Changes in microvascular density differentiate metabolic health outcomes in monkeys with prior radiation exposure and subsequent skeletal muscle ECM remodeling

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Running headline: Skeletal muscle architecture changes following irradiation

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Abstract

Radiation exposure accelerates the onset of age-related diseases such as diabetes, cardiovascular disease, and neoplasia and thus lends insight into in vivo mechanisms common to these disorders. Fibrosis and extracellular matrix (ECM) remodeling, which occur with aging, overnutrition, and following irradiation, are both risk factors for type-2 diabetes mellitus (T2DM) development. Our prior work demonstrated that monkeys with whole-body radiation exposure five to nine years prior had an increased incidence of skeletal muscle insulin resistance and T2DM. We hypothesized that irradiation-induced fibrosis alters muscle architecture predisposing irradiated animals to insulin resistance and overt diabetes. Rhesus macaques (Macaca mulatta; n=7-8/group) grouped as non-irradiated age-matched controls (NonRad-CTL), irradiated non-diabetic monkeys (Rad-CTL) and irradiated monkeys that subsequently developed diabetes (Rad-DM) were compared. Prior radiation exposure resulted in persistent skeletal muscle ECM changes including a relative overabundance of collagen IV and a trend of increased transforming growth factor beta 1 (TGFβ1). Notably, preservation of microvascular markers differentiated the irradiated diabetic and non-diabetic groups. Rad-DM had lower microvascular density, plasma nitrate, and heat shock protein 90 levels compared to Rad-CTL. These results are consistent with a protective effect of abundant microvasculature in maintaining glycemic control within radiation-induced fibrotic muscle.
Introduction

The long-term effects of ionizing irradiation include an increased risk of type 2 diabetes mellitus (T2DM). This is documented in patients exposed to radiation as children (16, 30) and patients that were treated with abdominal radiation for Hodgkin’s lymphoma (33). Following nuclear disasters such as Chernobyl and Hiroshima, increased rates of diabetes mellitus are reported, but the distribution of Type 1 and Type 2 diabetes are generally not clear (17, 46). Metabolic disturbance as a delayed effect of acute whole body irradiation (WBI) has also recently been documented in mice (34) and nonhuman primates (25).

The hallmark of metabolic decline and T2DM is peripheral insulin resistance in tissues such as skeletal muscle, which is responsible for the vast majority of insulin-stimulated peripheral glucose metabolism (7). While musculoskeletal damage has been described post-irradiation, physical function has generally been the clinically studied outcome (41). Considering the importance of muscle metabolism and the pronounced effects of irradiation, it is imperative that the link between prior irradiation and the pathogenesis of metabolic disease be defined. To date, there has been little attention to this important area of study.

Well-known biological effects of radiation exposure include the induction of reactive oxygen species and resulting inflammatory cascades, mediated by transforming growth factor beta 1 (TGFβ1), which lead to tissue fibrosis (28, 36, 45). Fibrosis in skeletal muscle, defined as the accumulation of collagen and other extracellular matrix (ECM) components, is linked to overall metabolic dysfunction in humans (2, 37). Further, mechanistic studies in rodent models demonstrate that skeletal muscle ECM expansion is a contributing factor to the development of
insulin resistance (19–22). It is possible that fibrosis of skeletal muscle plays a role in the increased incidence of metabolic disease following WBI.

Balanced ECM synthesis and degradation is required for capillary bed maintenance (13). Radiation may disturb this balance, effectively decreasing accessible muscle metabolic surface area. Radiation causes a decrease in capillary density in cardiac muscle (39) and brain tissue (10), and thus we hypothesized that skeletal muscle was likely similarly affected. Human studies have demonstrated that increased capillary density in skeletal muscle is associated with better health status (32), while loss of capillary density has been associated with disease states such as T2DM (11). Of particular importance in the development of metabolic disease, perhaps including following radiation exposure, is dysfunctional microvascular responses to insulin as extensively reviewed by Keske and others (26).

