Late Effects of Total-Body Gamma Irradiation on Cardiac Structure and Function in Male Rhesus Macaques

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Heart disease is an increasingly recognized, serious late effect of radiation exposure, most notably among breast cancer and Hodgkin's disease survivors, as well as the Hiroshima and Nagasaki atomic bomb survivors. The purpose of this study was to evaluate the late effects of total-body irradiation (TBI) on cardiac morphology, function and selected circulating biomarkers in a well-established nonhuman primate model. For this study we used male rhesus macaques that were exposed to a single total-body dose of ionizing gamma radiation (6.5–8.4 Gy) 5.6–9.7 years earlier at ages ranging from ~3–10 years old and a cohort of nonirradiated controls. Transthoracic echocardiography was performed annually for 3 years on 20 irradiated and 11 control animals. Myocardium was examined grossly and histologically, and myocardial fibrosis/collagen was assessed microscopically and by morphometric analysis of Masson's trichrome-stained sections. Serum/plasma from 27 irradiated and 13 control animals was evaluated for circulating biomarkers of cardiac damage [N-terminal pro B-type natriuretic protein (nt-proBNP) and troponin-I], inflammation (CRP, IL-6, MCP-1, sICAM) and microbial translocation [LPS-binding protein (LBP) and sCD14]. A higher prevalence of histological myocardial fibrosis was observed in the hearts obtained from the irradiated animals (9/14) relative to controls (0/3) ($P = 0.04$, χ^2). Echocardiographically determined left ventricular end diastolic and systolic diameters were significantly smaller in irradiated animals (repeated measures ANOVA, $P < 0.001$ and $P < 0.008$, respectively). Histomorphometric analysis of trichrome-stained sections of heart tissue demonstrated $14.9 \pm 1.4\%$ (mean $\pm$ SEM) of myocardial area staining for collagen in irradiated animals compared to $9.1 \pm 0.9\%$ in control animals. Circulating levels of MCP-1 and LBP were significantly higher in irradiated animals ($P < 0.05$). A high incidence of diabetes in the irradiated animals was associated with higher plasma

triglyceride and lower HDLc but did not appear to be associated with cardiovascular phenotypes. These results demonstrate that single total-body doses of 6.5–8.4 Gy produced long-term effects including a high incidence of myocardial fibrosis, reduced left ventricular diameter and elevated systemic inflammation. Additional prospective studies are required to define the time course and mechanisms underlying radiation-induced heart disease in this model. © 2016 by Radiation Research Society

INTRODUCTION

Radiation-induced heart disease (RIHD) is a serious side effect of radiotherapy and/or accidental radiation exposure, characterized by a heterogeneous group of acute and/or chronic physiologic or pathological abnormalities in the heart. Acute conditions include pericarditis, pericardial effusions and arrhythmias. Delayed abnormalities, appearing months to years later, include cardiomyopathy, coronary artery disease, valve defects, conduction abnormalities and myocardial fibrosis. The exact etiology of the acute tissue damage leading to long-term risks is not fully understood and may result from direct and indirect effects. Potential contributors include endothelial injury and dysfunction, DNA damage and altered cardiac gene expression, acute and chronic oxidative stress, microvascular damage, inflammation and other factors (1–6). Long-term cardiovascular disease (CVD) incidence data collected from survivors of accidental, malicious and therapeutic exposures highlight the need for well controlled studies to improve our understanding of RIHD pathophysiology so that targeted treatment and prevention strategies can be successfully developed and implemented.

From the Life Span Study research program, which has followed Hiroshima and Nagasaki atomic bomb survivors for more than 50 years, it was found that an estimated excessive relative risk for heart disease was 14% per Gy of exposure. Excess risk was observed even at relatively low doses of radiation (0.5–2 Gy), suggesting the threshold for increased risk to the heart (7) is much lower than previously

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thought. More recently, a dose-dependent increase in RIHD has been observed in residents of contaminated areas after the Chernobyl accident, (8), supporting previous findings of increased CVD nationwide in Belarus compared to the pre-Chernobyl disaster era (9).

