oral mucosa and tongue were scored for percent ulceration. In subgroups, animals were injected with intravenous BuDR according to the Materials and Methods. At the time of sacrifice, BuDR-injected mice were analyzed for percent of cells in the mucosal surface of the oral cavity, which had taken up BuDR as an indication of cell cycling. The dual antibody staining technique used an anti-BuDR antibody and a second antibody with an alkaline phosphatase tag, facilitated an alkaline phosphatase immunohistochemical analysis of percent positive cells. As shown in Table I with data at 48 hours after irradiation, mice receiving HA-

Table I. Effect of HA-MnSOD-PL, WR2721 or both radioprotectors on percent of mucosal cells showing BuDR incorporation 48 hours after 19 Gy irradiation to the oral cavity and oropharynx of C3H/HSNsd mice.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>19 Gy Radiation Alone</th>
<th>19 Gy + HA-MnSOD-PL</th>
<th>19 Gy + HA-MnSOD-PL + WR2721</th>
<th>19 Gy + WR2721</th>
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<tr>
<td>Mean±Std. Error</td>
<td>15.7±0.8</td>
<td>23.5±4.9</td>
<td>16.2±1.4</td>
<td>14.0±0.7</td>
<td>22.6±2.8</td>
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Groups of 10 mice were treated with WR2721, HA-MnSOD-PL or LacZ-PL, combinations of agents, or nothing prior to 19 Gy irradiation to the head and neck region. WR2721 was given i.p. at 150 mg/kg 30 minutes before irradiation and 100 μl HA-MnSOD containing 200 μg plasmid DNA was given 24 hours prior to irradiation. All mice were injected with BuDR 48 hours after irradiation. Then, after an additional hour, all mice were sacrificed. Tissues were removed, sectioned and analyzed for percent BuDR uptake in at least 10 sections of oral mucosa and tongue in at least 5 mice per group. The results are presented in percent BuDR-positive cells in the basal mucosal cell layers. After performing t-tests, several statistically significant differences were found between several different groups. Irradiation-treated mice as well as WR2721 or LacZ-PL-treated mice showed increased BuDR uptake over control, p=0.0293, 0.0016 or 0.0072, respectively. However, HA-MnSOD-PL or HA-MnSOD-PL plus WR2721 treatment did not show a significant increase in the irradiation-induced BuDR uptake (p=0.1181 and =0.7453, respectively).

Figure 1. Method of HA-MnSOD administration to non-anesthetized mouse. The mice are held by the scruff of the neck and tail and are injected orally using a 1 cc tuberculin-type syringe connected to a feeding tube inserted into the oral cavity. The mice received a uniform volume of 100 μl of plasmid/liposome complexes.

Figure 2. Quantitation of ulceration in tissue sections from oral cavity and tongue from mice in each group described in Table 1 at nine days after 30 Gy irradiation. The data show significant ulceration of the tongue in groups induced by irradiation. HA-MnSOD-PL or HA-MnSOD-PL + WR2721 treatment decreased irradiation-induced ulceration, which was significant (*) with the HA-MnSOD group (p<0.05). WR2721 alone did not significantly decrease ulceration. H&E Stain (x 1000).
MnSOD-PL 24 hours prior to irradiation showed a decrease in percent of mucosal cells with irradiation-induced BuDR uptake (14.0±0.7) compared to mice receiving irradiation alone (23.5±4.9), \( p=0.006 \). In contrast, WR2721 administration on the day of irradiation did not significantly decrease irradiation-induced cell cycling as shown by BuDR uptake similar at 48 hours to irradiation alone (21.9±1.5). Mice receiving both HA-MnSOD-PL and WR2721 showed a decrease in irradiation-induced cell cycling (16.2±1.4) in a manner similar to that observed with HA-MnSOD-PL therapy alone. Figure 2 shows the data for quantitation of tongue ulceration in groups of mice receiving HA-MnSOD-PL before irradiation compared to WR2721 alone, both radioprotective agents, and control irradiated mice nine days after 30 Gy. Mice receiving HA-MnSOD-PL before irradiation compared to irradiation alone showed a decrease in ulceration of the tongue compared to other groups of mice. These results indicated that HA-MnSOD-PL administration decreased transgene expression of epitope-tagged protein in the oral cavity and oropharynx while WR2721 alone did not have a detectable protective effect at this level.