Taken together, current evidence suggests that skeletal muscle is an underappreciated organ for determination of metabolic health outcomes following irradiation. The relative importance of architectural changes of muscle following irradiation and the ultimate progression of T2DM are not fully understood. Our prior work demonstrated that radiation exposure was associated with muscle tissue insulin resistance even in objectively healthy, non-diabetic monkeys many years after exposure (25). Our aim for this study was to evaluate whether the architectural properties of skeletal muscle could explain why some irradiated animals with skeletal muscle insulin resistance differentiate into overt diabetes, while others do not. We tested the hypothesis that impaired muscle insulin action corresponds to remodeling of skeletal muscle architecture following radiation.
Materials and Methods

Animals

Rhesus macaques (*Macaca mulatta*) are part of the animal core within the consortium termed Radiation Countermeasures Centers of Research Excellence (RadCCORE), an animal resource used to collectively and collaboratively increase possible agents to detect, mitigate and treat acutely those people exposed to deterministic doses of radiation (www.radccore.org). As previously reported, these animals originated from different institutions and are survivors of exposure to a single sub-lethal dose of gamma WBI ranging from 6.5 to 8.4 Gy prior to their arrival at Wake Forest University (25). Exposure ranged from five to nine years prior to this report (Table 1). Animals (n = 7-8/group) that were non-irradiated controls (NonRad-CTL), irradiated non-diabetic monkeys (Rad-CTL), and irradiated monkeys that subsequently developed diabetes (Rad-DM) were further characterized for skeletal muscle architecture changes as described below. All animals within this study had equivalent housing with limited opportunities to exercise. All monkeys have been maintained since 2010 on a diet similar in nutritional profile to what is typically consumed by people of westernized nations (Monkey Diet 5L0P; LabDiet®, St. Louis, MO), with the exception of the diabetic monkeys which were switched to a standard laboratory chow diet (Monkey Diet 5038) immediately following diagnosis. Food was available ad libitum and consumption is not quantitated.

Monkeys were diagnosed with diabetes one to four years prior to the start of this investigation using the current American Diabetes Association criteria (49). Those with diabetes had all combination insulin administration withdrawn for at least 24 hours, and regular insulin withdrawn for at least 12 hours prior to assessment. Monkeys were all fasted for a minimum of
12 hours prior to assessments. Animals were anesthetized with intramuscular ketamine (10 to 15 mg/kg) to allow for sample and data collection. Each animal was weighed. Blood samples were collected by percutaneous venipuncture of the femoral vein into ethylenediaminetetraacetic acid (EDTA). The blood was held on ice until it could be processed. After processing, samples were stored at -80°C until analysis. Fasting blood glucose was determined by the glucose oxidase method, and fasting plasma insulin concentration was determined by enzyme linked immunosorbent assay (ELISA; Mercodia, Uppsala, Sweden) from the plasma sample. Whole blood was used to determine the glycated hemoglobin (HbA1c) using high-performance liquid chromatography methodology (Primus PDQ, Primus Diagnostics, Kansas City, MO).

Triglyceride (TG), high density lipoprotein cholesterol (HDLc) and total plasma cholesterol (TPC) concentrations were measured enzymatically. Muscle biopsies were collected from the biceps femoris and fixed in 4% paraformaldehyde for 24 hours, then transferred to 70% ethyl alcohol until processing and embedding for histology, or frozen in liquid nitrogen for protein extractions.

All animal procedures were performed according to the protocol approved by the Wake Forest University Institutional Animal Care and Use Committee in compliance with the USDA Animal Welfare Act and Animal Welfare Regulations (Animal Welfare Act as Amended; Animal Welfare Regulations) and according to recommendations in the Guide for Care and Use of Laboratory Animals (Institute for Laboratory Animal Research).

**Extracellular Matrix**

**Collagens**
Collagens type I, III, and IV were assessed in formalin-fixed paraffin-embedded monkey muscle sections. Slides were stained using the Leica Bond Max IHC stainer. Heat-induced antigen retrieval was performed by incubating slides in Epitope Retrieval Solution 2 (Leica) for 20 minutes. Slides were then incubated in either anti-Collagen I (1:100; Abcam ab138492), anti-Collagen III (1:100; Abcam ab6310), or anti-Collagen IV (1:600; Abcam ab6586) antibodies for one hour. The Bond Polymer Refine Detection system was used to produce localized, visible 3,3’-diaminobenzidine staining. Slides were finally dehydrated, cleared, and cover slipped.

Images were captured at 200x total magnification using a Nikon DS-Ri2 camera mounted on a Nikon AZ100M upright, wide-field microscope. Image analysis was performed using a custom, automated macro in ImageJ. Briefly, 3,3’-diaminobenzidine (DAB) staining was isolated using color deconvolution and then a variation of the isodata algorithm was used for automatic thresholding. Finally, the % area of the section occupied by DAB staining was measured. An investigator blinded to group identity performed all image acquisition and analyses.