Radiation-induced heart disease is also a late and relatively common serious side effect of radiotherapy and has received increasing attention since it was reported that the heart was at least 10 times more radiosensitive than previously thought (10). RIHD is most prevalent among patients treated for breast, chest and thoracic cancer, as well as Hodgkin's disease patients who have received radiotherapy as a part of their medical intervention (1, 5, 11). CVD after radiotherapy for Hodgkin's disease is the third leading cause of death in Hodgkin's patients (1), and the primary cause of non-cancer death among 5-year childhood cancer survivors (12). In epidemiological studies such as Surveillance Epidemiology and End Results (SEER) (13) and Early Breast Cancer Trialists Collaborative Group (EBCTG) (14), it was found that while adjuvant radiotherapy reduced the risk of death from cancer it failed to confer significant survival benefit due to increases in CVD-related morbidity and mortality. Currently, the risk of dying from CVD exceeds the risk of dying from initial or recurrent cancer for many cancer survivors (15–17). The relative risk values generally reported in Hodgkin's disease survivors range from 2 to 5 for cardiovascular morbidity (18–20), with one study reporting a relative risk of 7.2 (21). A recent population-based case-control study in women who received radiotherapy for breast cancer between 1958 and 2001 in Denmark and Sweden showed that the rate of major coronary events increased by 7.4% per Gy of incidental exposure to the heart with no apparent threshold for increased risk (22). A meta-analysis of eight randomized trials involving breast cancer survivors showed a 62% increase in cardiac death among patients who were treated with radiation therapy (23). Hardenbergh *et al.* (24) reported that subclinical cardiac damage occurred in greater than 50% of breast cancer survivors who had received radiation therapy.

Currently, there are no approved treatments or reliable biological markers for RIHD. Traditional markers of cardiac damage or dysfunction do not appear to be useful for the evaluation of radiation-induced cardiac damage (25). Therefore, the discovery and development of biomarkers useful in identifying patients likely to experience RIHD would provide the healthcare community with useful tools to target populations for monitoring and therapy. The exact mechanisms underlying RIHD are still largely unclear (26); much of what is known has been derived from *in vitro* studies of tissue and cell responses and from *in vivo* studies in small animal models that do not fully approximate human physiology (19). Old world monkeys such as the rhesus macaque (*Macaca mulatta*) have close evolutionary ties to humans, making them an

invaluable model to study physiologic and pathologic conditions (27, 28). The utility of the rhesus macaque in investigation of radiation countermeasures is well established (29–32). In an effort to understand the susceptibility of the macaque to cardiac pathologies similar to those experienced in the human population, and in a long-term effort to identify pathways important in the initiation and progression of RIHD, we investigated the long-term effects of total-body irradiation (TBI) on cardiovascular structure, function and selected circulating biomarkers in adult rhesus macaques.

MATERIALS AND METHODS

Animal Acquisition and Housing

Male rhesus nonhuman primates (NHP) were obtained from the Armed Forces Radiobiology Research Institute (AFRRI), the University of Maryland (UMD) and the University of Illinois at Chicago (UIC). Animals were comprised of a group that had been total-body irradiated with a single dose (6.5–8.4 Gy) approximately 5.6–9.7 years earlier, and a group of age-matched, nonirradiated controls. Animals were screened by intradermal tuberculin testing to exclude tuberculosis, and by immunoassay to exclude simian retroviruses (Washington National Primate Research Center, Seattle, WA). Upon arrival animals were aged based on dentition, weighed, examined by a veterinarian and quarantined for 60 days per standard institutional protocol. All experimental procedures were conducted in compliance with the Wake Forest School of Medicine (WFSM) Institutional Animal Care and Use Committee requirements and followed the Guide for Care and Use of Laboratory Animals.

