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ORIGINAL ARTICLE

Simvastatin mitigates increases in risk factors for and the occurrence of cardiac disease following 10 Gy total body irradiation

Marek Lenarczyk[1], Jidong Su[1], Steven T. Haworth[2], Richard Komorowski[3], Brian L. Fish[4], Raymond Q. Migrino[5], Leanne Harmann[6], John W. Hopewell[7], Amy Kronenberg[8], Shailendra Patel[9,10], John E. Moulder[4] & John E. Baker[1,11,12]

[1]Division of Cardiothoracic Surgery, Medical College of Wisconsin, Milwaukee, Wisconsin
[2]Department of Medicine, Medical College of Wisconsin, Milwaukee, Wisconsin
[3]Department of Pathology, Medical College of Wisconsin, Milwaukee, Wisconsin
[4]Department of Radiation Oncology, Medical College of Wisconsin, Milwaukee, Wisconsin
[5]Phoenix Veterans Affairs Health Care System, Phoenix, Arizona
[6]Division of Cardiovascular Medicine, Medical College of Wisconsin, Milwaukee, Wisconsin
[7]Green Templeton College and Particle Therapy Cancer Research Institute, University of Oxford, Oxford, United Kingdom
[8]Lawrence Berkeley National Laboratory, Berkeley, California
[9]Division of Endocrinology, Medical College of Wisconsin, Milwaukee, Wisconsin
[10]Clement J. Zablocki Veterans Affairs Medical Center, Milwaukee, Wisconsin
[11]Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, Wisconsin,
[12]Children's Research Institute, Children's Hospital of Wisconsin, Milwaukee, Wisconsin

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Correspondence
John E. Baker, Division of Cardiothoracic Surgery, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226. Tel: (414) 955-8706;
Fax: (414) 955-6545;
E-mail: jbaker@mcw.edu

Present address
Marek Lenarczyk, Department of Pharmaceutical Sciences, University of Tennessee, Memphis, Tennessee

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Abstract

The ability of simvastatin to mitigate the increases in risk factors for and the occurrence of cardiac disease after 10 Gy total body irradiation (TBI) was determined. This radiation dose is relevant to conditioning for stem cell transplantation and threats from radiological terrorism. Male rats received single dose TBI of 10 Gy. Age-matched, sham-irradiated rats served as controls. Lipid profile, heart and liver morphology and cardiac mechanical function were determined for up to 120 days after irradiation. TBI resulted in a sustained increase in total- and LDL-cholesterol (low-density lipoprotein-cholesterol), and triglycerides. Simvastatin (10 mg/kg body weight/day) administered continuously from 9 days after irradiation mitigated TBI-induced increases in total- and LDL-cholesterol and triglycerides, as well as liver injury. TBI resulted in cellular peri-arterial fibrosis, whereas control hearts had less collagen and fibrosis. Simvastatin mitigated these morphological injuries. TBI resulted in cardiac mechanical dysfunction. Simvastatin mitigated cardiac mechanical dysfunction 20–120 days following TBI. To determine whether simvastatin affects the ability of the heart to withstand stress after TBI, injury from myocardial ischemia/reperfusion was determined in vitro. TBI increased the severity of an induced myocardial infarction at 20 and 80 days after irradiation. Simvastatin mitigated the severity of this myocardial infarction at 20 and 80 days following TBI. It is concluded simvastatin mitigated the increases in risk factors for cardiac disease and the extent of cardiac disease following TBI. This statin may be developed as a medical countermeasure for the mitigation of radiation-induced cardiac disease.

Abbreviations

Abca1, ATP-binding cassette, subfamily A, member 1; ALT, Alanine transaminase; ANOVA, analysis of variance; AST, Aspartate transaminase; BUN, blood urea

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nitrogen; bw, body weight; FDA, food and drug administration; H&E, hematoxylin and eosin; HDL, high density lipoprotein; Hmgcr, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; LDH, lactate dehydrogenase; LDLR, low-density lipoprotein receptor; LVDP, left ventricular developed pressure; Ppara, peroxisome proliferator-activated receptor alpha; Scarb1, scavenger receptor class B, member 1; Srebf1, sterol regulatory element binding transcription factor 1; TBI, Total body irradiation.

Introduction

Exposure to ionizing radiation results in the production of short-lived free radicals, predominantly from the ionization of water molecules, which causes damage to the DNA of cells. This damage to DNA can either be repaired and cells function normally, be imperfectly repaired and cells function abnormally, or cells may die as a result of this damage either directly, or is as more usual, indirectly at cell division. In the context of hematopoietic stem cell transplantation, total body irradiation (TBI) is used at a high dose to eliminate the cells of the recipient's own immune system, preventing immunological rejection of transplanted donor bone marrow stem cells (Thomas 1982). Hematopoietic stem cell transplantation is the primary treatment option for many children with blood disorders from cancer.

Recipients of hematopoietic stem cell transplantations following TBI are at an increased risk of developing high blood pressure, diabetes, and high cholesterol (Armenian et al. 2012). Adult survivors of childhood cancer are also at an increased risk from adverse cardiovascular outcomes, including congestive heart failure, myocardial infarction, pericardial disease, and valvular abnormalities as late as 30 years after transplantation (Mulrooney et al. 2009; Meacham et al. 2010; Armenian et al. 2012). The cardiovascular risks of transplantation have been associated, at least in part, with radiation exposure. High dose exposure to whole body irradiation may also occur outside a clinical setting. One such scenario would involve radiation exposure from a terrorist attack using an improvised nuclear device. In a radiation terrorism event, children would account for a significant portion of the affected population.

The TBI doses used in hematopoietic stem cell transplantation typically range from 10 to >12 Gy. A single TBI dose of 10 Gy has been determined to be relevant to radiologic terrorism (Coleman et al. 2003), since in such an incident, the entire body or a significant portion of the body would receive a single dose of radiation. Without intensive medical care, the median lethal dose of sparsely ionizing radiation (such as gamma rays or X-rays) for whole body exposure of an adult individual is estimated to be 4.5 Gy (Mole 1984). This dose is also referred to as the $LD_{50/60}$ or the dose that kills 50% of the exposed population within 60 days of exposure.

The impact of TBI exposure to 10 Gy, a potentially survivable dose in a radiation accident or radiological terrorism event (Coleman et al. 2003), on late injury to a child's cardiovascular system has not been known until recently (Baker et al. 2009). These authors demonstrated that a single TBI exposure to 10 Gy in the immature (5-week-old) WAG/RijCmcr rat, representative of a paediatric population, increases serum total cholesterol, low-density lipoprotein (LDL)-cholesterol, and triglycerides, all of which are biomarkers for an increased risk of developing cardiac disease. Hypercholesterolemia is associated with morphological injury to the vascular endothelium resulting in stenosis, decreased density of the smaller diameter coronary vessels, and a decrease in ventricular function at 120 days following TBI. Functionally, this is manifest as a decline in global radial and circumferential strain (Baker et al. 2009). However, the impact of TBI with 10 Gy on early cardiac events in children remains poorly understood.

There is an *urgent* and *immediate* need for practical therapies to mitigate against radiation injury to the heart following conditioning for hematopoietic stem cell transplantation or a radiologic terrorism event (Coleman et al. 2003), and to devise appropriate medical countermeasures using currently available pharmaceuticals. In the scenario of a radiation/nuclear incident, pretreatment of individuals prior to exposure will not be possible. Therefore, any medical countermeasures need to be effective when administered after radiation exposure.

