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Influence of Endothelin 1 Receptor Inhibition on Functional, Structural and Molecular Changes in the Rat Heart after Irradiation

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Abstract

Radiation-induced heart disease is a severe side effect of thoracic radiotherapy. Studies suggest that mast cells play a protective role in radiation-induced heart disease and that the endothelin (ET) system mediates protective effects of mast cells in other disorders. This study examined whether mast cells modulate the cardiac ET system and examined the effects of ET receptor inhibition in a rat model of radiation-induced heart disease. Mast cell-deficient (Ws/Ws), mast cell-competent (+/+) and Sprague-Dawley rats received 18 Gy irradiation to the heart. Left ventricular mRNA of ET1 and its receptors (ETA and ETB) was measured in Ws/Ws and +/+ rats at 1 week and 3 months. Sprague-Dawley rats were treated with the ETA/ETB antagonist bosentan, and at 6 months cardiac changes were assessed using the Langendorff perfused rat heart preparation, immunohistochemistry and real-time PCR. Ws/Ws and +/+ rat hearts did not differ in baseline mRNA. In contrast, +/+ rats hearts exhibited up-regulation of ET1 after irradiation, whereas Ws/Ws rats hearts did not, suggesting the possibility of interactions between mast cells and the cardiac ET system. Bosentan induced reductions in left ventricular systolic pressure, developed pressure and $+dP/dt_{max}$ but did not affect fibrosis. Because of the known opposing effects of ETA and ETB, studies with selective antagonists may clarify the role of each receptor.

INTRODUCTION

Radiation-induced heart disease is a potentially life-threatening side effect of radiotherapy of thoracic and chest wall tumors whenever all or part of the heart is included in the radiation field. Radiation-induced heart disease presents clinically several years after irradiation, and the disease process is progressive. Clinical manifestations include accelerated atherosclerosis, conduction abnormalities, injury to cardiac valves, and pericardial and myocardial fibrosis (1–3). Randomized studies show a significant increase in cardiac events in patients who were treated with radiotherapy for thoracic and chest wall tumors, including those resulting from Hodgkin's disease or breast cancer (4,5). In fact, the heart continues to be a major organ at risk

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SUPPLEMENTARY INFORMATION

Individual relative mRNA levels of mast cell-deficient (Ws/Ws) and mast cell-competent (+/+) hearts at 1 week and 3 months after local heart irradiation or sham irradiation. <http://dx.doi.org/10.1667/RR1093.1.S1>

in thoracic radiotherapy despite recent advances in radiation delivery and treatment planning techniques. Nonetheless, there is currently no method or approach for minimizing, preventing or reversing radiation-induced heart disease.

In a previous preclinical study on the role of mast cells in radiation-induced heart disease, mast cell-deficient rats showed more severe structural and functional changes in the heart in response to radiation (6). This suggests that mast cells, in contrast to what has been the prevailing assumption, play a predominantly protective role in radiation-induced heart disease. In addition, mast cell-deficient rats show more severe adverse remodeling in other cardiac disease models (7,8), suggesting that mast cells are protective.

Mast cells express a wide variety of mediators, including histamine, several growth factors and cytokines, and proteases by which they are able to activate or inactivate cell surface receptors (9) or cellular mediators in the extracellular space. Some mast cell mediators are preformed and stored in granules ready for immediate release, whereas others are synthesized on demand (10). While mast cell activation leads to the release of mediators that are mainly pro-inflammatory and pro-fibrogenic, non-activated mast cells play a critical role in regulating tissue homeostasis and responses to tissue injury.

Several observations suggest that the protective effects of mast cells may involve crosstalk with the ET system. Radiation-induced tissue injury is associated with up-regulation of endothelin 1 (ET1) (11). ET1, which was first discovered to be a potent vasoconstrictor, has a broad range of other properties, including pro-inflammatory and pro-fibrotic effects (12,13). ET1 exerts its effects through two receptors, ETA and ETB, which are expressed by a wide variety of cell types (13,14). The relationship between mast cells and the ET system is complex. *In vitro*, mast cells both produce and degrade ET1 (15–17). In addition, mast cells express ETA, which upon activation by ET1 induces mast cell degranulation (18). *In vivo*, these interactions between mast cells and the ET system appear to have the following net effects: In the rat heart, ET1 increases matrix metalloproteinase activity through increased mast cell maturation and activation (19,20). Systemic administration of ET1 activates mast cells to release proteases that degrade ET1. Mast cells thereby reduce systemic toxicity of ET1 (16).