Basic Clinical Care and Clinical Pathology Assessments

Monkeys were assessed daily for food consumption, defecation, urination and evidence of discomfort or illness. Blood counts and chemistry panels were collected quarterly after quarantine. In the event of low absolute neutrophil counts ($<250/\mu\text{L}$), animals were given parenteral prophylactic antibiotic treatment, consisting of enrofloxacin (10 mg/kg) or ceftiofur sodium (30 mg/kg), until the white blood cell count returned to the normal range and any clinical illness, if present, resolved. Supportive fluid therapy, analgesics (e.g., ketoprofen) and symptomatic care were given as needed based on clinical pathology. Signs of illness were monitored using the methods of Uckun *et al.*, as modified from the Children's Cancer Group Clinical Toxicity Criteria (33). We have used these procedures with success in previous primate trials of bone marrow-suppressive agents (34, 35). In the event that an animal became moribund or reached grade 4 toxicity by our criteria, they were humanely euthanized following the American Veterinary Medical Associations Guidelines on Euthanasia (36).

Diet

Prior to arrival at WFSM (and during the time of irradiation), animals consumed a standard laboratory primate chow diet containing 18% protein, 13% fat and 69% carbohydrates, with a low cholesterol content of 0.023 mg/cal (Monkey Diet 5038; LabDiet®, St. Louis, MO). After arrival at WFSM, animals were transitioned to a moderately atherogenic Western diet developed by WFSM and Purina® (Typical American Diet, TAD; Purina LabDiet as NHP Diet) which contained 18.4% protein, 36.2% fat and 45.4% carbohydrates as percentage of calories provided, and a cholesterol content of 0.18 mg/cal. Animals that became diabetic [hemoglobin A1c (HgbA1c) $>6.5\%$] during the study were transitioned back to standard laboratory chow in the interest of health preservation.

Echocardiography

Echocardiography was successfully performed annually on 20 irradiated and 11 control animals using a SonoSite MicroMaxx® Ultrasound System (Bothell, WA). Cardiac morphometric and functional phenotypes included left ventricular diameter at end diastole (LVDd), left ventricular diameter at end systole (LVDs), left ventricular wall fractional shortening percentage (LVDFS%) calculated as $[(LVDd - LVDs)/LVDd] \times 100$ and ejection fraction percentage (EF%). Body surface area (BSA, m²) was calculated as $BSA = BW^{2/3} \times 0.0969$, utilizing the animal's body weight (kg) obtained at the time of echocardiography to allow normalization of cardiac dimensions to BSA (37). Electrocardiography was performed biannually and blood pressure parameters were measured 8–10 times annually.

Lipids and Selected Circulating Biomarkers

Plasma lipids and selected biomarkers were measured longitudinally and/or cross-sectionally in irradiated and control animals. Timing of collections corresponded with the first annual echocardiographic evaluation in this study and had variable postirradiation intervals (~5–8 years, mean time since irradiation = 6 ± 0.14 years). Total plasma cholesterol (TPC), triglycerides (TG) and high-density lipoprotein (HDL) levels were assessed in all subjects as previously described (38). Serum/plasma from 27 irradiated and 13 control animals was evaluated for the selected biomarkers listed below. This set included all 20 irradiated and 11 control animals in the echocardiographic evaluation, along with an additional 7 irradiated and 2 control animals that died before the final echocardiographic time point reported in this study. Biomarkers included circulating biomarkers of cardiac damage [N-terminal pro b-type natriuretic protein (nt-proBNP 8-29) (ALPCO® Diagnostics, Salem, NH) and troponin-I (Kamiya Biomedical Company, Seattle, WA), inflammation (soluble intercellular adhesion molecule (s-ICAM) (monkey sICAM-1 Platinum ELISA; eBioscience/Bender MedSystems, Vienna, Austria), C-reactive protein (CRP) (ALPCO® Diagnostics), interleukin-6 (IL-6) and monocyte chemo-attractant protein-1 (MCP-1) (R&D Systems™, Minneapolis, MN)], microbial translocation (LPS-binding protein (LBP) (Hycult® Biotech, Uden, Netherlands) and soluble cluster of differentiation 14 (s-CD14) (R&D Systems) via commercially available ELISA assays.