Injury to the heart after TBI appears to be a largely indirect effect, with changes in non-thoracic organs causing or exacerbating an increase in the risk factors for cardiac disease, injury to the coronary vasculature and ventricular dysfunction (Baker et al. 2009). Direct injury to the heart from radiation requires higher doses (Fajardo

and Stewart 1970; Yeung and Hopewell 1985). To determine the role of abdominal organs in the genesis of cardiac injury following TBI, it has recently been shown that lower hemi-body irradiation, but *not* upper hemi-body irradiation, increased the risk factors for cardiac disease in a way that was quantitatively and qualitatively similar to that observed after TBI (Lenarczyk et al. 2013). There was evidence of abnormal liver function but no histological evidence of liver injury 120 days after TBI. These findings support the notion that injury to the heart following TBI appears to be an indirect effect, with injury to abdominal organs being responsible for the increased risk factors for and the occurrence of cardiac disease after TBI or lower hemi-body irradiation (Lenarczyk et al. 2013). Therefore, it was suggested that medical countermeasures that target these abdominal organs such as the liver could be effective in mitigating the development of cardiac disease following TBI (Lenarczyk et al. 2013).

Simvastatin has been used in paediatric patients to treat familial hypercholesterolemia (de Jongh et al. 2002), and restores endothelial function in hypercholesterolemic children and adolescents (Ferreira et al. 2007). However, the ability of a statin to mitigate the development of radiation-induced damage to the child's heart is unknown. It is proposed that TBI-induced increases in the risk factors for, and the occurrence of cardiac disease, will be mitigated by targeting the increased synthesis of cholesterol by the liver with simvastatin.

Simvastatin was chosen for this study as it protects against radiation enteropathy in rats (Hauer-Jensen 2007), improves endothelial function (O'Driscoll et al. 1997), and promotes vasculogenesis and increase thrombomodulin expression by a nitric oxide-dependent mechanism (Llevadot et al. 2001; Shi et al. 2003; Dimmeler et al. 2005). In a previous study (Baker et al. 2009), it was shown that 10 Gy TBI decreases protein levels for constitutive NOS (endothelial nitric oxide synthase (eNOS)), inducible NOS isoforms, and nitric oxide generation in the immature rat heart. Furthermore, simvastatin decreases severity of injury from an induced myocardial infarction (Bao et al. 2009) and improves postischemic ventricular function (Lefer et al. 1999). Taken together, these studies suggested that, in addition to its classical role of lowering cholesterol, simvastatin would be effective in mitigating increased risk for and occurrence of cardiac disease following exposure to radiation.

The objectives of the present study were to determine whether simvastatin (1) mitigates against TBI-induced increases in the risk factors for cardiac disease, (2) mitigates the occurrence of cardiac disease after exposure to radiation, and (3) increases the ability of the heart to withstand an acute ischemic stress following TBI. The term "mitigation" refers to therapies that are effective when administration is started after irradiation but before there is pathophysiological evidence of radiation injury (Stone et al. 2004).

Materials and Methods

Compliance with design and statistical analysis requirements

Group sizes were equal. The numbers in each experimental group were based on our previous experience with the same measurements in similar studies. Animals were randomized to each group. The identity of the animal in each experimental group was known to the investigator responsible for initiating and continuing with an intervention, for example, irradiation or drug treatment. The identity of the animal under study was not known to the investigator performing the experimental measurement or the analysis. These investigators were not the same person. Liver gene expression, radial and circumferential strain, infarct size, and recovery of left ventricular developed pressure (LVDP) were expressed as a percentage of an internal control. The study tested the hypothesis that simvastatin mitigates increases in risk factors for and the occurrence of cardiac disease following 10 Gy TBI.

Experimental animals

Young, 5–7 week-old, male WAG/RijCmcr rats were used in this study. They were maintained on sterilized rat chow and water ad libitum in a moderate-security barrier facility at the Biomedical Resource Center of the Medical College of Wisconsin, Milwaukee, Wisconsin, USA. The research was conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The Animal Care and Use Committee at the Medical College of Wisconsin approved all protocols.

Total body irradiation

Rats (9/group) received TBI with a single dose of 10 Gy. TBI irradiation was carried out using a posterior-anterior field at a dose-rate of 1.73 Gy/min. Irradiation was with 320 kVp orthovoltage X-rays. The half value layer of the beam used for irradiation was 1.4 mm of Cu. The radiation dosimetry has been described in detail previously (Moulder et al. 2014). Sham-irradiated rats (9/group) served as controls. The model used is relevant to a radiological terrorism scenario and a dose relevant to the use of radiation prior to bone marrow transplantation. One to 2 h after TBI, rats were given fresh isogenic bone marrow cells using techniques previously published (Moulder and Fish 1989). Rats were irradiated and maintained in the barrier facility throughout the study. The start of the study was defined as the time rats were irradiated or sham treated.

Simvastatin administration

The dose of simvastatin that was not toxic to the liver of WAG/RijCmcr rats was initially determined by adding simvastatin to the diet for 20 days in nonirradiated rats (3–4/group) to achieve doses of 10, 20, and 80 mg/kg bw/day. For the mitigation studies, simvastatin was added to the diet continuously from 9 days after TBI or to sham-irradiated rats until the end of the study. Simvastatin-containing rat chow was produced by Harlan Teklad (Madison, WI) by mixing 125 g of the drug with 1 kg standard diet (TD.110011) prior to pelleting. The simvastatin concentration used in the mitigation study was 10 mg/kg bw/day.

Liver function

Serum was taken from rats prior to treatment and then at 12 and 20 days for the simvastatin dose–response study or 120 days after TBI. This was used to assess liver function (i.e., aspartate transaminase [AST], alanine transaminase [ALT], alkaline phosphatase enzyme activity, and total bilirubin) (Dynacare Laboratories, Milwaukee, WI).

Lipid profile

Blood was taken just prior to irradiation (or sham) and then at 20, 40, 60, 80, 100, and 120 days after TBI alone, TBI plus simvastatin administration, simvastatin administration alone, and from sham-irradiated control rats. Serum was than analyzed for lipid levels (i.e., total cholesterol, LDL cholesterol, high density lipoprotein [HDL] cholesterol, and triglycerides) (Dynacare Laboratories).

Kidney function

Blood was also taken just prior to irradiation (or sham) and then at 20, 40, 60, 80, 100, and 120 days after TBI alone, TBI plus simvastatin administration, simvastatin administration alone, and from sham-irradiated control rats. Serum was analyzed for blood urea nitrogen (BUN) and creatinine levels (Dynacare Laboratories).

Ischemia/reperfusion studies in vitro

Isolated hearts were perfused retrogradely as described previously (Lam et al. 2012b). Briefly, hearts were perfused with modified Krebs-Henseleit buffer (120 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 4.7 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.20 mmol/L $MgSO_4$, 11 mmol/L glucose, and 1.8 mmol/L $CaCl_2$) bubbled with 95% O_2-5% CO_2 for a 40-min stabilization period and subjected to 25 min of global no-flow ischemia, followed by 180 min of reperfusion. Before use, all perfusion fluids were filtered through cellulose acetate membranes with a pore size of 5.0 μm, to remove particulate matter. The hearts were kept in temperature-controlled chambers to maintain a myocardial temperature of 37°C. A balloon, connected to a pressure transducer was inserted into the left ventricle to monitor cardiac function. During the initial 40-min reperfusion period, coronary effluent was collected and analyzed for lactate dehydrogenase (LDH) activity and recovery in mechanical function was measured as LVDP under steady-state conditions and expressed as a percentage of pre-ischemic LVDP. At the end of the 3 h reperfusion period, the hearts were processed and stained with 2,3,5-triphenyltetrazolium chloride dye the determine the size of the infarct.

Cardiac echocardiography

Segmental myocardial viability was assessed using two dimensional strain echocardiography in TBI alone, TBI plus simvastatin, simvastatin alone, and sham-irradiated rats (3–6/group) after 20, 80, and 120 days. The operator was blinded to treatment allocation. An echocardiograph Vivid 7 (General Electric, Waukesha, WI) was used with a M12L (11-MHz) linear-array transducer. Closed-chest imaging was performed in the short-axis view at the mid-LV level (level of papillary muscles). The image depth was 2.5 cm, acquisition was at 236 frames/sec with secondary harmonic imaging and electrocardiographic gating (Migrino et al. 2007).

