Radiation Induces Endothelial Dysfunction in Murine Intestinal Arterioles via Enhanced Production of Reactive Oxygen Species

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Objective—Endothelial dysfunction and vascular dysregulation contribute to the pathological effects of radiation on tissues. The objectives of this study were to assess the acute effect of irradiation on acetylcholine (Ach)-induced dilation of gut submucosal microvessels.

Methods and Results—Rats were exposed in vivo to 1 to 9 cGy in 3 fractions per week on alternate days for 3 successive weeks for a total dose of up to 2250 cGy. Submucosal microvessels were isolated after varying levels of irradiation. Diameters of isolated vessels were measured using videomicroscopy, and the dose-response relationship to Ach was determined. Dihydroethidine and 2′, 7′-dichlorodihydrofluorescein diacetate fluorescent probes were used to assess reactive oxygen species (ROS) production. After constriction (30% to 50%) with endothelin, dilation to graded doses of Ach (10⁻⁸—10⁻⁴ M) was observed in control vessels (maximal dilation [MD] 87 ± 9%; n = 7). However, Ach-induced dilation was reduced in vessels from irradiated rats (MD = 3 ± 9%; n = 7; P = < 0.05 versus controls). Significant increases in superoxide and peroxides were observed in irradiated microvessels. Irradiated microvessels pretreated with superoxide dismutase–mimetic demonstrated significant improvement in Ach-induced vasodilation compared with irradiation alone, suggesting that superoxide contributes to impaired dilation to Ach after irradiation.

Conclusions—Radiation induces acute microvascular dysfunction in the resistance arterioles of the intestine. Enhanced ROS contribute to this dysfunction and therefore may represent a novel therapeutic target to minimize radiation toxicity in the gut.

Key Words: radiation ■ endothelial dysfunction ■ microvessels ■ dihydroethidine ■ 2′, 7′-dichlorodihydrofluorescein diacetate

Radiation injury to normal tissues was first reported by Roentgen and Pierre Curie as delayed skin healing after exposure to radium. Over the next 20 years, the suppressive effects of radiation on tumor growth were discovered, laying the foundation for widely successful therapy for cancer.

Despite these successes, radiation is associated with acute and long-term toxicity to normal tissue. During the typical 5- to 6-week treatment course of radiotherapy for pelvic cancer, ~80% of patients will develop gastrointestinal complications. Long-term toxicity of pelvic radiotherapy is divided into 2 types of disorders: those that are life-threatening and, more commonly, those manifested with chronic changes in bowel behavior. The life-threatening conditions, such as sepsis, stenosis, fistulation, intestinal failure, perforation, transfusion-dependent bleeding, and secondary cancer development, are estimated to occur in 4% to 8% of patients after 5 to 10 years. On the other hand, there are chronic changes in bowel behavior in almost all irradiated patients. In addition, reports suggest up to 78% of long-term survivors have gastrointestinal symptoms after radiotherapy for gynecological, bladder, and rectal cancers.

The precise sequence of pathological changes that leads to postradiation symptoms is poorly understood. Complications are a function of the volume of the irradiated field and the treatment techniques (eg, overall treatment time, fraction size, radiation energy, and total dose) as well as the properties of the tissue treated (eg, rate of cell division). Surgical wounds within irradiated tissue are subject to an increased incidence of postoperative complications, including delayed wound healing. Several lines of evidence suggest that these postirradiation complications are attributable to capillary damage, microvascular occlusion, and increased fibrosis. In large vessels, this injury involves induction of atherosclerosis with resulting thromboembolic events or stenosis. In smaller vessels, the vascular effects are evident by the development of telangiectasias in skin and submucosal membranes.

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However, the effect of radiation on vasomotor function of microvessels has not been extensively studied.

The vascular endothelium plays an important role in the regulation of vascular tone by releasing 3 major vasoactive compounds: NO, prostacyclin, and endothelium-derived hyperpolarizing factor. Each of these compounds serves a dual role as anti-inflammatory mediators. Thus, the functional status of the endothelium is an important factor regulating the microcirculation. Release of these mediators leads to vasorelaxation and inhibition of platelet aggregation, thus preventing ischemia and vascular occlusion associated with endothelial dysfunction.

Previous studies suggest that radiation impairs endothelial cell function. Sugihara et al were the first to demonstrate impaired endothelium-dependent dilation to acetylcholine (Ach) and A23187 (a Ca$^{2+}$ ionophore that stimulates endothelial release of vasodilator compounds) in irradiated human cervical arteries. These findings are consistent with the results from animal models reported previously. However, no studies have examined the effect of irradiation on submucosal arterial vasomotor function, which could have significant importance on tissue perfusion in this organ with heightened susceptibility to the effects of irradiation.