Necropsy

Animals that died or were euthanized due to morbidity underwent a complete necropsy for collection and processing of all major systems. Major organs were removed, weighed and tissues were preserved either by snap-freezing in liquid nitrogen, by fixation in 4% paraformaldehyde or by freezing in OCT compound. Gross lesions were photographed at time of necropsy and interpretation of pathologic findings was provided by a board-certified veterinary pathologist (JMC).

Histopathologic Analysis of Cardiovascular and Pulmonary Tissues

Gross evaluation of the thoracic and abdominal aorta, iliac arteries, carotid arteries, coronary arteries, cerebral arteries, left ventricle (LV), right ventricle (RV) and interventricular septum (IVS) was performed. Masson's trichrome was used to stain sections of LV, RV and IVS for estimation of fibrosis. Stained sections were digitally imaged using a Hamamatsu NanoZoomer (Hamamatsu, Japan). Histomorphometric analysis of these digitized slides was performed using ImagePro Premier® (Media Cybernetics Inc., Rockville, MD). Whole sections were evaluated using computer-assisted digitization defining representative colors of stained collagen (blues) to determine the collagen stain positive fraction. Briefly, the region of interest was manually traced to determine the area in pixels². The image was manually color thresholded to select pixels meeting "blue" staining criteria, which

TABLE 1
Frequency Distribution of Cardiac Fibrosis in Irradiated and Nonirradiated Rhesus Macaques

	No fibrosis	Fibrosis	Total
Control	3	0	3
Irradiated	5	9	14
Total	8	9	17
Odds ratio	-	-	12
<i>P</i> value	-	-	0.04^a

^a Significance ($P < 0.05$) indicated in bold face.

were then automatically counted to obtain the stained area of the section in pixels². Collagen area fraction was then determined using the following formula: percentage area collagen positive = (blue area/total area) $\times$ 100.

Statistics

The effect of radiation exposure on incidence rates was tested using a chi-square analysis. Physical, echocardiographic and blood pressure phenotypes were analyzed via analysis of variance (ANOVA) or repeated measures ANOVA (RMANOVA). The effect of radiation exposure on these parameters at individual time points was evaluated post hoc using Bonferroni correction. Circulating markers and plasma lipids were analyzed using independent two-tailed *t* tests. In the event that two groups had unequal standard deviations, a Welch's *t* test correction was used. Data are expressed as means $\pm$ standard error of the mean (SEM). The above statistical tests were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Pearson's correlation coefficients were calculated to assess relationships between biomarkers and echocardiographic and blood pressure phenotypes across and within the control and irradiated groups. All pairwise correlation analyses were performed using Statistica version 12 (StatSoft Inc., Carlsbad, CA). $P < 0.05$ was considered significant.

RESULTS

Cardiac Structural and Functional Phenotypes

Fourteen irradiated and 3 control animals were euthanized during the study and their tissues were available for analysis at the time of this report. Elapsed time between irradiation and death was 5.6 ± 0.59 years (range 2.2–9.25). Figure 1A and B are photomicrographs of trichrome-stained sections of the LV, illustrating loss of normal cardiomyocyte architecture and replacement with collagen and fat in the irradiated animal. Compared to the myocardium of control animals, irradiated myocardium often exhibited separation of cardiac myofibers by intervening fibrous connective tissue, loss of normal architecture, with deranged cellular orientation and enlargement of cardiac myocytes (Fig. 1C–F). Gross and qualitative microscopic histological analysis of the 17 hearts available from euthanized animals revealed a significantly higher incidence of myocardial fibrosis in 9 of 14 irradiated animals compared to 0 of 3 control animals ($P = 0.04$, χ^2) (Table 1). Histomorphometric analysis of trichrome-stained sections of myocardium from these animals revealed a mean of 14.9% (± 1.35) percentage collagen in irradiated compared to 9.1% (± 0.86) in control animals ($P = 0.17$, *t* test).