The role of ET1 in cardiovascular pathology has been studied extensively. Short-term up-regulation of ET1 and its receptors may serve as a mechanism to maintain cardiac function in certain cardiovascular diseases (21,22). Long-term up-regulation of the ET system, on the other hand, may have detrimental effects due to the vasopressor, pro-hypertrophic and pro-fibrotic properties of ET1 (14,23). Hence effects of ET1 receptor inhibition on cardiovascular disease differ, depending on several factors, including the type of disease and the timing and selectivity of the receptor antagonist used (24,25).

Levels of ET1 often correlate with levels of calcitonin gene-related peptide (CGRP), a neuropeptide released by sensory nerves (26,27). CGRP is not only a potent vasodilator but is also known to play a protective role in myocardial injury (28,29). In the guinea pig heart, a negative feedback loop has been proposed to occur in which sensory nerve stimulation leads to CGRP release, which stimulates mast cells to release histamine. Histamine in turn inhibits the release of CGRP from sensory nerves (30).

In this study, we examined the effects of mast cells on the cardiac ET system after local heart irradiation and used the dual ETA/ETB antagonist bosentan (31) to further explore the role of ET1 in the development of molecular, structural and functional manifestations of radiation-induced heart disease in the rat.

MATERIALS AND METHODS

Animal Models

A total of 36 male Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Twenty-six male mast cell-deficient (Ws/Ws) rats and 26 mast cell-competent (+/+) littermates were obtained from Japan SLC (Hamamatsu, Japan). Ws/Ws animals originate from a rat of the BN/fMai strain with a spontaneous 12-base deletion in one locus of the c-kit gene. This Ws/+ rat was crossed with normal (+/+) rats of the Donryu strain (32). The offspring of the resulting hybrids include +/+ and Ws/Ws rats. As a result of their homozygous c-kit mutation, Ws/Ws rats lack functional mast cells as well as melanocytes and interstitial cells of Cajal in the intestine, while their +/+ littermates show no abnormalities for these cell types (33).

All procedures in this study were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences. All animals were maintained in our Division of Laboratory Medicine on a 12:12-h light-dark cycle with free access to food and water. Sprague-Dawley rats (280–320 g, 2 months of age) and Ws/Ws and +/+ rats (320–460 g, 4 months of age) received localized heart irradiation with a single dose of 18 Gy or 0 Gy as described below. Ws/Ws and +/+ rats were killed humanely at 1 week and 3 months after irradiation or sham treatment. Cardiac structural changes were assessed with immunohistochemistry (0-Gy groups: $n = 3$ for each time, 18-Gy groups: $n = 4$ for each time), and gene expression changes were examined with real-time PCR ($n = 3$ for each radiation dose and time). In the ET1 receptor inhibition experiment, Sprague-Dawley rats were treated with the dual ETA/ETB receptor antagonist bosentan or with vehicle from 1 week before until 6 months after irradiation (see below). Six months after irradiation or sham treatment, left ventricular function was measured using the Langendorff perfused rat heart preparation (0-Gy groups: $n = 4$, 18-Gy groups: $n = 6$), structural changes were assessed with (immuno) histochemistry (0-Gy groups: $n = 4$, 18-Gy groups: $n = 5$), and gene expression changes were examined with real-time PCR ($n = 4$ for each group). Ws/Ws and +/+ rats were 4 months of age at time of irradiation and Sprague-Dawley rats were 2 months of age at the time of irradiation to ensure that their hearts were of comparable size at the end of the experiments.

Rat Heart Irradiation

All rats received local heart irradiation or sham irradiation with a method used previously (6, 34). Rats were anesthetized with isoflurane and irradiated with a Seifert Isovolt 320 X-ray machine (Seifert X-Ray Corporation, Fairview Village, PA) operated at 250 kV and 15 mA with 3 mm aluminum and 1.85 mm copper added filtration at a dose rate of 1.17 Gy/min. A single dose of 18 Gy or 0 Gy was administered locally to the heart using parallel opposed fields (anterior-posterior 1:1) with a 19-mm diameter, while the rest of the animal was shielded with lead plates.