The purpose of this study was to determine the effects of irradiation on submucosal microvascular endothelial function in rat intestine and the contribution of reactive oxygen species (ROS) to this process.

Materials and Methods

The Medical College of Wisconsin institutional animal care and use committees approved all protocols. Submucosal gut arterioles were dissected from gut specimens obtained from irradiated and sham “normal control” rats used for this study. After resection, tissue samples were preserved as reported previously.

General Protocol for Irradiation

Sprague-Dawley rats were given total body irradiation with 1 to 9 equal fractions of 250 cGy for a total dose up to 2250 cGy; the radiation fractions were given on a Monday/Wednesday/Friday schedule. Rats were irradiated without anesthesia while restrained in a specially constructed plastic jig. Radiation dosimetry was based on measurements done in a tissue-equivalent plastic phantom with Farmer-type ionization chamber. Irradiation was done with a posterior to anterior field using 300-kV x-ray films with a half-value layer of 1.2 mm Cu and a dose rate of 243 cGy/min (defined at midline).

Videomicroscopy

Videomicroscopy was performed as reported previously. Briefly, isolated microvessels from control and irradiated rats were carefully dissected from the submucosal surface of the bowel tissue and transferred to a 20-mL organ culture chamber containing Krebs solution of the following composition (in mmol/L): 118 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, 20 NaHCO$_3$, 0.026 Na$_2$EDTA, and the following composition (in mmol/L): 118 NaCl, 4.7 KCl, 2.5 glucose, pH 7.4. Each end of the arteriole was secured to a separate glass micropipette (20 to 40 μm internal diameter) filled with Krebs buffer and transferred to the stage of an inverted microscope (CK2; Olympus) coupled to a charge-coupled device camera (VV-BL200; Panasonic) and video micrometer (VIA-100K; Boeckeler Instruments, Inc.). Internal vascular diameters were measured throughout the experiment with a manually adjusted videomicrometer as described previously.

Micropipettes were connected to a hydrostatic reservoir, and the vessel was pressurized to 60 mm Hg. The chamber solution was continuously recirculated at 30 mL/min, aerated with 20% O$_2$, 5% CO$_2$, and 75% N$_2$, and warmed to 37°C by an external heat changer. All pharmacological agents were added to the external bathing solution.

Experimental Protocols

Rat submucosal arterioles do not develop substantial myogenic tone. Therefore, after a 60-minute stabilization period, vessels were constricted to 30% to 50% of maximal passive diameter (at 60 mm Hg) by administration of endothelin-1 (ET-1; 10$^{-10}$ to 5×10$^{-10}$ M) or the thromboxane mimetic 9, 11-dideoxy-11α, 9α-epoxy methanoprostaglandin (U-46619; 10$^{-6}$ to 10$^{-3}$ M). Vascular responses to cumulative logarithmic increases in the concentration of Ach (10$^{-6}$ to 10$^{-4}$ M) in the external bathing media were examined. At the end of each experiment, the passive vessel diameter was determined by adding papaverine (10$^{-4}$ M). After recording the dose–diameter relationship, the chamber was washed with 300 mL of fresh buffer for 20 to 30 minutes. Inhibitors or vehicle was added to the circulating bath, and the vessel was stabilized for an additional 30 to 60 minutes. Arterioles were reconstricted and a second dose-response relationship generated. The addition of pharmacological agents produced <1% change in the volume of the circulating bath.

Fluorescence Detection of ROS

The oxidative fluorescent dye hydroethidium (HE; 5×10$^{-6}$ M; Molecular Probes) was used to evaluate in situ production of superoxide according to methods described previously. HE is cell permeable and, when oxidized by superoxide, is converted to 2-hydroxyethidium that is trapped within the nucleus. Fresh arterioles were exposed to HE in a light-protected chamber for 10 minutes at 37°C, then washed with physiological salt solution and examined under a fluorescence microscope equipped with a krypton/argon laser. Ethidium was excited at 488 nm with an emission spectrum of 610 nm. Fluorescence was detected with a 585-nm long-pass filter. In each case, a control vessel treated with manganese tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP; 10$^{-7}$ M; superoxide dismutase [SOD]–nimatect; Sigma) for 1 hour was run on parallel to determine the baseline fluorescence intensity of a vessel in the presence of minimal superoxide. All vessels in the study were tested the same day, with the same microscopic light intensity settings.