Echocardiography image analysis

The images were processed using EchoPAC Q analysis software (General Electric). The method has been previously described (Migrino et al. 2007). The endocardial border was manually traced in an end-systolic frame and then the software automatically selected six equidistant tissue-tracking regions of interest in the myocardium. The outer border was adjusted to approximate to the epicardial border. The software provided a profile of radial (myocardial deformation toward the center) and circumferential (myocardial deformation along the curvature) strain (%) with time. End systolic radial and circumferential strain was obtained for each of the six segments and the global strain calculated from the average of the values. Three consecutive heart beats were measured and the average used for the analysis.

Histology

To evaluate tissue damage at 120 days after irradiation, the entire heart and liver (3/group) were taken for the

following groups: TBI alone, TBI plus simvastatin, simvastatin alone, and from sham-irradiated control rats, and fixed in 10% formalin (v/v). Fixed tissue samples were embedded in paraffin. 5 μm-thick sections cut from each block and stained with hematoxylin and eosin (H&E) or Masson-trichrome, according to standard methods. Ten sections from each heart and liver were evaluated.

Coronary vessel imaging

At 120 days after irradiation, hearts (3/group) from rats receiving TBI alone, TBI plus simvastatin, simvastatin alone, and sham-irradiated were isolated and perfused in a retrograde manner at 37°C with bicarbonate buffer (Baker et al. 2008) containing high potassium (16 mmol/L) and low calcium (0.8 mmol/L) at a constant pressure (85 mmHg) for 2 min to wash out blood from the coronary vasculature and to arrest the heart in diastole. Each heart was then perfused with contrast media and processed for micro-computed tomography as described previously (Baker et al. 2009). The density of coronary vessels was then assessed in hearts from each group of animals.

Gene expression in liver

The liver was examined at 20 and 60 days after TBI for the expression of genes responsible for the synthesis, metabolism or transport of cholesterol (low-density lipoprotein receptor [LDLR], peroxisome proliferator-activated receptor alpha [Ppara], sterol regulatory element binding transcription factor 1[Srebf1]) and 3-hydroxy-3-methylglutaryl-Coenzyme A reductase [Hmgcr], and for cholesterol efflux (scavenger receptor class B member 1 [SR-B1] and ATP-binding cassette, subfamily A, member 1[Abca1]).

Real-time RT-polymerase chain reaction

Total RNA was isolated from the liver samples using the TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. The concentration of total RNA was measured by absorbance at 260 nm using an UV1240 spectrophotometer (Shimadzu, Kyoto, Japan). The purity was estimated by the 260/280 nm absorbance ratio. One microgram of total RNA was subjected to reverse transcription using an oligo-$(dT)_{15}$ primer (Promega, Madison, Wisconsin, USA) and M-MuLV reverse transcriptase (New England Biolabs, Ipswich, UK) according to the manufacturers' instructions. Details of the primers used are described in the table. Real-time polymerase chain reaction (PCR) amplification and detection were performed on a Stratagene Mx3000P qPCR system (Stratagene, Santa Clara, California, US) using SYBR Green technology. The reaction mixture was composed of 12.5 μL of SYBP Premix Ex Taq (Takala, Dalian, China), forward and reverse primers (10 μmol/L, 0.5 μL each), 0.5 μL of ROX reference Dye II, 10 μL of nuclease-free water, and the cDNA sample (1 μL). PCR primers (Table 1) were designed using Primer Premier 5.0 software from the sequence identified by the GenBank accession number (http://www.nvbi.nlm.nih.gov). The housekeeping gene β-actin was used as an internal control. The differences of efficiencies in amplification between the target genes and β-actin were all less than 5%. The PCR amplification protocol was 95°C for 15 min followed by 40 cycles of 94°C for 5 sec, 50/56°C for 15 sec, and 72°C for 10 sec. After PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product, which was displayed as a single peak (data not shown). Every sample was analyzed in triplicate. The relative expression ratio (R) of the target gene was expressed for the sample versus the control in comparison to the β-actin gene. R is calculated based on the following equation (Pfaffl 2001): $R = 2^{-\Delta\Delta C_t}$, where C_t represents the cycle at which the fluorescence signal is first significantly different from background and $\Delta\Delta C_t$ is $(C_{t,target} - C_{t,actin})_{treatment} - (C_{t,target} - C_{t,actin})_{control}$ (Zhang et al. 2008) Data from the individual parameters were compared by analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple comparison test when applicable. A $P < 0.05$ was considered significant for all tests (Niesen et al. 2008).

Statistical analysis

All values are expressed as the mean $\pm$ standard deviation. For the lipid and liver function, densitometry and echocardiography analysis statistical significance was determined by performing a one-way ANOVA using a Bonferroni's multiple comparison as the post hoc test (Cabral 2008). The threshold for statistical significance ($P < 0.05$) was prospectively identified and not varied in the data analysis.

Results

Simvastatin dose–response studies in sham-irradiated control rats

A dose–response study was performed to define the daily dose of simvastatin that was not toxic to the liver. Graded doses of simvastatin were added to the diet in four groups of nonirradiated rats. Rats receiving simvastatin at a dose of 80 mg/kg bw/day became morbid 20 days after the start of drug administration. This dose of simvastatin increased ALT activity to 406 $\pm$ 61 U/L at 12 days after the start of administration compared with the value of

Table 1. Sequences of primers for real-time RT-PCR amplification.

Target gene	GenBank accession no.	Primer sequences	Product length (bp)	T_m (°C)
LDLR	NM_175762	F: GGCTATGAGTGCCTATGTC R: GTGAAGAGCAGAAACCCTA	209	56.0
Ppara	NM_013196	F: TGAAAGATTCGGAAACTGC R: TCCTGCGAGTATGACCC	110	56.0
Srebf1	NM_001276707	F: TGGAGCGAGCATTGAA R: CGACAGCGTCAGAACAG	117	50.0
Hmgcr	NM_013134	F: GAGAATAAACCAAACCCAG R: ATCAGCTATCCAGCGACT	94	56.0
Scarb1	NM_031541	F:ACCGTCTTCACAGGTGTCCAGAT R:TCATGTTGCACTGTTCCGAATGCC	103	60.0
Abca1	NM_178095	F: CCCGGCGGAGTAGAAAGG R: AGGGCGATGCAAACAAAGAC	67	56.0
β-actin	NM_031144	F: TCGTGCGTGACATTAAAGAG	134	56.0

PCR, polymerase chain reaction; LDLR, low-density lipoprotein receptor; Ppara, peroxisome proliferator-activated receptor alpha; Srebf1, sterol regulatory element binding transcription factor 1; Hmgcr, 3-hydroxy-3-methylglutaryl- Coenzyme A reductase; Scarb1, scavenger receptor class B, member 1; Abca1, ATP-binding cassette, subfamily A, member 1; F, forward primer; R, reverse primer.

40 ± 11 U/L in control untreated rats. Reducing the simvastatin content of the diet to 20 mg/kg bw/day and 10 mg/kg bw/day resulted in no morbidity and the increase in ALT activity at 12 days was 132 ± 31 and 44 ± 12 U/L, respectively. Since the lowest dose of simvastatin of 10 mg/kg bw/day was not associated with morbidity and had no significant effect on the activities of ALT, alkaline phosphatase or total bilirubin, this dose of simvastatin was used for the mitigation studies.