Multiple administrations of HA-MnSOD-PL over a 30-day course of 24 fractions of irradiation show continuous transgene uptake and expression. Since transgene expression of HA-MnSOD is known to persist in normal murine tissues for 48-72 hours (28), we reasoned that multiple administrations would be required every 3-4 days for effective protein expression during a prolonged 7- to 8-week clinical radiotherapy treatment course. To translate radioprotective HA-MnSOD-PL gene therapy to clinical protocols of fractionated irradiation for patients with head and neck cancer, multiple administrations of the radioprotective PL would be required. Previous studies have demonstrated persistence of transgene mRNA and protein in epithelial tissues for 48-72 hours (19, 28). A protocol for evaluation of multiple fraction radioprotective gene therapy is shown in Figure 3. We also sought to test whether administration of WR2721 to mice receiving HA-MnSOD would inhibit or increase transgene uptake and expression. Groups of mice received 3 Gy irradiation fractions for the head and neck, daily, for 5 days per week for 5 ½ weeks to deliver a total of 24 fractions. Subgroups of animals received HA-MnSOD-PL intraoral administration twice per week as described in Figure 3. A second subgroup received daily i.p. WR2721 injections during the 24-fraction irradiation study. Another group received both HA-MnSOD-PL and WR2721. The mice were evaluated for weight loss and survival. Subgroups of mice were sacrificed at several time points, and the percent of epitope-tagged mucosal surface cells was quantitated in each group. The 3 Gy/fractionation protocol shown in Figure 3 was well tolerated. In another protocol, we used 4 fractions of 900 cGy.

As shown in Figure 4, HA-MnSOD-PL administration twice per week demonstrated a significant expression of epitope-tagged protein in mucosal basal layer cells over the 900 cGy x 4-fraction experiment time period. Mice receiving multiple HA-MnSOD-PL administrations as the only radioprotective agent showed a consistent 30-50% of mucosal cells positive for epitope-tagged transgene product. Mice receiving a single administration of HA-MnSOD-PL alone with no subsequent administration showed disappearance of epitope-tagged protein after 72 hours. These results confirm the previous studies showing transgene expression of mRNA for transgene MnSOD when delivered by PL and also
expression of transgene product (28). The data establish that multiple administrations of HA-MnSOD-PL to mice receiving a daily fractionated irradiation result in successful sequential uptake and expression of transgene during fractionated radiotherapy protocol. Control, irradiated mice and irradiated mice receiving WR2721 showed no detectable HA-MnSOD epitope-tagged protein (Figure 4). Mice receiving multiple administrations of HA-MnSOD-PL and concomitant daily WR2721 showed persistence of HA-MnSOD epitope-labeled protein in oral cavity mucosal basal layers (Figure 4). These results establish that administration of WR2721 did not detectably decrease the uptake of subsequent HA-MnSOD-PL administrations.

**HA-MnSOD-PL and WR2721 combined therapy provides protection of both mucosal surfaces and salivary glands.** The salivary gland radioprotective effect (but relative lack of mucosal protection) of WR2721 in patients receiving fractionated radiotherapy for head and neck cancer may be attributable to specifically increased salivary gland uptake (7, 8). In a prior publication, a significant WR2721 mucosal radioprotective effect was not detected in a murine model for irradiation-induced mucositis (18), while HA-MnSOD-PL provided significant protection from oral cavity mucosa. These data suggested that a combined agent radioprotection protocol might be additive or synergistic if properly sequenced.

In the next studies, mice received HA-MnSOD-PL prior to irradiation, alone, or in combination with WR2721 i.p. administration before the 19 or 30 Gy irradiation. We compared two radiation doses, 19 Gy and 30 Gy, the latter dose being large enough to produce mucosal acute injury and death. The 19 Gy dose was chosen to allow better mucosal recovery in all groups but also to allow expression of salivary gland toxicity quantitated as prolonged xerostomia. The animals were followed for survival and toxicity. As shown in Figure 5A, administration of WR2721 alone or in combination with HA-MnSOD-PL resulted in increased survival compared to 19 Gy irradiation alone or HA-MnSOD-PL administration prior to irradiation. However, in the larger dose 30 Gy experiment, mice receiving MnSOD-PL had increased survival compared to 30 Gy-irradiated mice or mice given WR2721 prior to irradiation (Figure 5B). At the lower dose, we conclude that WR2721 may protect the salivary glands from irradiation, reflected as an increase in survival, while not preventing mucosal ulceration (Figure 2). We conclude that HA-MnSOD-PL, while protecting the mucosa at 19 Gy, did not provide salivary gland protection,
which caused xerostomia after 10 days and limited survival. In contrast, at the 30-Gy-dose, WR2721 was not effective in protecting salivary gland output or mucosal integrity and, therefore, we did not see an increase in survival. The application of MnSOD-PL protected the oral mucosa from 30 Gy, resulting in increased survival over the shorter time course of 12 days. The data show critical lethal target cell shifting from salivary glands at 19 Gy to mucosal epithelial cells at 30 Gy. Therefore, both MnSOD-PL and WR2721 may be important in the protection of oral cavity mucosal epithelium and salivary glands from irradiation-induced damage.