To evaluate the in situ production of peroxide, vessels were loaded with 5×10$^{-6}$ M of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). Similar to HE, microvessels were incubated with and without MnTBAP. Dichlorofluorescein diacetate oxidizes rapidly to the highly fluorescent 2′,7′-difluorescein in the presence of peroxides. DCFH-DA was excited at 488 nm with an emission spectrum of 510 nm. Paired images were analyzed for fluorescence within a user-defined region of an arteriolar segment (maximal traceable area of the central portion of the vessel) using the public domain NIH Image program. Artifactual autofluorescent regions were manually eliminated from analysis. Relative average fluorescence intensity was normalized for surface area and compared between control and experimental microvessels. All settings were maintained constant throughout the experiment.

Measurement of Endothelial NO Production

To evaluate the production of NO, vessels were loaded with 10×10$^{-6}$ M of 4-amino-5 methylamino-2′, 7′-difluorescein diacetate (DAF-FM DA, Molecular Probe, Inc.), a pH-insensitive fluorescent dye that fluoresces after the reaction with an active intermediate of NO formed during spontaneous oxidation of NO. Vessels were incubated for 45 minutes at room temperature, followed by washing using Krebs solution for 15 minutes. Similar to ROS detection methods, microvessels were incubated with and without MnTBAP. DAF-FM was excited at 488 nm with an emission spectrum of 515 nm. Paired images were analyzed using the NIH Image program. Fluorescence intensity was analyzed similar to the methods described for ROS detection.
Table delineates diameter change in response to Ach (10^{-4} mol/L; in control and irradiated vessels, mean luminal diameter, number of vessels, and ET-1 or U-44619 dose (constrictors), grouped by condition (control and irradiated). The luminal diameter is maximal passive diameter at 60 mm Hg pressure. Data shown as mean±SD. *P<0.05 vs control.

Materials
ET-1 (Peninsula Laboratories, Inc) was prepared in saline with 1% BSA. U-46619 was purchased from Cayman. A stock solution of U-46619 was made by dissolving in a mixture of 100% ethanol and sodium carbonate. MnTBAP was dissolved in ethanol. DAF-FM was purchased from Molecular Probes Inc. and prepared in dimethyl sulfoxide. Other agents were prepared in distilled water. Final molar concentrations of agents in the organ chambers are reported. None of the pharmacological antagonists produced significant changes in baseline microvessels diameter. All the other chemicals were obtained from Sigma Chemical Co.

Statistical Analysis
Percent dilation was calculated as the percent change from the constricted diameter to the maximal passive diameter (maximal diameter in the experiment at 60 mm Hg, generally the diameter after papaverine). Percent constriction was determined by calculating the percent reduction from maximal diameter after the application of ET-1. Comparisons of percent vasodilation under different treatments were performed using a 2-factor repeated-measures ANOVA using the proc mixed modules from the SAS statistical package with auto-regressive covariance assumptions. Both computations were followed by a Bonferroni correction when significant differences were noted. Maximal percent dilation values were compared by Student’s paired t test. Statistical significance was defined as P<0.05. All data are described as mean±SEM; n indicates the number of microvessels.

Results
Animal Preparation and Microvessel Isolation
A total of 30 Sprague-Dawley rats (20 after irradiation and 10 without radiation; sham controls) were used. Submucosal intestinal arterioles from control and irradiated rats were isolated with an average resting diameter of 129.2±20.3 μm at 60 mm Hg before pharmacological constriction was induced. The number of vessels used for each protocol, mean luminal diameter, preconstrictor dose, and irradiation dose are summarized in the Table.

Endothelium-Dependent Dilation in Irradiated Rats
Vessels isolated from normal rat bowel demonstrated dose-dependent dilation to Ach (maximal dilation [MD] 87±3%;
significant differences were found in the dosage of ET-1 or U-44619 required to obtain 30% to 50% constriction or the dilation to Ach after constriction with either of the 2 preconstricting agents (Table).

**Endothelium-Independent Dilation in Irradiated Rats**

Although the dosages of preconstrictors needed for constriction of vessels from control and irradiated rats were similar, it is possible that radiation may have impaired the ability of the vascular smooth muscle to relax. We therefore assessed whether responses to cumulative doses of papaverine (an endothelium-independent vasodilator) were preserved. As seen in Figure 3, papaverine caused dose-dependent dilation that was similar in the irradiated and control rats.