Transforming Growth Factor Beta (TGFβ1)

ELISA was performed to measure TGFβ1 in monkey muscle. Quantities in muscle lysates were determined using the human TGFβ1 ELISA (Quantikine® Kit R&D System) according to the manufacturer’s instructions. Samples were acid-activated using a protocol provided by the manufacturer. Quantities were estimated based on a standard curve generated with recombinant TGFβ1.

Matrix Metalloproteinase Activity

Gelatin zymography was performed to measure matrix metalloproteinase 9 (MMP9) activity. Monkey skeletal muscle samples were mechanically homogenized (Bullet Blender, Next
Advance) in a pH 7.5 buffer containing 100mM Tris HCl, 10mM EDTA, and 0.5% Triton X-100. Homogenates were centrifuged at 13,000 rpm for 20 minutes at 4°C. Supernatants (500 ug protein) were then incubated with gelatin-Sepharose beads for 2 hours at 4°C to purify and concentrate gelatinases. After 3 washes, gelatin-sepharose beads were resuspended in non-reducing Laemmli buffer and loaded onto 10% zymogram gels for electrophoresis. Conditioned medium from HT-1080 cells was used as a positive control. Following electrophoresis, gels were washed in a renaturing buffer (Invitrogen, Carlsbad, CA) to restore gelatinolytic activity. Gels were then incubated in a developing buffer (Invitrogen) overnight at 4°C. Finally, the gels were stained with SimplyBlue SafeStain (Thermo Fisher; Waltham, MA) to reveal areas of gelatin digestion, which appear clear against a blue background. The gels were then imaged and digestion band intensity was quantified by densitometry.

Skeletal Muscle Triglycerides

Muscle triglyceride (mTG) content was measured as previously described (24).

Microvasculature-Related Parameters

CD31+ Endothelial Cells

Slides of muscle tissue were embedded for immunohistochemistry. CD31+ endothelial cells were stained with PECAM-1 (M-20) goat anti-mouse polyclonal antibody (Santa Cruz Biotechnology, Inc). Blinded assessors counted the total number of cells that stained positively for CD31 in 20 fields at 20x magnification in duplicate. Representative images are shown in Figure 2 C-E.
**Plasma Nitrate**

Nitrate (NO₃) levels were measured in the plasma samples, using an Eicom NOx analyzer (eNO-20) per the manufacturer’s instructions as previously described (40).

**Vascular Endothelial Growth Factors**

Vascular endothelial growth factor (VEGF) and tumor necrosis factor related ligand 1A (TNFR1A) in muscle tissue were measured using ELISA (MyBioSource, San Diego, CA) according to the manufacturer’s protocols.

**Endothelial Nitric Oxide Synthase**

Quantification of nitric oxide synthase (eNOS) mRNA in muscle was measured by quantitative real-time polymerase chain reaction (qRT-PCR). Primer sequences for eNOS were forward: 5′-gaaggctggcatctggaa -3′ and reverse: 5′- cagagccctggccttttc-3′. Data are expressed in arbitrary units normalized by the qRT-PCR signal obtained in the same cDNA preparation for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The primer sequences used for GAPDH were forward: 5′-caccaactgettagcaccce - 3’ and reverse: 5′- tggteatgagtcctcagc - 3’.

**Heat Shock Protein**

Heat shock protein 90 (HSP90) in muscle homogenate was quantified in duplicate using a sandwich ELISA with the HSP90 Alpha ELISA Kit (StressMarq Biosciences Inc., Victoria BC CANADA) per the manufacturer’s instructions.

**Data Analysis**

Data were first inspected for significant outliers and winsorized as necessary to three standard deviations. Data were also inspected for deviations from normality. Non-normal parameters were
log transformed if required to achieve statistical assumptions of normality. Data are expressed throughout as mean ± standard error of the mean. Group differences were analyzed using one-way analysis of variance (ANOVA) with alpha level set at 0.05 for statistical significance and p = 0.10 for denoting trends. We additionally examined for the main effects of radiation and diabetes by ANOVA. Outcome measures were co-varied by the monkeys’ body weights at assessment. Post-hoc analyses were conducted using Tukey’s honest significant differences testing. Correlation coefficients were determined by Pearson’s R statistics for association. All statistical testing was performed using Statistica V10 (StatSoft Inc., Carlsbad, CA).