Simvastatin mitigates the risk of cardiac disease after X-irradiation

Simvastatin and lipid profile

From >60 days after TBI, the total cholesterol, LDL-cholesterol and triglyceride levels were significantly elevated compared to those in age-matched sham-irradiated control rats. In sham-irradiated rats, simvastatin alone significantly decreased total cholesterol levels at 20 days after the start of the study and throughout the duration of the study (Fig. 1A). From 80 to 120 days after TBI, simvastatin consistently mitigated this increase in total cholesterol (Fig. 1A). At these time intervals, simvastatin administration after TBI also reduced the increase in LDL-cholesterol, triglyceride levels, and HDL cholesterol levels compared with TBI alone. There were no mortalities in irradiated or sham-irradiated groups over the 120 day study period.

Simvastatin effects on renal function

BUN and creatinine levels remained stable over the duration of the study period in nonirradiated rats (Fig. 1B). Treatment of rats with simvastatin alone did not induce any changes in the BUN or creatinine levels (Fig. 1B). TBI significantly increased BUN levels from 19 ± 3 mg/dL at the start of the study to 112 ± 21 mg/dL after 120 days. Simvastatin significantly mitigated these TBI-induced increases in BUN at 80 and 120 days, but not at 100 days after TBI (Fig. 1B). TBI significantly increased creatinine levels from 0.3 ± 0.1 mg/dL to 1.2 ± 0.4 mg/dL at 120 days after TBI. The increase in creatinine following TBI was significantly mitigated by simvastatin after 100 and 120 days (Fig. 1B).

Simvastatin and liver function

Serum AST activity at 120 days after TBI, was decreased compared with sham-irradiated rats. Simvastatin restored AST levels in irradiated rats to sham-irradiated control levels, and had no significant effect on sham-irradiated rats. Serum ALT levels were unaffected by TBI or simvastatin administration (Fig. 2A). Total bilirubin levels were increased 120 days after TBI compared with sham-irradiated rats. Simvastatin completely mitigated this increase in bilirubin levels in irradiated rats back down to control levels. Simvastatin had no effect alone on sham-irradiated rats. Serum alkaline phosphatase activity was decreased at 120 days following TBI, compared with sham-irradiated rats. Simvastatin partially reversed this decline in alkaline phosphatase activity in irradiated rats and had no effect on sham-irradiated rats (Fig. 2A).

To determine whether TBI resulted in gross liver injury at 120 days after the start of the study, liver sections were analyzed by a pathologist (R. A. K.) who was not told the treatment. There was no histological evidence of necrosis or inflammation in any treatment group, as compared to the control sham-irradiated group (Fig. 2B).

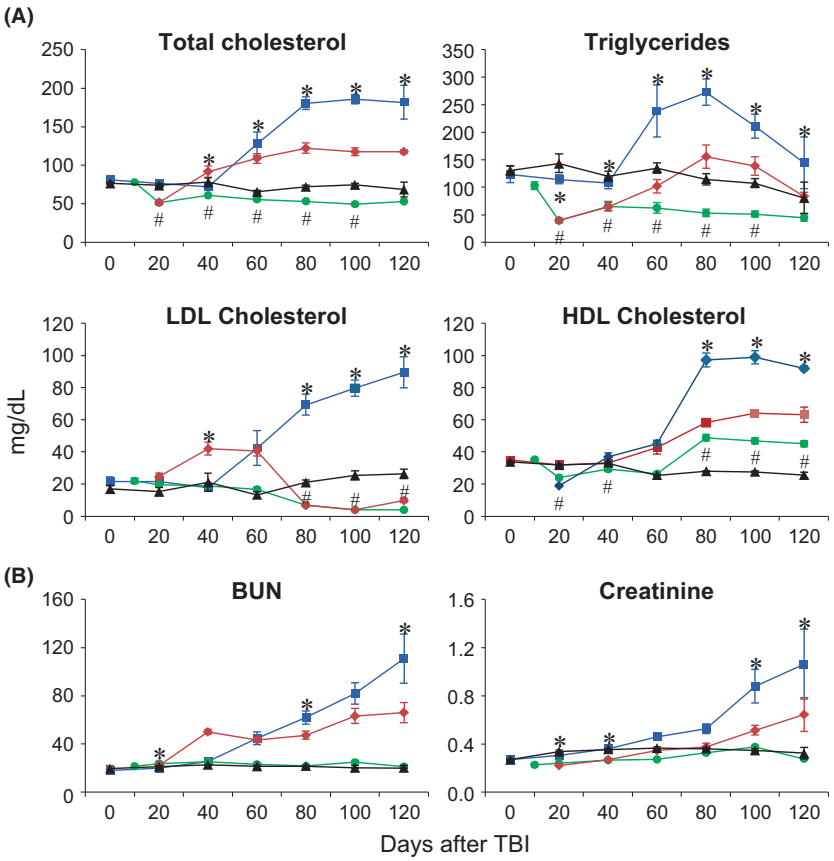

Figure 1. Simvastatin mitigates increases in risk factors for (A) cardiac disease and (B) kidney injury. Time-related changes in total cholesterol, triglycerides, LDL-cholesterol, HDLcholesterol, BUN and creatinine after TBI (■) and the impact of simvastatin (Sim) alone (●) or in combination with TBI (◆) compared with age-matched, sham-irradiated controls (▲). Data shown as means + SD, n = 9–15/group. #$P < 0.05$, Sim vs. control, *$P < 0.05$, TBI vs. TBI + Sim. LDL, low-density lipoprotein; HDL, high density lipoprotein; BUN, blood urea nitrogen; TBI, total body irradiation.

In normal humans and rats, an intricate balance is maintained between the biosynthesis, utilization and transport of cholesterol, keeping its harmful deposition to a minimum. Exposure to ionizing radiation appears to uncouple this balance, resulting in a late sustained elevation in total cholesterol, LDL-cholesterol as well as triglycerides (Baker et al. 2009). In support of the idea that ionizing radiation affects cholesterol metabolism, expression levels of liver genes were determined at 20 and 60 days after 10 Gy of X-rays using Reverse transcription (qRT)-PCR to determine when radiation-driven changes started to appear. Expression of the genes for the LDLR, Ppara, and Hmgcr were increased at 60 days after irradiation (Fig. 2C).

Simvastatin mitigates cardiac disease after X-irradiation

Simvastatin and cardiac histology

TBI resulted in an increase in peri-arterial sclerosis of small caliber intramural coronary vessels at 120 days, compared with age-matched controls. Affected vessels had partial to complete luminal sclerosis due to concentric laminar thickening of the vessel walls due to the accumulation of amphophilic matrix material between layers of hyperplastic and vacuolated smooth myocytes. Cardiomyocytes from TBI-treated rats remained normal in appearance (Fig. 3). Trichrome staining revealed peri-arterial fibrosis and irregular collagen deposition around the penetrating coronary vessels of hearts from irradiated rats. Control hearts had symmetrical penetrating vessels with only less collagen and fibrosis (Fig. 3). Simvastatin partially mitigated the appearance of TBI-induced fibrosis of the penetrating coronary vessels. Simvastatin alone had no effect on the density of these coronary vessels.