Administration of Bosentan

Sprague-Dawley rats were treated with bosentan or vehicle from 1 week before until 6 months after irradiation. Bosentan was kindly provided by Actelion Pharmaceuticals (Allschwil, Switzerland) and was added to the standard rodent chow (TD8640 from Harlan-Teklad, Madison, WI) at 750 mg/kg chow. With this concentration of bosentan, an oral dose of 70 mg/kg per day was obtained at the start of the experiment. TD8640 chow without bosentan was used as the vehicle. Rats were randomly divided into four treatment groups: sham irradiation + vehicle, sham irradiation + bosentan, irradiation + vehicle, and irradiation + bosentan.

Langendorff Perfused Rat Heart Preparation

Hearts were isolated from Sprague-Dawley rats in each of the four treatment groups and immediately perfused through the aorta with an oxygenated Krebs-Henseleit solution (37°C) composed of 118.0 mM NaCl, 27.1 mM NaHCO₃, 3.7 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, 1.0 mM KH₂PO₄, and 11.1 mM glucose at a flow rate of 12.0 ml/g heart per min. Both atria were removed, and the ventricles were paced electrically with platinum contact electrodes positioned on the interventricular septum to obtain a heart rate of 250 beats/min. A fluid-filled balloon catheter connected to a pressure transducer was placed in the left ventricle, and the heart was enclosed in a humidified, temperature-controlled chamber. Cardiac function was monitored by measuring left ventricular diastolic pressure, peak systolic pressure, +dP/dt_{max} (rate of contraction), and -dP/dt_{max} (rate of relaxation) at various preload balloon volumes (60–300 µl, a range that elicited maximum contractility in all preparations). Coronary pressure was monitored continuously with a Statham pressure transducer. In addition to a polygraph recording, all data were digitized and analyzed using acquisition and analysis software (CODAS, DataQ Instruments, Akron, OH).

Histology and Immunohistochemistry

Hearts from Ws/Ws, +/- and Sprague-Dawley rats were examined by immunohistochemistry. For this purpose, hearts were fixed in methanol Carnoy's solution (60% methanol, 30% chloroform, 10% acetic acid) and embedded in paraffin to obtain 5-µm cross sections.

For determination of mast cell numbers, sections were stained with 0.5% Toluidine Blue (Sigma-Aldrich, St. Louis, MO) in 0.5 N HCl for 20 min followed by a 10-min incubation in 0.7 N HCl. The total number of mast cells in 10 ocular fields with a 10× objective was determined. This area covered almost the entire left ventricular area. Mast cell density was calculated as number of mast cells per area (mm²).

Immunohistochemistry for extracellular matrix-associated transforming growth factor β (TGF-β, TGFB), ETA, ETB and collagen I and III was performed with methods established and optimized in our laboratory. Sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked with 1% H₂O₂ in methanol for 30 min at room temperature. Nonspecific antibody binding was reduced by 10% normal rabbit serum or 10% normal goat serum (Vector Laboratories, Burlingame, CA) in 3% dry powdered milk in Tris-buffered saline (TBS) for 30 min. Sections were then incubated with pan-specific rabbit anti-TGF-β (R&D, Minneapolis, MN) at 1:300, or rabbit anti-ETA (Abcam, Cambridge, MA), rabbit anti-ETB (Abcam), goat anti-collagen I, or goat anti-collagen III (both Southern Biotechnology Associates, Birmingham, AL), each at 1:100, for 2 h at room temperature. This was followed by a 30-min incubation with biotinylated goat anti-rabbit IgG or biotinylated rabbit anti-goat IgG (both Vector Laboratories), each at 1:400. Sections were then incubated with preformed avidin-biotin-peroxidase complex (Vector Laboratories) for 30 min. For ETA and ETB immunostaining, bound peroxidase was enhanced with biotin-labeled tyramide followed by streptavidin-bound horseradish peroxidase (TSATM Biotin System, PerkinElmer, Shelton, CT). All peroxidase binding was visualized with 0.5 mg/ml 3,3-diaminobenzidine tetrahydrochloride solution (Sigma-Aldrich) and 0.003% H₂O₂ in TBS.

Quantitative assessment of immunoreactivity was performed with computerized image analysis (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). Areas positive for extracellular matrix-associated TGF-β, interstitial collagen I, or collagen III were measured in 20 fields per section (40× objective) and calculated as area per 100 µm².