Results

Demographic and metabolic characteristics of NonRad-CTL, Rad-CTL, and Rad-DM monkeys are shown in Table 1. There were no significant differences in age between groups. Amongst irradiated monkeys there were no significant differences in irradiation dose, age at irradiation, and years since irradiation. The mean post-WBI delay to assessment was 9.0 years. In this species, this is the human equivalent of approximately 20 years, a significant period of life which would span childhood to adulthood. In all subsequent analyses, dose of irradiation, time since irradiation, and age at irradiation were not related to any outcome measures. No results violated outlier criterion.

Body weights of the Rad-DM monkeys were not overweight nor obese and comparable to NonRad-CTL animals. The Rad-CTL monkeys were significantly leaner (~40%) compared to the other groups. Waist circumference showed a similar pattern to body weight (Table 1) as did previously documented body fat percentage by computed tomography (25). No differences in
systolic or diastolic blood pressure were noted between groups. Other related health and inflammatory markers within this cohort have been previously published (6, 25).

Diabetic monkeys were hyperglycemic and hyperinsulinemic as expected. The degree of hyperinsulinemia was highly variable within the diabetic group, reflecting different stages of compensatory insulin secretion. The diabetic monkeys, expectantly, had significantly higher HbA1C and plasma TG compared to the non-diabetic monkeys. Rad-CTL monkeys had significantly less circulating insulin than those in the Rad-DM group likely due to their decreased adiposity (18). Previous work within this cohort included mixed meal tolerance testing, intravenous glucose tolerance testing, and skeletal muscle insulin signaling. Briefly, as expected tolerance tests were significantly worse in diabetic animals compared to non-diabetic groups. Interestingly, all irradiated animals showed poorer skeletal muscle insulin signaling response as evidenced by decreased phosphorylation of protein kinase B and insulin receptor substrate-1 in insulin-stimulated muscle biopsies (25).

Years after radiation exposure, monkeys had a relative overabundance of collagen type IV deposition, as reflected by a significantly greater ratio of collagen 4:1 (Figure 1A, p = 0.04), and a trend for an increase in TGFβ1 (Figure 1B; p = 0.10). There were no differences between groups in ECM breakdown as measured by MMP9 activity (Table 2). Skeletal muscle of Rad-DM had both greater mTG content (Table 2) and greater absolute collagen IV deposition (Table si2) than non-diabetic monkeys.

CD31+ cells (Figure 2A; p = 0.02), and plasma NO3 (Figure 2B; p = 0.04) were more abundant in Rad-CTL compared to Rad-DM. Rad-CTL was 150% and 160% greater than NonRad-CTL respectively. The NO3 results have been repeated in an additional cohort of monkeys consistently
demonstrating Rad-CTL monkeys had higher values then NonRad-CTL (data not shown). VEGF and TNFR1A, showed no significant differences between groups (Table 3). In addition, there were no significant differences in the quantity of eNOS mRNA (Table 3). Irradiated monkey muscle had significantly less HSP90 (Figure 3A). Both HSP90 and TNFR1A positively correlated with plasma NO3 (Figure 3B; \( r = 0.44, p = 0.04 \) and Figure 3C; \( r = 0.44, p = 0.05 \)).

The relationship between groups with respect to MMP9 activity and TNFR1A is similar to the group pattern seen with CD31\(^+\) endothelial cells and NO3. Rad-CTL monkeys had 1.8-fold greater mean MMP9 activity and 1.7-fold greater mean TNFR1A, which is similar to the 2-4 fold greater observed CD31\(^+\) cells and NO3 when compared to Rad-DM.

Evidence of an interaction between ECM and microvasculature-related indices is demonstrated as TGFβ1 correlated negatively with both proangiogenic VEGF and eNOS chaperone, HSP90 (Figure 4A-B; \( r = -0.42, p 0.04 \) and \( r = -0.51, p = 0.01 \)). Additionally, greater muscle lipid content was associated with increased fibrotic signaling (Figure 4C; \( r = 0.48, p = 0.018 \)) and increased collagen IV deposition (Figure 4D; \( r = 0.49, p = 0.019 \)).

**Discussion**

Our study is the first to examine the late effects of irradiation induced changes in skeletal muscle architecture and their relationship with metabolic health. It is unique in the description of these changes in nonhuman primates, a model which very closely matches the pathogenesis of human metabolic disease (44). The observed preservation of microvascular abundance in non-diabetic monkeys exposed to radiation is in line with previous studies showing a link between capillary density and metabolic disease (1, 12, 35). We also demonstrate that radiation is associated with changes to muscle ECM and leads to suppression of HSP90 levels many years after radiation.
exposure. This reduction in HSP90 may reduce skeletal muscle perfusion through decreased eNOS activity in response to insulin (42).