Simvastatin and coronary vessel density

TBI resulted in a decrease in the density of the smaller diameter coronary vessels (<50 μm) compared with non-

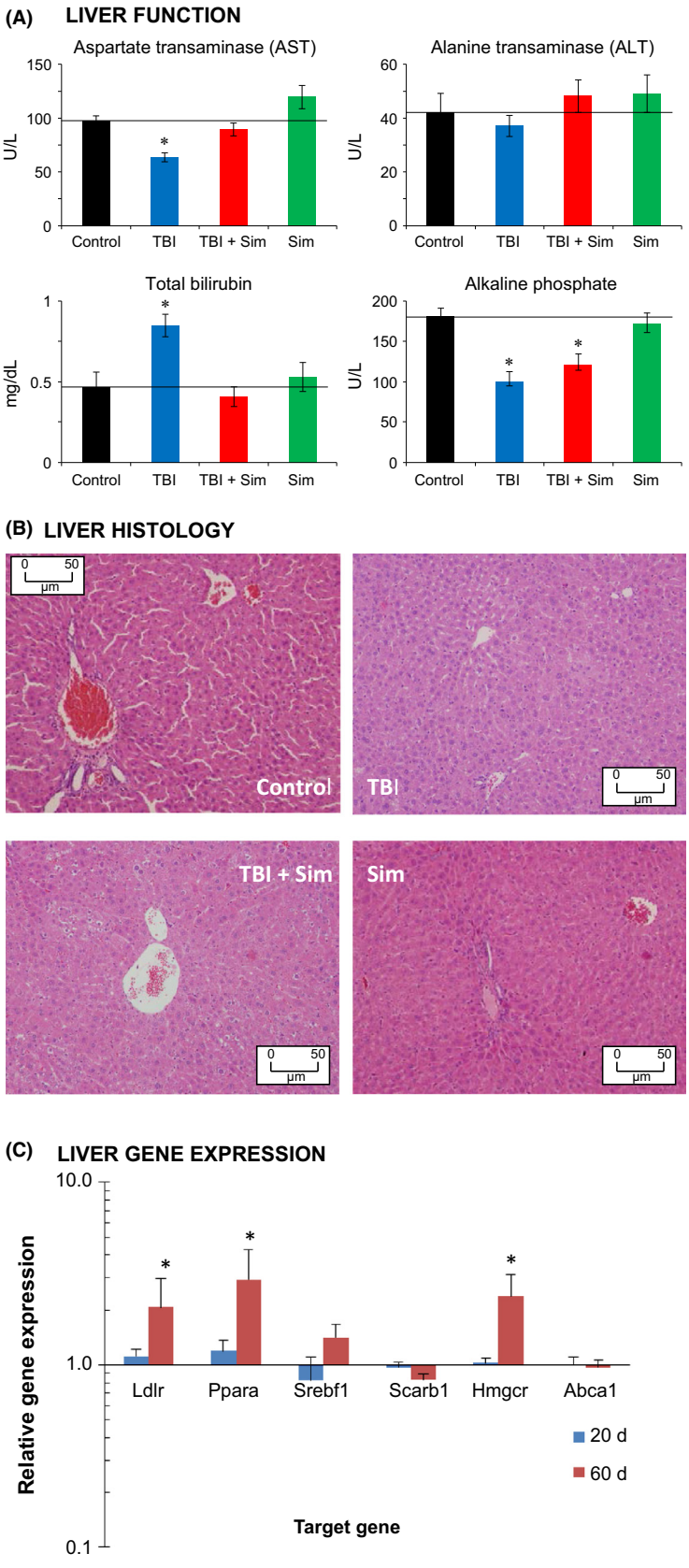

Figure 2. Simvastatin and liver function. (A) Liver function at 120 days after TBI compared with age-matched sham-irradiated control animals. The horizontal line represents value for age matched, sham-irradiated control rats. (B) Morphological changes in the liver at 120 days after TBI (H&E staining). (C) Quantitative RT-PCR analysis of gene expression in liver after 10 Gy TBI. The identities of the genes are described in the table. Livers were examined at 20 and 60 days post irradiation. Data shown as means + SD, n = 3–6/group. *P < 0.05, vs. control. TBI, total body irradiation; H&E, hematoxylin and eosin; PCR, polymerase chain reaction.

irradiated controls after 120 days (Fig. 4). The density of large diameter coronary vessels was unchanged following a TBI dose of 10 Gy. Simvastatin did not mitigate the decrease in the density of the smaller diameter coronary vessels induced by radiation (Fig. 4). Simvastatin administration did not change the density of small diameter vessels in nonirradiated control rats.

Simvastatin and cardiac mechanical function

To determine whether TBI resulted in mechanical injury to the heart, global radial and circumferential strains were determined using 2D echocardiography in intact rats. At 20 days after TBI, hearts had a significantly increased global radial strain and circumferential strain compared with age-matched controls (Fig. 5). Administration of simvastatin after TBI mitigated this effect. Simvastatin alone had no effect on mechanical function. At 80 days after TBI, hearts showed reduced global radial strain and circumferential strain compared with age-matched controls (Fig. 5). Simvastatin mitigated this reduction in radial and circumferential strain following TBI. Simvastatin alone decreased global circumferential strain after 80 days. At 120 days after TBI, global radial and circumferential strain was decreased to a greater extent than observed at 80 days. Simvastatin administered after TBI again mitigated this decrease in cardiac function at 120 days. Simvastatin alone increased radial and circumferential strain in nonirradiated rat hearts (Fig. 5).

Simvastatin mitigates acute stress from myocardial ischemia/reperfusion after X-irradiation

To determine whether simvastatin affects the ability of the heart to withstand an acute stress after TBI with

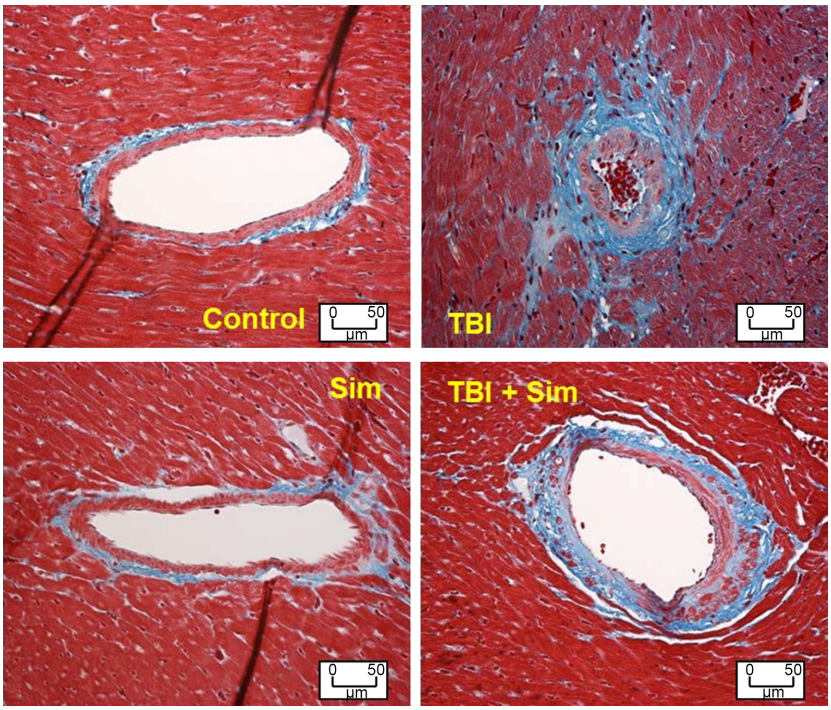

Figure 3. Simvastatin mitigates cardiac morphological changes. Sections of the heart stained with Trichrome showing increased peri-arterial fibrosis in small caliber coronary vessel 120 days after TBI with 10 Gy, compared with a comparable vessel in an age-matched nonirradiated control rat. Fibrosis appears as blue using trichrome staining. TBI exposure to 10 Gy, followed by Sim treatment (TBI + Sim), mitigated against increased mural fibrosis in coronary arteries and blockage of the vessel lumen. Three hearts were studied in each group. TBI, total body irradiation.