RNA Isolation and Real-Time PCR

Steady-state mRNA levels were measured in hearts from Ws/Ws, +/+ and Sprague-Dawley rats. Hearts were snap-frozen in liquid nitrogen and stored at -80°C . Frozen tissue samples from the left ventricle were homogenized in UltraspecTM RNA reagent (Biotecx Laboratories, Houston, TX) according to the manufacturer's instructions. After treatment with RQ-DNase I (Promega, Madison, WI) at 37°C for 30 min, cDNA was synthesized using the High Capacity cDNA Archive KitTM (Applied Biosystems, Foster City, CA).

Steady-state mRNA levels were measured with real-time quantitative PCR (TaqManTM) using the ABI Prism 7700 Sequence Detection System, TaqMan mastermix and TaqMan polymerase, and the following pre-designed TaqMan Gene Expression AssaysTM: rat interleukin 6 (IL6, Rn00561420_m1), TGF- β 1 (Rn00572010_m1), connective tissue growth factor (CTGF, Rn00573960_q1), ET1 (Rn00561129_m1), ETA (Rn00561137_m1), ETB (Rn00569139_m1), CGRP (Rn00569199_m1), manganese superoxide dismutase (MnSOD, Rn00563570_m1). Eukaryotic 18S rRNA (Hs99999901_s1) and rat glyceraldehyde 3-phosphate de-hydrogenase (GAPDH, Rn99999916_s1) were used as internal references (all Applied Biosystems). mRNA levels were calculated relative to levels in vehicle-treated sham-irradiated rat hearts using the $\Delta\Delta\text{Ct}$ method.

Statistical Analysis

Data from experiments with Ws/Ws and +/+ rats were analyzed with a stratified Wilcoxon Mann-Whitney test using observation time (1 week and 3 months) as strata. Stratified analysis is commonly used for assumption-free, non-parametric control of confounding variables. Hence the stratified Wilcoxon Mann-Whitney test allowed the generation of a single P value referring to the difference between Ws/Ws and +/+, rats, while controlling for observation time. This test was performed with StatXact 8 (Cytel, Cambridge, MA), calculating exact two-sided P values (i.e., not asymptotic or Monte Carlo simulated). This software package is designed specifically for distribution-free non-parametric inference and is particularly suited for analysis of small data sets. Langendorff data were tested with repeated-measures ANOVA and all other data were tested with two-way ANOVA, with radiation (0 Gy or 18 Gy) and treatment (vehicle or bosentan) as fixed factors. ANOVA tests were performed using the software package NCSS (NCSS, Kaysville, UT). The criterion for significance was a $P < 0.05$. Data are reported as means $\pm$ SEM.

RESULTS

Effects of Radiation on Mast Cell-Deficient and Mast Cell-Competent Rats

As expected, absence of metachromatic staining with Toluidine Blue in Ws/Ws rat hearts confirmed that these hearts were devoid of mast cells. Figure 1 shows cardiac mast cell numbers per mm^2 in +/+ rats. While we have shown previously that mast cell hyperplasia is present in radiation-induced heart disease (6), the increase in mast cells 3 months after irradiation of +/+ animals compared to time-matched controls did not reach statistical significance ($P = 0.06$) in the current study.

Immunohistochemical staining revealed a particularly strong ETA immunoreactivity on mast cells (Fig. 2A). ETB expression was found mainly on vascular smooth muscle cells in both +/+ and Ws/Ws rats (Fig. 2B). The intensity of ETB staining did not change after irradiation.

No significant differences were observed in the mRNA levels of ET1, ETA and ETB in sham-irradiated Ws/Ws rat hearts compared to sham-irradiated +/+ rat hearts (ET1: 1.5 ± 0.3 in Ws/Ws rats relative to +/+ rats, ETA: 1.3 ± 0.1 , and ETB: 1.2 ± 0.2). Table 1 shows the mRNA levels of ET1, ETA and ETB in irradiated Ws/Ws and +/+ rat hearts relative to time-matched

sham-irradiated controls. Individual sample data of this experiment are presented in a supplementary file. Radiation induced an increase in ET1 mRNA in $+/+$ rat hearts but not in Ws/Ws rat hearts, leading to a difference between the two genotypes ($P = 0.02$). ETA and ETB mRNA did not change after irradiation in either Ws/Ws or $+/+$ rat hearts. To ensure that 18S rRNA was appropriate as internal reference for the real-time PCR assays, *ET1* gene expression in Ws/Ws and $+/+$ rats at 1 week after irradiation was measured once more with GAPDH as the internal reference. ET1 mRNA in irradiated Ws/Ws rats relative to sham-irradiated Ws/Ws rats was 1.0 ± 0.4 . On the other hand, relative ET1 mRNA in irradiated $+/+$ rats was 3.0 ± 0.6 , confirming the data obtained with 18S rRNA as the internal reference.