FIG. 1. Effects of prior exposure on indices of cardiac fibrosis: Panels A and B: Trichrome-stained sections of myocardium from the left ventricle of a control animal (panel A) and of a 6.5 Gy irradiated animal 2.5 years earlier (panel B) (scale bars = 5 mm). Panels C–F: Longitudinal and cross-sectional H&E stained myocardium. Panels C and D: control myocardium. Panels E and F: Irradiated myocardium exhibiting marked separation of cardiac myofibers by intervening fibrous connective tissue, loss of normal architecture, cellular orientation and enlargement of cardiac myocytes. Photomicrographs were taken at 40 $\times$. Scale bar for panels C–F = 50 μ m.

Echocardiographic Phenotypes

Selected outcomes for echocardiographic phenotypes are shown in Fig. 2. Thirty-one animals were evaluated annually for 3 consecutive years using echocardiography. The estimated mean age at the final assessment was 11.2 ($\pm$ 0.3) years for the 11 control animals, compared to 11.8 ($\pm$ 0.5) years for the 20 irradiated animals. At the final echocardiographic assessment monkeys had an average postirradiation interval of 8.6 $\pm$ 0.2 years and had been consuming the Western diet for an average of 3.7 $\pm$ 0.1 years. Echocardiographic LVDd and LVDs were significantly smaller in irradiated animals compared to controls (LVDd: P = 0.001 and LVDs: P = 0.008, RMANOVA; Fig. 2A and B). Correction for body surface area (BSA) (39) did not significantly alter outcomes (LVDd: P = 0.007 and LVDs: P = 0.017, RMANOVA, data not shown). Irradiated

animals tended to have greater LVDFS% (P = 0.054, RMANOVA) (Fig. 2C). Radiation had no effect on EF% (P = 0.930, RMANOVA; data not shown). Irradiated animals tended to have higher mean annual heart rate (P = 0.06, RMANOVA), but were not different from controls in systolic, diastolic or mean arterial blood pressure (all P > 0.35, Table 2).

Biomarkers

Cardiac. Circulating troponin-I and nt-proBNP fragment (8–29) were below the detection limits of the ELISA assays, which were 0.156 ng/ml for cardiac troponin-I and 171 pmol/l for nt-proBNP.

Inflammation. Significantly higher levels of circulating MCP-1 were observed in the irradiated animals (P = 0.016) (Fig. 3A). Irradiated animals showed a trend towards

FIG. 2. Effects of prior exposure on cardiac function via echocardiographic assessments. Panels A and B: Echo left ventricular diastolic and systolic diameters (LVDD and LVDs, respectively) were significantly smaller in irradiated animals (RMANOVA, $P = 0.001$ and $P = 0.008$). Panel C: Fractional shortening percentage (LVDFS%) tended to be greater in irradiated animals (RMANOVA, $P = 0.054$). Data are mean $\pm$ SEM. Control ($n = 11$); irradiated ($n = 20$) across all years. Unlike letters denote significance between control and irradiated animals within time points with P values < 0.05 .

FIG. 3. Effects of prior exposure on circulating MCP-1 and LBP. Monocyte chemoattractant protein-1 (MCP-1; panel A) and LPS-binding protein (LBP; panel B) were significantly higher in irradiated animals (t test, $P = 0.016$). Data are mean $\pm$ SEM. Unlike letters denote significance between control and irradiated animals with P values < 0.05 .

TABLE 2
Effects of Prior Irradiation on Heart Rate and Blood Pressure in the Echocardiographic Subset of Animals

	Time point 1		Time point 2		Time point 3		P value		
	Control	TBI	Control	TBI	Control	TBI	Time	TBI	Time $\times$ TBI
n =	11	21	11	21	11	21			
Heart rate (beats per min)	138.7	151	128	137	125	132	0.0001^a	0.06	0.31
SEM	4.4	3.1	4.6	2.8	4.4	2.8			
Systolic BP (mm Hg)	128	127	126	123	126	127	0.067	0.83	0.27
SEM	2.9	2.8	2.9	2.6	2.9	3.1			
Diastolic BP (mm Hg)	68.2	73.3	67.4	69.9	70.8	72.4	0.10	0.36	0.42
SEM	1.8	2.3	3.3	2.0	2.4	2.5			
Mean BP (mm Hg)	91.6	94.4	88.6	90.8	91.6	92.3	0.06	0.57	0.73
SEM	2.3	2.4	3.2	2.1	1.9	2.5			

Notes. Time points 1, 2 and 3 correspond to years 1, 2 and 3. Data were evaluated by repeated measures ANOVA. SEM = standard error of the mean. BP = blood pressure.