**Role of ROS in the Impaired Endothelium-Dependent Vasodilation After Irradiation**

We directly assessed ROS (superoxide and peroxides) production in the intact microvessels using intravital dyes and fluorescence microscopy. Dihydroethidine and DCFH-DA24 staining were used to visualize ROS production as described briefly in methods. We observed minimal production of superoxide anion and 2’,7’-dichlorofluorescein (DCF) fluorescence in control microvessels (Figure 4AC and 4AD). In contrast, irradiated gut microvessels demonstrated high levels of superoxide and peroxides (Figure 4AA and 4AB). Using image analysis software to compare relative production of ROS, significant increases in superoxide (322.6±14.7 arbitrary units; P<0.05) and DCF fluorescence production (374.2±112.2 arbitrary units; P<0.05) were observed in irradiated versus nonirradiated gut, respectively, after the ninth dose of irradiation (Figure 4B). To evaluate the cumulative effect of radiation on ROS production, we assessed superoxide and DCF fluorescent production after each irradiation dose (Figure 4C). Vessels isolated from irradiated gut began to demonstrate elevated ROS after the second dose. This stepped increase in ROS immediately precedes the attenuation in Ach-induced dilation as observed in Figure 2, consistent with causal role for ROS in the endothelial dysfunction.

**Effect of MnTBAP (an ROS scavenger) on Irradiation-Induced Endothelium Dysfunction**

To determine whether the excess vascular superoxide found in irradiated gut contributed to the diminished Ach-induced dilation, experiments were performed in the presence of a cell-permeable SOD–mimetic MnTBAP. Irradiated microvessels pretreated with MnTBAP (10⁻⁴ M; 60-minute incubation) demonstrated significant improvement in Ach-induced vasodilation (2.44±8.21% versus 67.9±8.02%; P<0.05; Figure 5A). Furthermore, arterioles were assessed for superoxide and DCF fluorescence ROS production from vessels from irradiated rats pretreated with MnTBAP. In these conditions, we observed a significant reduction in superoxide and DCF fluorescence ROS production in MnTBAP pretreated compared with irradiated arterioles (Figure 5B). Using image analysis software to determine relative levels of superoxide and peroxides, a significant decrease in superoxide (206.6±14.7 versus 322.6±19.4 arbitrary units; P<0.05) and DCF fluorescence ROS (211±8.8 versus 374.2±112.2 arbitrary units; P<0.05) were observed in vessels from irradiated rats pretreated with MnTBAP compared with irradiated vessels without MnTBAP (Figure 5B).

**Effect of Preventive Therapy With Tempol on Ach-Induced Dilation and ROS Production in Irradiated Rats**

We further investigated whether oral treatment with Tempol (water-soluble SOD-mimetic) throughout the irradiation period (3 weeks) could prevent the impairment of endothelium-dependent dilation. Microvessels dissected from irradiated rats pretreated with Tempol (10⁻⁴ M) demonstrated significant improvement in Ach-induced vasodilation (44.2±5.5% versus 8.6±4.4%, respectively; P<0.05; Figure 5C), similar to the improvement observed after a short incubation with MnTBAP. We further assessed the production of superoxide and DCF fluorescent ROS in vessels from irradiated rats with and without Tempol. In these conditions, we observed a significant reduction in superoxide after chronic therapy with Tempol compared with radiation alone (data not shown), similar to arterioles pretreated with MnTBAP.

**Scavenging of NO**

To further examine the role of ROS in radiation-induced endothelial dysfunction, we tested NO production in control and after radiation with and without MnTBAP, using the NO-specific fluorescent dye DAF-FM diacetate. This membrane-permeant probe is converted by intracellular esterases to DAF-FM, which reacts with NO to form a green-fluorescent product. Figure 5D shows images of arterioles loaded with DAF-FM diacetate. Compared with the control, vessels treated with radiation (2250 cGy) revealed a dramatic decrease in DAF-FM fluorescence, which was partially re-
versed by MnTBAP, suggesting that ROS-induced endothelial dysfunction may be attributable to quenching of NO by superoxide. Using image analysis software to determine relative levels of NO, a significant decrease in NO were observed in vessels from irradiated rats, which was restored partially after treatment with MnTBAP (213±4; 160±15 and 198±12, respectively; arbitrary units; *P*<0.05).

**Discussion**

There are 4 major new findings of this study. First, there is a significant loss in Ach-induced NO-mediated vasodilation in submucosal microvessels isolated from irradiated gut. Second, this loss in vasodilatory capacity is attributable to impairment of endothelial not the vascular smooth muscle function. Third, radiation-induced endothelial dysfunction is an early phenomenon associated with increased of oxidative stress. Fourth, early radiation-induced microvascular dysfunction can be restored or prevented using antioxidant compounds with restoration of Ach-induced NO production. These findings increase our understanding of radiation toxicity in the bowel and suggest novel treatment strategies for improving vascular function after radiation treatment to this area.