**Microvasculature**

Staining for endothelial cell marker CD31 showed that microvascular abundance differentiated diabetic and non-diabetic irradiated animals, as Rad-DM had significantly less CD31+ cells than Rad-CTL. Surprisingly, there were no significant differences in VEGF and TNFR1A between groups suggesting a similar balance of vascular remodeling between groups. Studies evaluating delayed effects of irradiation on skeletal muscle microvascular abundance are limited. Mouse studies suggest the response is dependent on dose. Mouse cardiac muscle decreased capillary density following exposure to radiation at doses comparable to our study (≥ 8 Gy) (39). Conversely, Mathis et al. found an increase in CD31+ cells in mouse cardiac muscle following irradiation when using a low exposure dose of 2 Gy (29).

There is evidence that T2DM is associated with reduced insulin-mediated muscle capillary recruitment even without changes in capillary density(4). Therefore, indicators of endothelial function were further examined within this study. Capillary endothelial cells contain an enzyme, nitric oxide synthase, which produces NO in response to shear stress and growth factors. Insulin increases eNOS activity in endothelial cells to promote microvascular recruitment, which subsequently improves hormone (e.g. insulin) and nutrient (e.g. glucose) delivery to tissues (5). There were no significant differences in eNOS gene expression between groups. There was, however, a significant decrease in plasma NO3, a NO metabolite, in Rad-DM monkeys. This may suggest decreased function of eNOS in Rad-DM, which is in agreement with the findings of Kashyap et al. where nitric oxide synthase (NOS) activity increased 2.5 fold in controls in
response to insulin, however insulin failed to stimulate NOS activity in people with diabetes (23).

HSP90 influences perfusion by associating with eNOS and augmenting the production of NO (9). Irradiated monkeys had significantly lower amounts of HSP90 in muscle tissue compared to non-irradiated monkeys, with Rad-DM also being significantly lower than both groups without diabetes. In rats that developed fibrosing alveolitis following irradiation, HSP90 levels decreased more than 90% in lung parenchymal cells (14). One mechanism for decreased amounts of HSP90 in irradiated tissues could be repressed gene expression by p53 as noted in UV-irradiated cells (47). Microvascular function is impaired in pro-inflammatory states such as obesity and ageing (26). Here we provide evidence of reduced HSP90 as a possible mechanism of endothelial impairment and predisposition to insulin resistance following radiation exposure. A limitation to our study is a lack of direct measurement and comparison of microvascular responsiveness between groups.

**Extracellular Matrix**

TGFβ1 is widely recognized as a major regulating cytokine of tissue wound healing and fibrosis. It is involved in the deposition of fibrotic products through proliferation of fibroblasts and enhanced collagen synthesis, and negatively influences the breakdown of these products (28). We observed a trend toward increased amounts of TGFβ1 in the irradiated monkeys. This suggests there is ongoing initiation of fibrosis which is consistent with documented ongoing inflammatory stimuli previously reported in this same cohort of monkeys (6).

Collagen IV is associated with the pericapillary basement membrane (BM) in tissues (27). We found a significantly greater abundance of collagen IV deposition in the muscle of diabetic
monkeys when compared to those without diabetes. This is not surprising as collagen IV deposition and BM thickening are known microvascular characteristics of patients with chronic diabetes, most clinically notable as diabetic retinopathies and nephropathies (43). Suggestive of ECM remodeling, all irradiated monkeys had a relative overabundance of collagen IV, consistent with what has been noted acutely with low doses of irradiation in mouse cardiac muscle (29) and chronically in lung tissue following larger doses of irradiation (31). Of relevance, Kang et.al have demonstrated that inflammatory effects from a high fat diet lead to greater collagen IV deposition and subsequent insulin resistance in mice (19). Additionally, a genetic deletion of MMP9, a collagen degrading enzyme, exacerbates collagen IV deposition and insulin resistance in the skeletal muscle in these mice (21).