^a Significance ($P < 0.05$) indicated in bold face.

TABLE 3
Effects of Prior Radiation Exposure on Circulating Levels of hsCRP, IL-6, sCD-14 and sICAM-1

	Control (n = 13)	TBI (n = 27)	P value
hsCRP (ng/ml)	986	1,507	0.12
SEM	227	198	
IL-6 (pg/ml)	1.95	4.25	0.12
SEM	0.46	0.96	
sCD-14 (ng/ml)	1,150	1,286	0.25
SEM	77	71	
sICAM (U/ml)	24.4	30.6	0.53
SEM	3.0	6.7	

Note. Data are mean $\pm$ standard error of mean (SEM).

elevated levels of circulating CRP and IL-6 ($P = 0.12$), and there was no effect of radiation on s-ICAM (Table 3).

Microbial translocation. Significantly higher levels of circulating LBP were observed in the irradiated animals ($P = 0.016$) (Fig. 3B). Previous exposures to radiation had no effect on circulating levels of sCD14 at the time point evaluated in this study (Table 3).

Relationships between biomarkers and other phenotypes. Circulating levels of MCP-1 were positively correlated with circulating levels of LBP ($r = 0.37$, $P = 0.018$, $n = 40$). MCP-1 and LBP were not correlated with cardiac functional phenotypes (all $P > 0.05$, data not shown).

Diabetes, Body Weight and Plasma Lipids

Across the entire cohort of 59 subjects, a high incidence of type 2 diabetes mellitus (T2DM, defined as HgbA1c $> 6.5\%$) was observed in the irradiated animals (13 of 46), while there was no T2DM in the control animals (0 of 13) ($P = 0.03$, χ^2). Diabetic animals had significantly higher HbA1c values than controls ($P = 0.0001$) and irradiated nondiabetic monkeys ($P = 0.0001$), while controls were not different from irradiated nondiabetic monkeys ($P = 0.9$)

(Table 4). Body weight and plasma lipids were influenced by exposure and diabetes status. Irradiated nondiabetic animals had significantly lower body weights than control animals and irradiated diabetic animals (all $P < 0.05$). TBI animals with diabetes exhibited higher TG than controls ($P < 0.005$) and TBI nondiabetic monkeys ($P = 0.003$), and had lower HDLc compared to control ($P < 0.03$) and nondiabetic irradiated monkeys ($P < 0.002$). Further details on the pathophysiology of T2DM in irradiated macaques have been previously published elsewhere (40).

Effects of Diabetes on Individual Phenotypes

Cardiac phenotypes (ventricular diameters, heart rate and blood pressure) and nonlipid biomarkers did not differ between diabetic and nondiabetic irradiated animals (data not shown), and HgbA1c concentrations were not correlated with cardiac phenotypes (all $P > 0.05$), but were associated with body weight in animals in the echocardiographic subset (all $P < 0.01$).

DISCUSSION

Our data indicate that male rhesus macaques receiving total-body ionizing radiation (6.5–8.4 Gy) 5.6–9.7 years earlier exhibited the following: 1. Increased incidence of gross myocardial fibrosis at time of death ($P < 0.05$); 2. Smaller cardiac dimensions ($P < 0.01$); 3. Increased incidence of T2DM ($P = 0.03$); and 4. Higher levels of circulating MCP-1 and LBP than control animals. With the exception of a tendency for higher heart rates in irradiated animals, no significant differences were observed in standard clinical measures of cardiac health and function, such as systolic or diastolic blood pressure, with the caveat that these were conducted while animals were under anesthesia.