Interestingly, while CGRP mRNA levels did not differ significantly between sham-irradiated Ws/Ws and $+/+$ rat hearts (levels in Ws/Ws rat hearts relative to $+/+$ rat hearts were 1.2 ± 0.3), a trend of radiation-induced up-regulation in CGRP mRNA was found in $+/+$ rat hearts only, leading to a difference between the two genotypes ($P = 0.04$).

Effects of Radiation and Bosentan on Left Ventricular Function in Sprague-Dawley Rats

Figure 3 shows parameters of Langendorff isolated perfused hearts of Sprague-Dawley rats 6 months after local heart irradiation. Radiation did not significantly alter any of these parameters, although a trend toward an increased diastolic pressure was observed (Fig. 3A). Bosentan treatment resulted in a reduction in systolic pressure in both irradiated and sham-irradiated rat hearts (repeated-measures ANOVA: $F = 5.1$, $P = 0.04$). This reduction in systolic pressure led to a reduction in developed pressure (peak systolic–diastolic pressure) by bosentan treatment in both irradiated and sham-irradiated hearts (Fig. 3C, $F = 8.7$, $P = 0.01$). The rate of contraction, $+dP/dt_{\max}$, is a measure of left ventricular contractility. Bosentan induced a reduction in $+dP/dt_{\max}$ in both irradiated and sham-irradiated hearts (Fig. 3D, $F = 22.8$, $P < 0.001$). No differences were seen in $-dP/dt_{\max}$ or coronary pressure between any of the four treatment groups (data not shown).

Effects of Radiation and Bosentan on Cardiac Structure and Molecular Changes in Sprague-Dawley Rats

At 6 months after irradiation, significant increases were observed in interstitial collagen I ($F = 5.6$, $P = 0.03$) and collagen III ($F = 7.3$, $P = 0.02$) (Fig. 4), in extracellular matrix-associated TGF- β area ($P = 15.0$, $P = 0.001$) (Fig. 5), and in cardiac mast cell density ($F = 8.6$, $P = 0.01$) (Fig. 6). Bosentan had no significant effect on any of these parameters. Again, a particularly strong ETA immunoreactivity was found on mast cells, while ETB expression was found mainly on vascular smooth muscle cells (data not shown).

Table 2 shows left ventricular relative mRNA levels. Radiation induced significant increases in mRNA for ET1 ($F = 8.8$, $P = 0.01$), ETA ($F = 8.5$, $P = 0.01$), and CGRP ($F = 8.9$, $P = 0.01$). Gene expression of CTGF and IL6 was not affected by radiation but was up-regulated by bosentan ($F = 10.2$, $P = 0.01$ and $F = 5.4$, $P = 0.04$, respectively). In each ANOVA, the interaction term between bosentan and radiation was tested but was not significant. Finally, mRNA levels of ETB and TGF- β 1 did not change throughout the experiment.

DISCUSSION

In a previous experiment we have shown that mast cells, in contrast to what has been the prevailing assumption, play a predominantly protective role in radiation-induced heart disease. As shown previously, and again in the current study, cardiac mast cell numbers are reduced in the first weeks after irradiation but increase thereafter. Recent evidence suggests that the endothelin (ET) system may mediate the protective effect of mast cells in certain disorders. Upon mast cell activation by ETA, mast cells may reduce the toxicity of ET1 by the release of

proteases that are able to degrade ET1 (16). A recent study showed that acute increases in cardiac mast cell density due to chronic volume overload in a rat model were prevented by bosentan, demonstrating that cardiac mast cells are responsive to ET1 *in vivo* (20). In the current study, left ventricular mRNA levels of ET1 were increased by radiation at all times investigated but only in mast cell-competent (+/+) rats. Although the sample size in this experiment was small ($n = 3$), these results may suggest interactions between mast cells and the cardiac ET system in radiation-induced heart disease. To investigate the role of ET1 in radiation-induced heart disease, this study was focused on examining the effects of the ET1 receptor antagonist bosentan on cardiac radiation injury in rats.