BM thickening in skeletal muscle is also seen with advancing age and senescence (3). For example, key BM molecules (collagen IVα1, collagen IVα2, and laminin 2) were increased approximately two-fold in myofibroblasts differentiated from 32-month-old rats compared to those from 3-month-old rats (48). As age is the greatest risk factor for diabetes development, the parallel in these muscle changes are of note. Radiation is considered a model for aging as it accelerates the onset of many age-related diseases such as heart failure and cancer (38), thereby possibly further broadening the scope of relevancy of our findings to the natural aging processes and associated age-related metabolic dysfunction. Illustrative of this accelerated aging is our cohort’s average age of T2DM diagnosis being 13 years versus 19 years in other colonies (15).

High dose therapeutic radiation is prevalent and the persistent threat of malicious or accidental radiation exposure continues to exists, therefore results of this study are of significant public health interest. The monkeys in our study are long-term survivors of WBI and as such present a survivor bias which may provide particular relevance to humans who survive to develop delayed
late effects following radiation exposures. Additionally, the accelerated phenotype of metabolic
disease noted in clinical trials is evident within our cohort. It is likely our model shares relevant
pathogenesis, perhaps an exaggerated version, of radiation therapy associated increased T2DM risk.

This study is particularly novel as it utilizes a non-human primate model of delayed adverse
effects of irradiation. This model is superior as it has more human-like muscle architecture
compared to rodent models (8). The translatability of our findings is further bolstered by the
dietary environment of the monkeys which is similar to the diets consumed by people in
westernized nations. Our study is limited by the variability of individual monkey’s histories,
small sample size, lack of quantification of food intake and physical activity, and the
unavailability of a non-irradiated diabetic control group for comparison. The possibility of other
subclinical disease exists in our irradiated monkeys, such as cardiac fibrosis which has been
described in animals within this cohort (6). It is possible that fibrosis of other metabolically
active tissues, such as fat, could influence T2DM disease risk of the monkeys within this study.

**Perspectives and Significance**

Our study provides evidence that irradiation leads to persistent ECM muscle changes many years
after radiation exposure. This remodeling is associated with muscle lipid content and variable
microvascular abundance. Maintained or enhanced microvascular abundance in muscle appears
to protect against radiation-induced metabolic disease in the face of ECM changes and decreased
HSP90 levels. Future studies will include prospective monitoring and evaluation of irradiated
monkeys to determine when and how the trend for muscle metabolic defects progresses to
eventually overwhelm the ability to metabolize glucose. These studies will include functional
measurements of perfusion to build upon the noteworthy architectural differences of skeletal muscle microvasculature noted between groups within this study. Additionally, elucidation of potential therapeutic targets such as heat shock protein induction and other avenues to enhance muscle perfusion or decrease fibrosis will be pursued with an aim to alleviate the predisposition for and prevent the progression to overt T2DM following radiation exposure.

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Author Contributions

KK reviewed and edited the manuscript, led experimental design and generated/analyzed data. KF wrote the manuscript and analyzed data. AD, TP, IW, and BP generated data. IW additionally contributed in writing and editing the manuscript. DW assisted with experimental design and reviewed/edited the manuscript. JC reviewed/edited the manuscript.
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**Figure Legends**

**Figure 1.**

Panel A: Radiation exposure leads to relative overabundance of collagen type IV in skeletal muscle of rhesus macaques exposed to radiation years earlier. Mean and standard errors of the mean are shown for (NonRad-CTL; n=8 open bar), irradiated nondiabetic (Rad-CLT; n=8 lined bar), and irradiated type-2 diabetic (Rad-DM; N=8 black bar) rhesus macaques.

Panel B: The master signaling molecule, tissue growth factor beta (TGFβ), trends higher (p=0.1) in muscle with prior radiation exposure, consistent with altered collagen deposition shown in panel A. Mean and standard errors of the mean are shown for (NonRad-CTL; n=8 open bar), irradiated nondiabetic (Rad-CLT; n=8 lined bar), and irradiated type-2 diabetic (Rad-DM; N=8 black bar) rhesus macaques.

**Figure 2.**

Panel A: CD31⁺ endothelial cell counts per high-powered field (HPF) were used as biomarker of capillary density in skeletal muscle. Mean and standard errors of the mean are shown for (NonRad-CTL; n=8 open bar), irradiated nondiabetic (Rad-CLT; n=8 lined bar), and irradiated type-2 diabetic (Rad-DM; N=8 black bar) rhesus macaques. Data show that protection from type 2 diabetes (DM) post-radiation exposure was accompanied by greater capillary density in muscle. Unlike letters denote significant difference between groups (p<0.05).