TABLE 4
Effects of Prior Radiation Exposure and Diabetes on Plasma Lipids in the Echocardiographic Subset of Animals: Time Point 2 Only

	Control (n = 11)	TBI, no T2DM (n = 13)	TBI + T2DM (n = 8)	ANOVA P value	P values		
					Control vs. TBI, no T2DM	Control vs. TBI + T2DM	TBI, no T2DM vs. TBI + T2DM
HbA1c (%)	4.5	4.7	9.6	0.0001^a	0.9	0.0001^a	0.0001^a
SEM	0.1	0.1	0.6				
Body weight (kg)	12.9	8.8	14.2	0.0001^a	0.0004^a	0.45	0.0001^a
SEM	1.0	0.4	0.7				
TPC (mg/dl)	164	187	213	0.19	0.57	0.16	0.57
SEM	18	9	30				
TG (mg/dl)	88.9	76.5	484	0.002^a	0.99	0.005^a	0.003^a
SEM	9.1	3.5	179				
HDL (mg/dl)	71.6	80.6	47.8	0.003^a	0.49	0.03^a	0.002^a
SEM	7.6	4.1	5.9				

Notes. All lipid measurements were performed at a single time point. All lipids were evaluated using an unpaired two-tailed t test and ANOVA to evaluate the effect of T2DM on lipid measurements. Between group ANOVA P values. Post hoc analyses corrected for multiple comparisons via Tukey's HSD. TBI = total-body irradiation; T2DM = type 2 diabetes mellitus; SEM = standard error of the mean.

^a Significance ($P < 0.05$) indicated in bold face.

Gross histopathological evaluation of postmortem myocardium revealed a significant increase in incidence of myocardial fibrosis at time of death in previously irradiated animals. Image analysis of trichrome-stained sections of myocardium suggested a tendency for increased collagen deposition in irradiated animals. These results suggest that TBI in the 6.5 Gy and above range predisposed animals to the development of myocardial fibrosis as a late effect and expand on previously published data from Wondergem *et al.* (39) using a similar model.

Irradiated animals exhibited significantly smaller LVDd and LVDs diameters than controls before and after adjustment for BSA. Wondergem *et al.* (39) also observed smaller LVDd and LVDs in irradiated male and female rhesus macaques ($P = 0.0002$ and 0.038 , respectively), although correction for BSA in their study reduced significance for LVDs ($P = 0.07$). This modest difference between studies may be in part due to the inclusion of females and postirradiation treatment with bone marrow transplants (39), which were not used in our study (41–43). Similar observations were recently reported in a murine model of radiation exposure, where LVDs was significantly lower ($P < 0.05$) and LVDd tended to be lower ($P = 0.06$) (44). The smaller LV dimension measurements are consistent with a restrictive cardiomyopathy due to fibrosis, although reduced relaxation or blunted growth of the heart postirradiation could play a role.

Functional parameters suggest that systolic function (LV fractional shortening) was preserved in the irradiated cohort, as irradiated animals tended to have higher LVDFS% ($P = 0.06$). In the murine model noted above, fractional shortening also tended to be increased ($P = 0.10$) (44). The mechanisms underlying the tendency towards increased fractional shortening are unclear, but may represent a compensatory response to promote adequate cardiac output and blood flow.

In our previous work with female cynomolgus monkeys, we showed that rapid dose-dependent increases in circulating cytokines including MCP-1 soon after irradiation (42). MCP-1 is an important mediator of inflammation and monocyte recruitment. In humans, increased serum levels of MCP-1 are associated with several cardiovascular conditions including subclinical atherosclerosis, acute coronary syndromes, congestive heart failure and peripheral arterial disease (45–48). In a previous study unrelated to radiation effects, we found that MCP-1 was significantly correlated with atherosclerosis in coronary, carotid and iliac arteries of ovariectomized female cynomolgus macaques consuming an atherogenic Western diet (49). The current finding that MCP-1 and LBP were elevated several years postirradiation (5–8 years) is suggestive of a persistent pro-inflammatory state, which may have a long-lasting impact on many tissues, including the heart. Increased incidence of T2DM and higher circulating MCP-1 levels were also observed in a subset of the animals in the current study, although these

phenotypes did not appear to be correlated with one another or causally linked (40).