*MnSOD-PL and WR2721 improves salivary gland function in 24 x 3 Gy fractionated irradiated mice.* Mice that received the 24 X 3 Gy protocol or single fraction irradiation were analyzed for salivary gland function (Figure 6). Saliva output was preserved in the irradiated mice by MnSOD-PL administration in mice receiving 24 x 3 Gy fractions (Figure 6). In contrast, there was no significant preservation of saliva output by MnSOD-PL treatment prior to 19 Gy, while saliva output was preserved in the WR2721 or WR2721 plus MnSOD-PL-treated mice in the 19 Gy experiment (Figure 7). The data establish that irradiation-induced salivary gland dysfunction is a distinct toxicity from normal ulceration and
indicate that different radiation protection strategies may be required for each target organ.

MnSOD administration reduces irradiation-induced oral mucosal cell apoptosis. C3H/HeNHsd mice received intraoral MnSOD-PL or LacZ-PL and were irradiated along with control mice to 22.5 Gy to the oral cavity. Forty-eight hours after irradiation, the mice were sacrificed, tongues removed, frozen in OCT, sectioned and stained for detection of the percent of cells undergoing apoptosis (Figure 8). Mice receiving LacZ-PL or the control irradiated mice demonstrated a significant increase in the percent of apoptotic cells in the oral cavity mucosa ($p<0.0001$). No significant increase in the percent of cells undergoing apoptosis was detected in mice that received MnSOD-PL prior to 19 Gy irradiation. The data confirm that the MnSOD protective effect is at the level of the oral cavity mucosal cell compartment.

Discussion

Irradiation protection of normal tissues remains a major challenge in cancer therapy protocols designed to focus ionizing irradiation on tumor target volumes (1-6, 30). Strategies used have included sophisticated Physics treatment planning (1, 2), IMRT (1), beam shaping profiles (1) and biological methods for normal tissue protection (30-45). Agents that provide normal tissue protection, if suitable for clinical trial, must not also protect tumor tissue in the irradiation target volume (18, 31). MnSOD-PL gene therapy remains an attractive candidate for pharmacologic radioprotection perhaps, in part, because of the known difference in antioxidant handling by tumor compared to normal tissues (32-45). Most squamous and adenocarcinomas demonstrate a deficiency in MnSOD activity (38-41). Solid tumors in vivo or tumor cell lines in vitro, when transfected
to overexpress transgene SOD, demonstrate reduced ability to handle hydrogen peroxide, the product of superoxide dismutation (38-42). Several studies have demonstrated either altered or improved tumor eradication by radiotherapy in vitro and in vivo by overexpression of MnSOD in tumor cells, either alone or in the presence of normal tissue cells (31, 38). Thus, MnSOD transgene therapy is a potentially attractive and safe method by which to protect normal tissues during clinical radiotherapy.

The radioprotective agent, WR2721, has shown promise in clinical trials for irradiation protection; however, concerns have been raised that systemic administration of this agent may provide tumor radioprotection (8-10). The present studies were carried out in an attempt to determine whether the combination of both WR2721 and HA-MnSOD-PL gene therapy could provide synergistic or additive irradiation protection. The results demonstrated that oral mucosal cell cycling, induced by irradiation, was decreased by MnSOD transgene overexpression in normal oral cavity tissues. In contrast, while WR2721 provided protection of the salivary glands from irradiation damage, there was no significant decrease in irradiation-induced cell cycling of oral cavity mucosal cells. The combination of WR2721 and MnSOD-PL administration provided both salivary gland and oral cavity mucosal protection, as evidenced by enhanced saliva output after 10 days and decreased BuDR uptake, respectively. The results of the present studies provide an incentive to further explore protocols by which both WR2721 administration and MnSOD-PL gene therapy can be sequenced to optimize the radioprotection of normal tissues during radiotherapy of head and neck cancers.

Acknowledgements

The authors thank Megan Sylves for administrative assistance and typing of the manuscript. This research is supported by NIH grant #RO1-CA83876, U.S.A.

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Received January 19, 2004
Accepted March 30, 2004