Panel B: Plasma nitrate (NO₃⁻) in the T2DM monkeys was significantly lower than non-diabetic monkeys. Unlike letters denote significant difference (p<0.05) between groups. Mean and standard errors of the mean are shown for (NonRad-CTL; n=8 open bar), irradiated nondiabetic
Panels C-E: Representative images of CD31+ endothelial cell immunohistochemistry. NonRad-CTL, Rad-CTL, Rad DM respectively at X10 magnification with the scale bar = 100 um.

Figure 3.

Heat shock protein (HSP) 90 associates with endothelial nitric oxide synthase (eNOS) to improve its function.

Panel A: Radiation suppressed HSP90 levels (p=0.01 for NonRad-CTL versus Rad groups), which were most reduced in T2DM monkeys (p=0.03 for CTL groups versus DM). Unlike letters denote significant difference between groups by 1-way ANOVA. Mean and standard errors of the mean are shown for (NonRad-CTL; n=8 open bar), irradiated nondiabetic (Rad-CLT; n=8 lined bar), and irradiated type-2 diabetic (Rad-DM; N=8 black bar) rhesus macaques.

Panel B: HSP90 significantly correlated with plasma nitrate and Panel C. tumor necrosis factor related ligand 1A (TNFR1A).

Figure 4.

Greater fibrotic signaling as indicated by tissue growth factor beta (TGFβ) levels in skeletal muscle, correlated with less abundant heat shock protein 90 (HSP90; Panel A) and vascular endothelial growth factor (VEGF; Panel B). Increasing abundance of TGFβ1, and resultant collagen IV deposition, in skeletal muscle were both associated with greater muscle lipid as measured by triglyceride (mTG) content.
Table 1.

Demographic information and cardiometabolic endpoints for nonirradiated nondiabetic, irradiated nondiabetic, and irradiated type-2 diabetic rhesus macaques.

<table>
<thead>
<tr>
<th></th>
<th>NonRad-CTL</th>
<th>Rad-CTL</th>
<th>Rad-DM</th>
<th>Overall p-value</th>
<th>Radiation Effect p-value</th>
<th>Diabetes Effect p-value</th>
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</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>12.6 (0.4)</td>
<td>13.1 (0.9)</td>
<td>13.9 (1.0)</td>
<td>0.52</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>Age at exposure (years)</td>
<td>N/A</td>
<td>4.24 (0.67)</td>
<td>5.04 (0.76)</td>
<td>0.44</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Radiation dose (Gy)</td>
<td>N/A</td>
<td>7.14 (0.21)</td>
<td>6.98 (0.20)</td>
<td>0.57</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Years since irradiation (years)</td>
<td>N/A</td>
<td>8.9 (0.2)</td>
<td>9.0 (0.3)</td>
<td>0.76</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Body Weight (kg)</td>
<td>14.1 (1.3)</td>
<td>8.9 (0.6)</td>
<td>14.2 (0.9)</td>
<td>&lt;0.001</td>
<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>45.3 (3.1)</td>
<td>35.1 (2.5)</td>
<td>46.7 (3.6)</td>
<td>0.03</td>
<td>0.31</td>
<td>0.13</td>
</tr>
<tr>
<td>Blood Glucose (mg/dl)</td>
<td>62 (6)</td>
<td>66 (4)</td>
<td>237 (31)</td>
<td>&lt;0.001</td>
<td>0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin (µIU/ml)</td>
<td>35.3 (9.6)</td>
<td>14.8 (5.0)</td>
<td>62.7 (31.3)</td>
<td>0.02</td>
<td>0.37</td>
<td>0.09</td>
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<tr>
<td>Hemoglobin A1c (%)</td>
<td>4.5 (0.8)</td>
<td>4.6 (0.7)</td>
<td>9.5 (0.7)</td>
<td>&lt;0.001</td>
<td>0.11</td>
<td>0.0002</td>
</tr>
<tr>
<td>Blood Triglycerides (mg/dl)</td>
<td>45.6 (3.7)</td>
<td>37.0 (2.9)</td>
<td>154.1 (26.5)</td>
<td>&lt;0.001</td>
<td>0.35</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>High density lipoprotein cholesterol (mg/dl)</td>
<td>72.9 (4.4)</td>
<td>78.8 (4.3)</td>
<td>57.1 (5.8)</td>
<td>0.02</td>
<td>0.55</td>
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</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>139.9 (7.5)</td>
<td>153.1 (13.4)</td>
<td>141.5 (10.1)</td>
<td>0.69</td>
<td>0.58</td>
<td>0.72</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>73.8 (3.4)</td>
<td>72.7 (5.4)</td>
<td>70.0 (3.7)</td>
<td>0.80</td>
<td>0.61</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM.