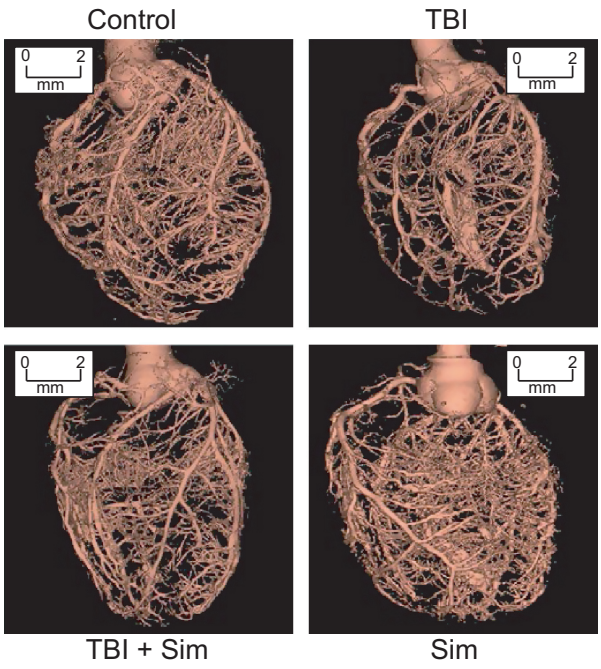

Figure 4. Simvastatin does not mitigate decreases in coronary vessel density. Representative micro-computed tomography images of changes in coronary vessel density of the heart obtained 120 days after exposure to TBI with 10 Gy, TBI followed by the administration of simvastatin (TBI + Sim) (10 mg/kg bw/day), and simvastatin administration alone (Sim) in comparison with heart from an age-matched, sham-irradiated rat (control). TBI resulted in a decrease in the density of the smaller diameter coronary vessels (<50 μm). The density of large diameter coronary vessels was unchanged following TBI with 10 Gy. Simvastatin did not affect the diameters of the smaller diameter coronary vessels. TBI, total body irradiation.

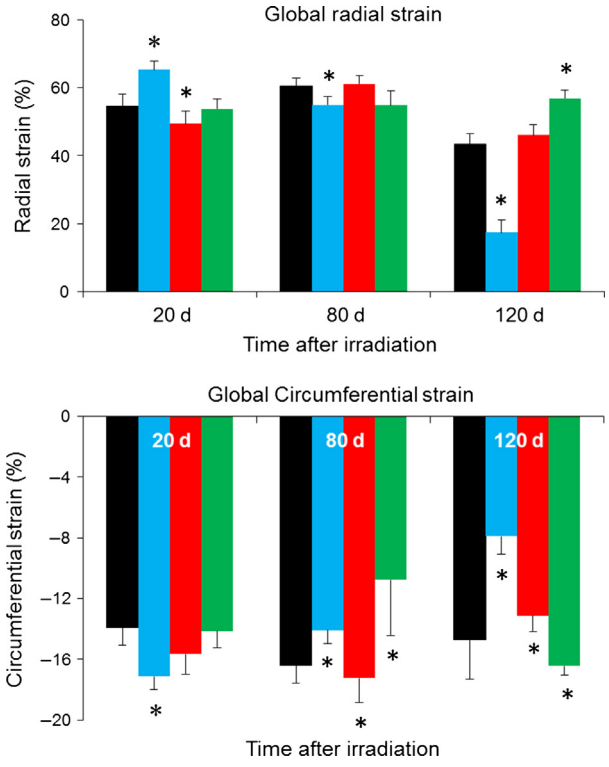

Figure 5. Simvastatin mitigates cardiac ventricular dysfunction. Cardiac mechanical function at 20, 80 and 120 days after 10 Gy TBI alone (blue), 10 Gy TBI plus simvastatin (10 mg/kg bw/day) (red), and simvastatin (10 mg/kg bw/day) alone (green) compared with age-matched, sham-irradiated control (black). Data shown as means + SD, n = 3–6/group. *P < 0.05 vs. control. TBI, total body irradiation.

10 Gy of X-rays, the severity of injury from an induced myocardial infarction was assessed in the isolated perfused heart. TBI increased the severity of myocardial infarction by 21% at 20 and 80 days after irradiation (Fig. 6).

Simvastatin mitigated the increased severity of myocardial infarction at both 20 days and 80 days after TBI (Fig. 6). Simvastatin alone decreased this induced infarct size at 80 days following sham treatment. TBI increased the post-ischemic leakage of LDH produced at 20 days and 80 days after exposure. Simvastatin mitigated LDH leakage at 20 days after TBI. TBI decreased the recovery of LVDP after 80 days. Simvastatin alone increased recovery of LVDP at 20 days TBI.

Discussion

Radiation exposure results in hypercholesterolemia (Baker et al. 2009) and injury to the coronary vasculature. Prolonged elevation of blood cholesterol following TBI suggests the development of abnormal liver cell function resulting from the increased synthesis or metabolism of cholesterol, or from decreased cholesterol efflux. The impact of ionizing radiation on the expression of genes in the liver responsible for this abnormal phenotype has not been examined. Cells that are exposed to ionizing radiation can either be repaired and function normally, be repaired and function abnormally, or die as a result of the damage. In support of this notion, persistent abnormalities in cell division in hepatocytes, induced by partial hepatectomy after irradiation, suggests abnormal liver cell function (Weinbren et al. 1960). The present study suggests that damage from a high, total body dose of sparsely ionizing radiation results in abnormal liver cell function for the following reasons: (1) radiation results in a sustained increase in expression of genes responsible for cholesterol synthesis (Ldlr, Hmgcr), (2) radiation results in a sustained increase in expression of genes responsible for lipid metabolism (Ppara), (3) radiation results in a phenotype of sustained increases in serum LDL and total cholesterol, and triglycerides, (4) radiation does not result in inflammation or necrosis of the liver upon histologic

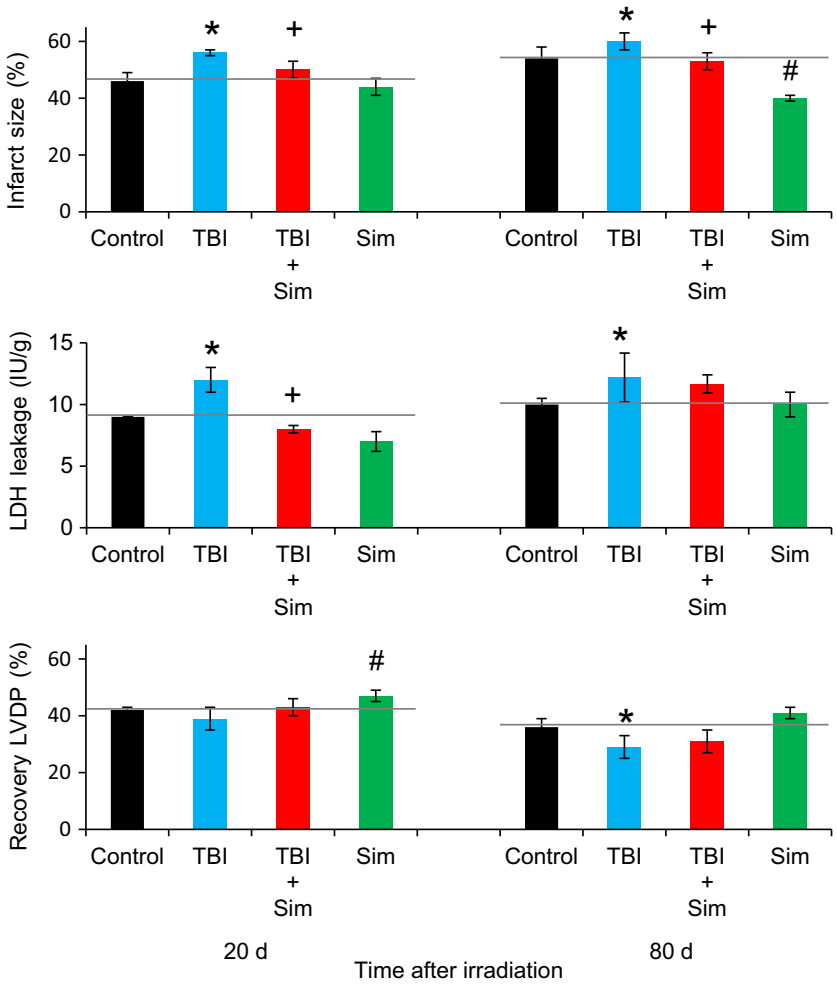

Figure 6. Increased susceptibility of the heart to the stress of ischemia/reperfusion following TBI and its mitigation by simvastatin. Severity of myocardial infarction, recovery of post ischemic LVDP, and post-ischemic leakage of LDH after 10 Gy TBI alone, 10 Gy TBI plus simvastatin (TBI + Sim) (10 mg/kg bw/day) or simvastatin (10 mg/kg bw/day) alone (Sim) compared with age-matched nonirradiated hearts (control). Horizontal line represents the value for age-matched, sham-irradiated hearts. Data shown as means + SD, $n = 6$/group. *$P < 0.05$, 10 Gy vs. age-matched, sham-irradiated controls. $^{+}P < 0.05$, TBI vs. TBI plus simvastatin. #$P < 0.05$, simvastatin vs. age-matched, sham-irradiated controls. TBI, total body irradiation; LVDP, left ventricular developed pressure; LDH, lactate dehydrogenase.

examination, and (5) radiation alters liver phenotypic function, manifest by decreased AST and alkaline phosphatase, and increased total bilirubin. Taken together, these novel findings supports the notion that liver cells have been repaired and function abnormally 120 days after irradiation.