This study showed that a single dose of radiation locally to the heart induced sustained up-regulation of ET1 mRNA together with an increase in cardiac mast cell numbers in Sprague-Dawley rats. The exact mechanisms responsible for these sustained increases in mast cell numbers and ET1 mRNA are not known. Recently, it has been shown that ionizing radiation induces long-term oxidative stress (35,36). Moreover, oxidative stress is known to affect mast cell function (37,38). Hence it may be speculated that sustained oxidative stress is involved in mast cell activation and up-regulation of ET1. In addition, ionizing radiation is known to induce prolonged endothelial dysfunction (loss of thromboresistance and increased expression of chemokines and cytokines) (39), which is likely involved in sustained changes in tissues after localized irradiation. A wide variety of cell types in the heart, including myocytes, fibroblasts, endothelial cells and mast cells, are capable of synthesizing ET1 (12). In a previous study using mast cell-deficient (Ws/Ws) and mast cell-competent (+/+) rats to examine cardiac transplantation, myocytes and mast cells were found to be the main locations of ET1 protein in +/+ rat hearts (8). Unequivocal identification of the source of ET1 mRNA and relative contributions by cardiac endothelial cells, myocytes, fibroblasts or mast cells may be done by *in situ* hybridization, similar to what has been performed previously (40).

This study examined the effects of the dual ETA/ETB antagonist bosentan on functional, structural and molecular changes in the Sprague-Dawley rat heart after irradiation. The cellular effects of ETA and ETB are highly diverse and are sometimes opposing. While activation of ETA on smooth muscle cells induces the well-known vasoconstrictive effects of ET1, for instance, ETB may be involved in both vasoconstriction and vasodilation (41,42). Both receptors, on the other hand, seem to promote a fibrogenic response in cardiac fibroblasts (43). Therefore, selective ET1 receptor antagonists do not always reduce experimental myocardial fibrosis (44). Because of the complexity of the ET system, a dual ETA/ETB antagonist was chosen for this initial study.

Bosentan reduced systolic pressure and $+dP/dt_{\max}$, which suggest reduced contractility of the left ventricle. Interestingly, bosentan induced reductions of these parameters in irradiated as well as sham-irradiated hearts. Few studies have reported the results of isolated perfused heart preparations after long-term treatment with bosentan or other dual ETA/ETB receptor antagonists in otherwise untreated or healthy animals. In a study on the effects of bosentan on cardiovascular function in diabetic rats, 7 weeks of bosentan treatment reduced developed pressure against left atrial filling pressure in isolated working hearts of all rats, including control rats (45). Long-term inhibition of ET1 receptors not only may directly inhibit the positive inotropic effects of ET1 on the heart but also may lead to activation of compensatory mechanisms in an attempt to reverse the cardiac effects of ET1 receptor inhibition.

This study used computerized image analysis for quantitative assessment of immunohistochemistry results. We have previously done extensive quality testing of our quantitative immunohistochemistry readings, including testing of batch-to-batch variability and variability among observers (46). Our technique showed to be highly reproducible. With this technique, the area of staining is considered and not the intensity of staining. This is because

studies have shown that staining intensity does not reflect protein levels (47). However, one may debate whether quantitative assessment of immunoreactivity is a true measure of protein expression. For instance, in the TGF- β immunostaining, no distinction is made between the latent and active form of the protein. One should keep these considerations in mind when interpreting the quantitative immunohistochemistry results.

Even though bosentan administration resulted in altered cardiac gene expression of several pro-inflammatory and pro-fibrogenic mediators, radiation-induced collagen deposition was not affected. This is a somewhat surprising result, considering the well-known anti-fibrotic properties of bosentan (48). Several explanations may account for these results. It is possible that ET1 simply is not involved in radiation-induced fibrosis in the heart. Although ET1 has been shown to be involved in microvessel dysfunction after exposure to ionizing radiation (11), to our knowledge a role for ET1 in radiation-induced late-occurring adverse tissue remodeling has not been reported. On the other hand, a dual ETA/ETB antagonist may not reveal the role of each receptor subtype, since the effects of ETA and ETB are diverse and are sometimes opposing. Also, a dual ETA/ETB antagonist such as bosentan may block clearance of ET1 by the ETB receptor, which could result in an increase in circulating ET1 levels (49). The use of selective antagonists of ETA and ETB may give further insight into the role of each receptor subtype. Finally, future studies may be designed in which the dose of bosentan is increased to at least 100 mg/kg per day, analogous to the dose that reduced myocardial fibrosis in chronic heart failure, induced by left coronary artery ligation in rats (50).