Unlike superscripted letters indicate significant differences between groups (p<0.05).
Table 2.

Radiation and diabetic effects on mean abundance of collagens, triglyceride, and matrix metalloproteinase activity in skeletal muscle of nonirradiated nondiabetic, irradiated nondiabetic, and irradiated type-2 diabetic rhesus macaques.

<table>
<thead>
<tr>
<th></th>
<th>NonRad-CTL</th>
<th>Rad-CTL</th>
<th>Rad-DM</th>
<th>Overall p-value</th>
<th>Radiation Effect p-value</th>
<th>Diabetes Effect p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COL 1 IHC</strong> (% stained)</td>
<td>5.79 (0.72)</td>
<td>4.50 (0.34)</td>
<td>4.48 (0.83)</td>
<td>0.27</td>
<td>0.09</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>COL 3 IHC</strong> (% stained)</td>
<td>3.47 (0.58)</td>
<td>4.44 (0.59)</td>
<td>3.44 (0.61)</td>
<td>0.38</td>
<td>0.50</td>
<td>0.46</td>
</tr>
<tr>
<td><strong>COL 4 IHC</strong> (% stained)</td>
<td>8.78 (0.67)</td>
<td>8.29 (0.43)</td>
<td>9.86 (0.64)</td>
<td>0.19</td>
<td>0.75</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>MMP9 Activity</strong> (AU)</td>
<td>0.90 (0.34)</td>
<td>1.35 (0.37)</td>
<td>0.74 (0.26)</td>
<td>0.39</td>
<td>0.80</td>
<td>0.32</td>
</tr>
<tr>
<td><strong>Muscle TG</strong> (ug/mg protein)</td>
<td>64.55 (12.7) (^a)</td>
<td>32.12 (3.54) (^a)</td>
<td>90.18 (25.19) (^b)</td>
<td>0.04</td>
<td>0.87</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM. COL, collagen.

Unlike superscripted letters indicate significant difference between groups (p<0.05).
Table 3.

The effect of radiation and diabetes on mean perfusion-related parameters in skeletal muscle.

<table>
<thead>
<tr>
<th></th>
<th>NonRad-CTL</th>
<th>Rad-CTL</th>
<th>Rad-DM</th>
<th>Overall p-value</th>
<th>Radiation effect p-value</th>
<th>Diabetes effect p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (pg/ug muscle protein)</td>
<td>11.45 (2.11)</td>
<td>10.05 (1.18)</td>
<td>11.49 (1.52)</td>
<td>0.78</td>
<td>0.74</td>
<td>0.71</td>
</tr>
<tr>
<td>TNFRI A (pg/ug muscle protein)</td>
<td>7.39 (0.98)</td>
<td>13.2 (3.68)</td>
<td>7.85 (2.91)</td>
<td>0.13</td>
<td>0.34</td>
<td>0.53</td>
</tr>
<tr>
<td>ln eNOS (AU)</td>
<td>1.012 (0.28)</td>
<td>1.069 (0.30)</td>
<td>1.634 (0.69)</td>
<td>0.90</td>
<td>0.93</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. Ln, natural log.
Figure 3

A

B

C

HSP90 (pg/μg muscle)

Non-Rad CTL Rad CTL Rad DM

HSP90 (pg/μg muscle protein)

Log NO₃ (μM)

r = 0.44, p = 0.04

TNFRA (pg/μg muscle)

Log NO₃ (μM)

r = 0.44, p = 0.05
Figure 4

A. HSP90 (pg/ug muscle protein) vs. TGFβ (pg/ug muscle protein). The correlation coefficient is $r = -0.51$, $p = 0.01$.

B. VEGF (pg/ug muscle protein) vs. TGFβ (pg/ug muscle protein). The correlation coefficient is $r = -0.42$, $p = 0.04$.

C. TG (ug/mg muscle protein) vs. TGFβ (pg/ug muscle protein). The correlation coefficient is $r = 0.48$, $p = 0.02$.

D. TG (ug/mg muscle protein) vs. Collagen 4 (% area stained). The correlation coefficient is $r = 0.49$, $p = 0.02$. 