Simvastatin is a prodrug of a specific inhibitor of Hmgcr, an enzyme essential for cholesterol biosynthesis in the liver (Del Puppo et al. 1995). It is widely used to reduce the risk of cardiovascular diseases including atherosclerosis (Grobbee and Bots 2003). Simvastatin is food and drug administration (FDA) approved and is widely available as an oral formulation. The present study demonstrates simvastatin administered post-irradiation mitigates TBI-induced increases in the risk factors for cardiac disease and the occurrence of cardiac injury. An unexpected finding was that simvastatin also partially mitigated radiation-induced renal injury.

Our findings show simvastatin mitigates against increases in risk factors for cardiac disease following 10 Gy TBI by a cholesterol-dependent mechanism. The beneficial effects of statins can also be mediated by cholesterol-independent (pleotropic) mechanisms, with upregulation of eNOS expression to preserve nitric oxide bioactivity in endothelium, and activation of endothelial progenitor cells to maintain and repair endothelium (Martinez-Gonzalez and Badimon 2007). In our study, simvastatin did not prevent the decrease in density of smaller diameter coronary vessels at 120 days following irradiation (Fig. 4). However, simvastatin did mitigate

against increased mural fibrosis in the penetrating coronary arteries; a hallmark of radiation injury (Fig. 3). Taken together, these findings suggest the actions of simvastatin in this study may not extend to the smaller diameter coronary vessels. Additional studies are needed to determine the underlying mechanisms by which simvastatin mitigates radiation injury to heart.

The 10 Gy dose of acute radiation exposure impairs the ability of the heart to function mechanically as a pump. Cardiac strain was increased at 20 days and decreased at 80 and 120 days following TBI. Cardiac strain is a very earlier predictor of heart failure and measures injury to the heart independently from the severity of myocardial infarction. Increased strain at 20 days following TBI may reflect a hyperdynamic state. This hyperdynamic state may be induced by dehydration from blood volume depletion (Swift and Taketa 1958) or sepsis (Duran-Struuck et al. 2008) present immediately following irradiation. Simvastatin was effective in mitigating cardiac mechanical dysfunction at 20–120 days following irradiation.

Severity of injury from an induced myocardial infarction was increased at 20 days and persisted up to at least 80 days following exposure to ionizing radiation. This finding of an increased severity of myocardial infarction and increased postischemic enzyme leakage as early as 20 days after TBI suggests the cardiac effects induced by ionizing radiation are manifest much earlier than shown in previous studies (Baker et al. 2009). To the knowledge of the present authors, this has not been described before. The present finding of an increased susceptibility to cardiac injury, as early as 20 days after TBI, is supported by the recent observation of decreased cardiac defenses against oxidative stress that become manifest as early as 1–7 days after TBI in rats (Mansour and Tawfik 2012). This response of the heart to an environmental stress following TBI is present after injury to the gastrointestinal and hematopoietic systems, but before there is pathophysiological evidence of lung or kidney injury (Moulder 2014). Simvastatin also decreased the severity of injury from an induced myocardial infarction at 20 and at 80 days following irradiation, further supporting its ability to function as a mitigator of radiation-induced injury to the heart.

Radiation injury to abdominal organs is responsible for increasing risk factors for, and the occurrence of, cardiac disease after 10 Gy TBI (Lenarczyk et al. 2013). Ionizing radiation alters the abundance of specific intestinal microbiota (Lam et al. 2012a) that persists at least 21 days following exposure to radiation. The present study shows an increased severity of injury from an induced myocardial infarction at 20 days following exposure to 10 Gy TBI. Increased severity of injury from an induced myocardial infarction persists at 120 days following TBI although the magnitude of the effect is slightly smaller than at 20 days. This persistence in effect suggests the composition and abundance of the intestinal microbiota remains altered at 120 days following irradiation and has yet to return to pre-irradiation values. Intestinal microbiota have been mechanistically linked to the severity of an induced myocardial infarction (Lam et al. 2012b). In this study, vancomycin, a nonabsorbed antibiotic, and *Lactobacillus plantarum 299v*, a probiotic bacterium that lives in the intestines and is beneficial to health, reduced the severity of an induced myocardial infarction. The probiotic bacteria *Lactobacillus plantarum 299v* also improved hemodynamic systolic and diastolic function in the rat model of heart failure, suggesting intestinal microbiota may be used in the treatment of heart failure (Gan et al. 2014). It is proposed that ionizing radiation alters the composition of the intestinal microbiota to indirectly increase severity of an induced myocardial infarction. The intestinal microbiota may be another target for mitigation of radiation-induced indirect injury to the heart.

At high doses, TBI affects many tissues and results in multiple organ dysfunction, including hematopoietic failure, pulmonary fibrosis, renal failure, accelerated coronary artery disease, and cardiac mechanical dysfunction. Similar findings can occur for high dose partial body exposure, as in the case of cancer therapy. Therefore, an effective approach to mitigate against radiation injury is to target several effects of radiation injury simultaneously instead of targeting just a single pathway. Furthermore, cardiac injury from radiation occurs by multiple mechanisms. Targeting multiple mechanisms may be the most effective strategy to reduce radiation-induced cardiac injury. Candidate drugs to mitigate against radiation injury in the setting of a radiation/nuclear incident should have the following qualities; (1) prior FDA approval, and (2) the ability to target multiple organs by affecting a common cell type (i.e., endothelium). Inhibition of Hmgcr by statins has led to an improvement in primary and secondary prevention of coronary artery disease. Since statins are approved by the FDA for other indications, are widely available as oral formulations, and can be safely administered over periods of years in people, including children, they could be highly effective if employed as countermeasures to mitigate radiation injuries to multiple organs under conditions where high radiation dose exposures have occurred.

In conclusion, simvastatin mitigates against radiation-induced increases in the risk factors for and the occurrence of cardiac disease following TBI with 10 Gy. This dose of radiation is relevant to the clinical setting, for example, hematopoietic stem cell transplantation, and for certain radiological terrorist scenarios where high dose

exposures are likely. This statin may be suitable for development as a medical countermeasure for the mitigation of radiation-induced cardiac disease.

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Author Contributions

Moulder and Baker participated in research design, Lenarczyk, Su, Haworth, Fish, Harmann conducted the experiments while Lenarczyk, Su, Haworth, and Komorowski performed data analysis, and Migrino, Hopewell, Kronenberg, Patel, Moulder, Baker wrote or contributed to the writing of the manuscript.

Disclosures

None declared.

References

Armenian SH, Sun CL, Vase T, Ness KK, Blum E, Francisco L, et al. (2012). Cardiovascular risk factors in hematopoietic cell transplantation survivors: role in development of subsequent cardiovascular disease. Blood 120: 4505–4512.