Significant elevations in LBP, a marker of microbial translocation and mediator of the innate immune response to microbial infection, may be indicative of chronic LPS exposure (50) due to damage to the gastrointestinal system, which is particularly radiosensitive and can become transiently or permanently leaky as a result of radiation exposure (51). LBP is an acute phase protein which recognizes bacterial LPS and complexes with sCD14, initiating production of pro-inflammatory interleukins and cytokines via Toll-like receptor (TLR) interactions (52, 53), and enhancing macrophage activation during acute injury processes (54). Recently, LBP has gained attention as a potential marker of coronary artery disease/atherosclerosis (55, 56) and cardiovascular-related mortality (57).

Circulating troponin-I and nt-proBNP data presented here were obtained using standard ELISA assays, which lack sensitivity to detect low, subclinical levels of these biomarkers. Previous studies of these markers in humans undergoing radiotherapy have produced mixed results. In a study of 30 patients undergoing thoracic radiotherapy with chemotherapy, no significant elevations in troponin, nt-proBNP or creatine kinase-myocardial band (CK-MB) were observed after radiotherapy (58). However, recent studies suggest BNP may be useful as an acute marker of early radiotherapy-related cardiovascular impairment in left-sided breast cancer patients (59), and in patients receiving thoracic radiotherapy with high-dose heart exposure (59, 60). Nevertheless, the majority of patients receiving greater than 20 Gy to the heart during thoracic radiotherapy did not exhibit increased levels of troponin I (60). The follow-up times in the above studies were shorter than those in the current study.

Inflammation and diabetes are well-established factors in the development and progression of CVDs (61). While the incidence of T2DM and the levels of inflammatory MCP-1 and LBP were increased in the irradiated cohort, we did not find evidence that either T2DM or these inflammation-associated markers were specifically associated with the cardiac structural or functional parameters in the study (data not shown). Ongoing evaluations of molecular and cellular phenotypes in the heart and coronary arteries of these and other subjects should provide insights into the determinants of atherosclerosis and cardiac damage.

Due to the opportunistic nature of this study, certain limitations were inherent and unavoidable. The irradiated animals used in this study were survivors of potentially lethal TBI, and perhaps possessed unique protective genotypes and/or phenotypes not representative of all rhesus macaques. The pro-inflammatory effects of TBI observed during this study may differ from that of localized therapy, as it represents a much broader exposure of multiple tissue types including the heart. Elevated circulating biomarkers may originate from

numerous tissues within the animal, and information is lacking as to the specific tissue origin of the elevated MCP-1, although LBP is presumed to be derived from the liver. Nevertheless, off-target effects of region-specific radiation treatment, either through scatter, pro-inflammatory and/or oxidative factors generated locally and distributed through the circulation is known to occur. The experimental design for this group of animals is not prospective in the sense that we have no baseline data or samples available prior to the time of exposure, similar to situations involving survivors of a radiation accident. Additionally, our animal cohort was consuming a standard low-fat chow diet at the time of irradiation, and was not transitioned to a Western diet until after arriving at our institution. The Western diet was utilized to better represent physiologic conditions present in a large proportion of Western society. Unfortunately, this design does not allow for an assessment of the effects of diet on radiosensitivity. Lastly, while the Western diet may have contributed to the development of diabetes in the irradiated animals, it is not possible to fully explore the interactions between diet, diabetes and cardiac or other phenotypes since the animals are generally placed on chow once diabetes becomes evident.

In summary, TBI given as single doses of 6.5–8.4 Gy produced long-lasting myocardial injury, altered heart structure and function and elevated levels of MCP-1 and LBP consistent with a pro-inflammatory state. Additional studies in the NHP model are required to more fully understand the sequence of events postirradiation, which can promote RIHD, in order to identify useful biomarkers and develop strategies for mitigating the process.

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