Interestingly, a trend toward increased expression of *CGRP* was found both in +/+ rats and in Sprague-Dawley rats. Indeed, levels of CGRP, a potent vasodilator, often correlate with levels of the vasoconstrictor ET1 (26,27). The fact that CGRP mRNA was not up-regulated in Ws/Ws rats suggests that mast cells were involved in regulation of cardiac CGRP levels after irradiation. The role of CGRP, a neuropeptide expressed and released by sensory nerves, has been studied in radiation-induced intestinal injury (51). Like radiation-induced up-regulation in the heart, intestinal CGRP is up-regulated after localized irradiation. Administration of full-length CGRP ameliorated radiation-induced intestine injury in rats, while a CGRP antagonist exacerbated injury (51). Since CGRP is known to play a protective role in myocardial injury (28,29), its role in the heart after irradiation deserves further investigation.

During localized heart irradiation in the current rat model, parts of the spinal cord and the lungs are included in the radiation field. Changes in these tissues may influence the radiation response in the heart. Levels of ET1 in rat spinal cords, for instance, are increased up to 6 months after a single dose of 15 Gy (11). Data on levels of circulating ET1, however, have not been reported. Therefore, it is not known whether sufficient quantities of ET1 would be released into the circulation to have effects in the heart. In our study, the animals did not show paralysis at any time after local heart irradiation, although this observation does not entirely rule out spinal cord injury. However, because irradiated volumes and radiation doses to these tissues were similar in each of the experimental groups, responses of these tissues likely do not have a significant impact on the comparisons made in the heart.

In conclusion, mast cell-competent but not mast cell-deficient hearts exhibited postirradiation up-regulation of ET1, suggesting the possibility of interactions between mast cells and the cardiac ET system. The dual ETA/ETB receptor antagonist, bosentan, induced reductions in left ventricular systolic pressure, developed pressure, and $+dP/dt_{\max}$ but did not affect radiation-induced cardiac remodeling. Moreover, bosentan did not significantly alter the cardiac radiation response in Sprague-Dawley rats in the current study. Further studies are needed to clarify the role of ET1 and each of its two receptors in radiation-induced heart disease.

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FIG. 1.

Effects of radiation on cardiac mast cell densities in +/+ rats determined in Toluidine Blue-stained tissue sections. A nonsignificant increase in mast cell number ($P = 0.06$) was observed 3 months after irradiation (irradiated +/+ compared to sham +/+; exact two-sided P value obtained with the Wilcoxon Mann-Whitney test). Means + SEM, $n = 3$ (0-Gy groups) or 4 (18-Gy groups).

FIG. 2. Representative images of ETA and ETB immunohistochemistry in +/+ rat hearts. Panel A: Both before and after irradiation, mast cells showed a particularly strong ETA expression. Panel B: Both before and after irradiation, ETB was found mainly in vascular smooth muscle cells. A similar staining pattern was found in Ws/Ws rats. Original magnification 40 $\times$.

FIG. 3.

Effects of balloon volume on left ventricular parameters in Langendorff perfused hearts isolated from Sprague-Dawley rats 6 months after irradiation with and without bosentan. Bosentan did not significantly affect diastolic pressure (panel A), but it reduced systolic pressure (panel B, $P = 0.04$), developed pressure (panel C, $P = 0.01$), and $+dP/dt_{\max}$ (panel D, $P < 0.001$) in both irradiated and sham-irradiated rats (repeated-measures ANOVA). Averages $\pm$ SEM, $n = 4$ (0-Gy groups) or 6 (18-Gy groups).

FIG. 4.