Baker J, Su J, Hsu A, Shi Y, Zhao M, Xu H, et al. (2008). Human thrombopoietin reduces myocardial infarct size, apoptosis and stunning following ischemia/reperfusion in rats. Cardiovasc Res 77: 44–53.

Baker JE, Fish BL, Su J, Haworth ST, Strande JL, Komorowski RA, et al. (2009). 10 Gy total body irradiation increases risk of coronary sclerosis, degeneration of heart structure and function in a rat model. Int J Radiat Biol 85: 1089–1100.

Bao N, Ushikoshi H, Kobayashi H, Yasuda S, Kawamura I, Iwasa M, et al. (2009). Simvastatin reduces myocardial infarct size via increased nitric oxide production in normocholesterolemic rabbits. J Cardiol 53: 102–107.

Cabral HJ (2008). Multiple comparisons procedures. Circulation 117: 698–701.

Coleman CN, Blakely WF, Fike JR, MacVittie TJ, Metting NF, Mitchell JB, et al. (2003). Molecular and cellular biology of moderate-dose (1-10 Gy) radiation and potential mechanisms of radiation protection: report of a workshop at Bethesda, Maryland, December 17-18, 2001. Radiat Res 159: 812–834.

Del Puppo M, Rauli S, Galli Kienle M (1995). Inhibition of cholesterol synthesis and hepatic 3-hydroxy-3-methylglutaryl–CoA reductase in rats by simvastatin and pravastatin. Lipids 30: 1057–1061.

Dimmeler S, Zeiher AM, Schneider MD (2005). Unchain my heart: the scientific foundations of cardiac repair. J Clin Invest 115: 572–583.

Duran-Struuck R, Hartigan A, Clouthier SG, Dyson MC, Lowler K, Gatza E, et al. (2008). Differential susceptibility of C57BL/6NCr and B6.Cg-Ptprca mice to commensal bacteria after whole body irradiation in translational bone marrow transplant studies. J Transl Med 6: 10.

Fajardo LF, Stewart JR (1970). Experimental radiation-induced heart disease. I. Light microscopic studies. Am J Pathol 59: 299–316.

Ferreira WP, Bertolami MC, Santos SN, Barros MR, de Matos Barretto RB, Pontes SC Jr, et al. (2007). One-month therapy with simvastatin restores endothelial function in hypercholesterolemic children and adolescents. Pediatr Cardiol 28: 8–13.

Gan XT, Ettinger G, Huang CX, Burton JP, Haist JV, Rajapurohitam V, et al. (2014). Probiotic administration attenuates myocardial hypertrophy and heart failure after myocardial infarction in the rat. Circ Heart Fail 7: 491–499.

Grobbee DE, Bots ML (2003). Statin treatment and progression of atherosclerotic plaque burden. Drugs 63: 893–911.

de Jongh S, Ose L, Szamosi T, Gagne C, Lambert M, Scott R, et al. (2002) Efficacy and safety of statin therapy in children with familial hypercholesterolemia: a randomized, double-blind, placebo-controlled trial with simvastatin. Circulation 106:2231–2237.

Lam V, Moulder JE, Salzman NH, Dubinsky EA, Andersen GL, Baker JE (2012a). Intestinal microbiota as novel biomarkers of prior radiation exposure. Radiat Res 177: 573–583.

Lam V, Su J, Koprowski S, Hsu A, Tweddell JS, Rafiee P, et al. (2012b). Intestinal microbiota determine severity of myocardial infarction in rats. FASEB J 26: 1727–1735.

Lefer AM, Campbell B, Shin YK, Scalia R, Hayward R, Lefer DJ (1999). Simvastatin preserves the ischemic-reperfused myocardium in normocholesterolemic rat hearts. Circulation 100: 178–184.

Lenarczyk M, Lam V, Jensen E, Fish BL, Su J, Koprowski S, et al. (2013). Cardiac injury after 10 Gy total body irradiation: indirect role of effects on abdominal organs. Radiat Res 180: 247–258.

Llevadot J, Murasawa S, Kureishi Y, Uchida S, Masuda H, Kawamoto A, et al. (2001). HMG-CoA reductase inhibitor mobilizes bone marrow–derived endothelial progenitor cells. J Clin Invest 108: 399–405.

Mansour HH, Tawfik SS (2012). Early treatment of radiation-induced heart damage in rats by caffeic acid phenethyl ester. Eur J Pharmacol 692: 46–51.

Martinez-Gonzalez J, Badimon L (2007). Influence of statin use on endothelial function: from bench to clinics. Curr Pharm Des 13: 1771–1786.

Meacham LR, Chow EJ, Ness KK, Kamdar KY, Chen Y, Yasui Y, et al. (2010). Cardiovascular risk factors in adult survivors of pediatric cancer–a report from the childhood cancer survivor study. Cancer Epidemiol Biomarkers Prev 19: 170–181.

Migrino RQ, Zhu X, Pajewski N, Brahmbhatt T, Hoffmann R, Zhao M (2007). Assessment of segmental myocardial viability using regional 2-dimensional strain echocardiography. J Am Soc Echocardiogr 20: 342–351.

Mole RH (1984). The LD50 for uniform low LET irradiation of man. Br J Radiol 57: 355–369.

Moulder JE (2014). 2013 Dade W. Moeller lecture: medical countermeasures against radiological terrorism. Health Phys 107: 164–171.

Moulder JE, Fish BL (1989). Late toxicity of total body irradiation with bone marrow transplantation in a rat model. Int J Radiat Oncol Biol Phys 16: 1501–1509.

Moulder JE, Cohen EP, Fish BL (2014). Mitigation of experimental radiation nephropathy by renin-equivalent doses of angiotensin converting enzyme inhibitors. Int J Radiat Biol 90: 762–768.

Mulrooney DA, Yeazel MW, Kawashima T, Mertens AC, Mitby P, Stovall M, et al. (2009). Cardiac outcomes in a cohort of adult survivors of childhood and adolescent cancer: retrospective analysis of the Childhood Cancer Survivor Study cohort. BMJ 339: b4606.

Niesen M, Bedi M, Lopez D (2008). Diabetes alters LDL receptor and PCSK9 expression in rat liver. Arch Biochem Biophys 470: 111–115.

O'Driscoll G, Green D, Taylor RR (1997). Simvastatin, an HMG-coenzyme A reductase inhibitor, improves endothelial function within 1 month. Circulation 95: 1126–1131.

Pfaffl MW (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.

Shi J, Wang J, Zheng H, Ling W, Joseph J, Li D, et al. (2003). Statins increase thrombomodulin expression and function in human endothelial cells by a nitric oxide-dependent mechanism and counteract tumor necrosis factor alpha-induced thrombomodulin downregulation. Blood Coagul Fibrinolysis 14: 575–585.

Stone HB, Moulder JE, Coleman CN, Ang KK, Anscher MS, Barcellos-Hoff MH, et al. (2004). Models for evaluating agents intended for the prophylaxis, mitigation and treatment of radiation injuries. Report of an NCI Workshop, December 3-4, 2003. Radiat Res 162: 711–728.

Swift MN, Taketa ST (1958). Effect on circulating blood volume of partial shielding of rat intestine during x-irradiation. Radiat Res 8: 516–525.

Thomas ED (1982). Kettering Award Lecture. The role of marrow transplantation in the eradication of malignant disease. Cancer 49: 1963–1969.

Weinbren K, Fitschen W, Cohen M (1960). The unmasking by regeneration of latent irradiation effects in the rat liver. Br J Radiol 33: 419–425.

Yeung TK, Hopewell JW (1985). Effects of single doses of radiation on cardiac function in the rat. Radiother Oncol 3: 339–345.

Zhang H, Shi Z, Liu Y, Wei Y, Dai J (2008). Lipid homeostasis and oxidative stress in the liver of male rats exposed to perfluorododecanoic acid. Toxicol Appl Pharmacol 227: 16–25.