This work is the first to demonstrate that Ach-induced endothelium-dependent dilation is markedly impaired in irradiated intestine, and the effect is dependent on the radiation dose delivered. The impairment (Figures 2 and 3) occurs as early as 1 week and after only 2 doses of 250 cGy. This finding is consistent with the results from human and animal models of irradiation in other vascular beds.15–17 Furthermore, our data exclude the possibility that irradiation might have induced a generalized dysfunction of the relaxant and contractile capacity of the arterial smooth muscle since the response to papaverine; U-44619 and ET-1 were unaffected in the gut arterioles after 2250-cGy radiation.

ROS (superoxide and hydrogen peroxide) are implicated in a number of pathologic processes, including tissue injury, inflammatory disorders,18,27,28 cardiovascular disease,21,29,30 pulmonary disease,30 and degenerative diseases associated with aging.31 We demonstrate dose-dependent elevations of superoxide anion and peroxides in gut arterioles from irradiated compared with control animals (Figure 3C). The specific
mechanism(s) whereby microvascular superoxide and peroxides generation is increased during radiation injury are not yet defined. This work documented in vitro and in vivo radioprotection using SOD-mimetic agents in irradiated rats (Figures 3B and 4B). This finding contrasts with animal models published previously by other investigators in which free radical scavengers (ie, SOD) did not restore the impaired endothelium-dependent relaxations in the vessels from irradiated rats.16 This effect of ROS on the endothelium may be a consequence of superoxide reacting with NO, which reduces bioavailability of NO. In accordance with this hypothesis, we observed a reduction in NO after radiation that is restored with MnTBAP (Figure 5D).

Interestingly, our group has observed that MnTBAP possesses superoxide and hydrogen peroxide quenching.32 Previous studies have demonstrated that manganese salts actively decompose hydrogen peroxide, thereby protecting cells and tissues from ROS-mediated injury.33–35 Furthermore, Day et al showed that manganic porphyrins are stable redox active compounds that protect endothelial cells because they can catalytically consume hydrogen peroxide.32 Thus, it is not clear whether superoxide or hydrogen peroxide is the ROS responsible for vascular damage. The beneficial effect of Tempol indirectly supports the primary role of superoxide because Tempol does not quench hydrogen peroxide, but more definitive studies are needed.

Study Limitations

DCFH is sensitive to hydrogen peroxide but is not specific for this ROS because other peroxide radicals may also be detected with this method.36,37 Therefore, it remains to be determined which peroxide species are increased after radiation. Ach was the only pharmacological endothelium-dependent dilator used in this study. However, Ach is an important vascular neurotransmitter stimulus that is released in response to parasympathetic nerve stimulation, a vital regulatory mechanism in gut function.38 It will be important to determine whether responses to other vasomotor agonists or shear stress are also impaired after radiation. Future
investigations will evaluate the late radiation effect in rat submucosal arterioles on responses to ACh and other agonists.

A unique aspect of radiation injury is the large bursts of ROS generated. Several potential sources of the ROS include NADPH oxidase, xanthine oxidase, endothelial NO synthase, mitochondria, and inflammatory cells (eg, neutrophils). However, the specific source(s) of vascular ROS production in response to radiation require future studies.

Clinical and preclinical evidence strongly suggest that endothelial dysfunction plays a critical role in the pathogenesis of early and delayed radiation injury of normal tissues, and that a dysfunctional thrombomodulin–protein C pathway may contribute to this condition. The mechanisms responsible for interruption of this pathway are multiple and include direct ROS-mediated mechanisms and indirect mechanisms secondary to radiation-induced inflammation. Hence, this pathway is a promising target for interventions aimed at preventing or treating radiation toxicity in normal tissues.

Summary

This investigation demonstrates profound microvascular endothelial dysfunction in irradiated gut. Excess levels of superoxide and peroxides were found to play a key role in this endothelial dysfunction. Defining the mechanisms that underlie microvascular dysfunction after irradiated tissue should yield important insight into the pathogenesis of radiation-induced gastrointestinal complications. Defining strategies to reverse microvascular dysfunction after radiation treatment may suggest novel therapeutic approaches for the prevention or treatment of radiation injury. More important, our data suggest that microvascular dysfunction is an early phenomenon, and that early treatment with SOD-mimetics may prevent the development of the permanent gastrointestinal complications and comorbidities related to this disease process.

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