Effects of radiation and bosentan on left ventricular interstitial collagen areas as measured by quantitative immunohistochemistry. Panel A: Radiation induced significant increases in collagen I. Panel B: Radiation induced significant increases in collagen III [two-way ANOVA with radiation (0 Gy or 18 Gy) and treatment (vehicle or bosentan) as fixed factors]. Bosentan had no significant effect on collagen areas. Averages + SEM, $n = 4$ (0-Gy groups) or 5 (18-Gy groups).

FIG. 5.

Effects of radiation and bosentan on left ventricular extracellular matrix-associated TGF-β area as measured by quantitative immunohistochemistry. Radiation induced a significant increase in TGF-β [two-way ANOVA with radiation (0 Gy or 18 Gy) and treatment (vehicle or bosentan) as fixed factors]. Bosentan had no significant effect on TGF-β area. Averages + SEM, $n = 4$ (0-Gy groups) or 5 (18-Gy groups).

FIG. 6.

Effects of radiation and bosentan on cardiac mast cell densities as examined from Toluidine Blue-stained tissue sections. Radiation induced a significant increase in cardiac mast cell numbers [two-way AN-OVA with radiation (0 Gy or 18 Gy) and treatment (vehicle or bosentan) as fixed factors]. Bosentan did not significantly affect cardiac mast cell number. Averages + SEM, $n = 4$ (0-Gy groups) or 5 (18-Gy groups).

TABLE 1
Effects of Radiation on Left Ventricular Relative mRNA Levels in Ws/Ws and +/+ Rats

	Ws/Ws sham	Ws/Ws 18 Gy	+/+ sham	+/+ 18 Gy
1 week after (sham) irradiation				
ET1	1.0 ± 0.1	2.2 ± 0.4	1.1 ± 0.7	9.2 ± 3.7
ETA	1.0 ± 0.1	0.9 ± 0.4	1.0 ± 0.03	3.9 ± 2.2
ETB	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	1.2 ± 0.2
CGRP	1.4 ± 0.9	1.2 ± 0.4	1.2 ± 0.5	3.6 ± 2.0
3 months after (sham) irradiation				
ET1	1.1 ± 0.4	1.4 ± 0.7	1.1 ± 0.3	3.0 ± 0.8
ETA	1.0 ± 0.2	1.0 ± 0.03	1.0 ± 0.1	1.9 ± 1.0
ETB	1.0 ± 0.2	0.8 ± 0.1	1.0 ± 0.2	1.5 ± 0.7
CGRP	1.1 ± 0.4	1.3 ± 0.3	1.1 ± 0.3	3.9 ± 1.0

Notes. Radiation induced an increase in ET1 mRNA in +/+ rat hearts but not in Ws/Ws rat hearts (irradiated +/+ compared to irradiated Ws/Ws: exact two-sided *P* value = 0.015, Wilcoxon Mann-Whitney test, stratified on time). Means ± SEM, *n* = 3 at each time.

TABLE 2

Left Ventricular Relative mRNA Levels in Sprague-Dawley Rats 6 Months after Sham Irradiation with Bosentan, Irradiation Alone, or Irradiation with Bosentan

	0 Gy, vehicle	0 Gy, bosentan	18 Gy, vehicle	18 Gy, bosentan
ET1	1.0 ± 0.3	1.5 ± 0.3	1.9 ± 0.2	2.3 ± 0.3
ETA	1.0 ± 0.4	1.7 ± 0.2	3.2 ± 0.7	3.6 ± 0.5
ETB	1.0 ± 0.6	1.4 ± 0.4	0.9 ± 0.6	1.5 ± 0.3
CGRP	1.0 ± 0.3	1.8 ± 0.2	2.2 ± 0.4	2.8 ± 0.2
CTGF	1.0 ± 0.4	2.0 ± 0.2	1.7 ± 0.2	2.6 ± 0.3
IL6	1.0 ± 0.3	1.9 ± 0.4	1.5 ± 0.4	3.1 ± 0.3
MnSOD	1.0 ± 0.6	2.2 ± 0.6	1.4 ± 0.7	2.7 ± 0.2
TGF-β1	1.0 ± 0.2	1.1 ± 0.1	1.2 ± 0.2	1.2 ± 0.1

Notes. Radiation induced a significant increase in ET1, ETA and CGRP mRNA; bosentan induced a significant increase in CTGF and IL6 mRNA [two-way ANOVA with radiation (0 Gy or 18 Gy) and treatment (vehicle or bosentan) as fixed factors]. Means ± SEM, $n = 4$.