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

Fanning KM, Pfisterer B, Davis AT, Presley TD, Williams IM, Wasserman DH, Cline JM, Kavanagh K. Changes in microvascular density differentiate metabolic health outcomes in monkeys with prior radiation exposure and subsequent skeletal muscle ECM remodeling. J Physiol Regul Integr Comp Physiol 313: R290–R297, 2017. First published July 12, 2017; doi:10.1152/ajpregu.00108.2017.—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 and overnutrition and following irradiation, are risk factors for development of type 2 diabetes mellitus. We previously demonstrated an increased incidence of skeletal muscle insulin resistance and type 2 diabetes mellitus in monkeys that had been exposed to whole body irradiation 5–9 yr prior. 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 nonirradiated age-matched controls (Non-Rad-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 toward increased transforming growth factor-β1. Preservation of microvascular markers differentiated the irradiated diabetic and non-diabetic groups. Microvascular density and plasma nitrate and heat shock protein 90 levels were lower in Rad-DM than Rad-CTL. These results are consistent with a protective effect of abundant microvasculature in maintaining glycemic control within radiation-induced fibrotic muscle.

irradiation; type 2 diabetes; monkey; muscle; fibrosis; microvasculature

LONG-TERM EFFECTS OF IONIZING IRRADIATION include an increased risk of type 2 diabetes mellitus (T2DM), which is documented in patients exposed to radiation as children (16, 30) and patients treated with abdominal radiation for Hodgkin’s lymphoma (33). After nuclear disasters such as Chernobyl and Hiroshima, increased rates of diabetes mellitus are reported, but the distribution of type 1 and type 2 diabetes is 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 postirradiation, physical function has generally been the clinically studied outcome (41). Because of 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. 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-β1 (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). Furthermore, mechanistic studies in rodent models demonstrate that skeletal muscle ECM expansion contributes 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 are 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); 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 that after radiation exposure, are 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 in muscle following irradiation and the ultimate progression of T2DM are not fully understood. Our previous work demonstrated that radiation exposure was associated with muscle tissue insulin resistance, even in objectively healthy, nondiabetic 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 exposure.

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 people exposed to deterministic doses of radiation (www.radcore.org). As previously reported, these animals originated from different institutions and are survivors of exposure to a single sublethal 6.5- to 8.4-Gy dose of gamma WBI before their arrival at Wake Forest University (25). The radiation exposure occurred 5–9 yr before this study (Table 1). Animals (n = 7–8/group) that were nonirradiated controls (Non-Rad-CTL), irradiated nondiabetic monkeys (Rad-CTL), and irradiated monkeys that subsequently developed diabetes (Rad-DM) were further characterized for skeletal muscle architecture changes (see below). All animals were housed equivalently and had limited opportunities to exercise. All monkeys have been maintained since 2010 on a diet similar in nutritional profile to that 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, LabDiet) immediately following diagnosis. Food was available ad libitum, and consumption was not quantitated.

Monkeys were diagnosed with diabetes according to the current American Diabetes criteria (1a) ranging from 1–4 yr before the start of this investigation All combination insulin administration was withdrawn from animals with diabetes for ≥24 h and regular insulin was withdrawn for ≥12 h before assessment. The monkeys were fasted for ≥12 h before assessment. Animals were anesthetized with intramuscular ketamine (10–15 mg/kg) to allow for sample and data collection. Each animal was weighed. Blood samples were obtained by percutaneous venipuncture of the femoral vein and collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes. The blood was held on ice until it could be processed. After processing was completed, samples were stored at ~8°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) of the plasma sample. Whole blood was used to determine glycate hemoglobin (HbA1c) using high-performance liquid chromatography methodology (Primus PDQ, Primus Diagnostics, Kansas City, MO). Triglyceride (TG), high-density lipoprotein cholesterol, and total plasma cholesterol concentrations were measured enzymatically. Muscle biopsies were collected from the biceps femoris, fixed in 4% paraformaldehyde for 24 h, and 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 US Department of Agriculture 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. Collagen I, III, and IV were assessed in formalin-fixed paraffin-embedded sections of muscle. Slides were stained using the Bond-MAX immunohistochemistry stainer (Leica). For heat-induced antigen retrieval, slides were incubated for 20 min in Epitope Retrieval Solution 2 (Leica). Slides were then incubated in anti-collagen I (1:100 dilution; ab138492, Abcam), anti-collagen III (1:100 dilution; ab6310, Abcam), or anti-collagen IV (1:600 dilution; ab6586F, Abcam) antibody for 1 h. The Bond Polymer Refine Detection system was used to produce localized, visible 3,3′-diaminobenzidine (DAB) staining. Slides were finally dehydrated and cleared, and coverslips were mounted. Images were captured at ×200 total magnification using a camera (model DS-Ri2, Nikon) mounted on an upright, wide-field microscope (model AZ100M, Nikon). Image analysis was performed using a custom, automated macro in ImageJ. Briefly, DAB staining was isolated using color deconvolution, and then a variation of the isodata algorithm was used for automatic thresholding. Finally, the percent area of the section occupied by DAB staining was measured. An investigator blinded to group identity performed all image acquisition and analyses.

Table 1. Demographic information and cardiometabolic end points for Non-Rad-CTL, Rad-CTL, and Rad-DM rhesus macaques

<table>
<thead>
<tr>
<th>Value</th>
<th>Non-Rad-CTL</th>
<th>Rad-CTL</th>
<th>Rad-DM</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>12.6 ± 0.4</td>
<td>13.1 ± 0.9</td>
<td>13.9 ± 1.0</td>
<td>0.52</td>
</tr>
<tr>
<td>Age at exposure, yr</td>
<td>N/A</td>
<td>4.24 ± 0.67</td>
<td>5.04 ± 0.76</td>
<td>0.44</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>
</tr>
<tr>
<td>Time since irradiation, yr</td>
<td>N/A</td>
<td>8.9 ± 0.2</td>
<td>9.0 ± 0.3</td>
<td>0.76</td>
</tr>
<tr>
<td>Body wt, kg</td>
<td>14.1 ± 1.3a</td>
<td>8.9 ± 0.6b</td>
<td>14.2 ± 0.9a</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>45.3 ± 3.1ab</td>
<td>35.1 ± 2.5a</td>
<td>46.7 ± 3.6a</td>
<td>0.03</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>62 ± 6a</td>
<td>66 ± 4a</td>
<td>237 ± 31b</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin, μIU/ml</td>
<td>35 ± 0.6ab</td>
<td>14.8 ± 5.0b</td>
<td>62.7 ± 31.3a</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hemoglobin A1c, %</td>
<td>4.5 ± 0.8a</td>
<td>4.6 ± 0.7a</td>
<td>9.5 ± 7.0a</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blood TG, mg/dl</td>
<td>45.6 ± 3.7b</td>
<td>37.0 ± 2.9b</td>
<td>154.1 ± 26.5a</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>72.9 ± 4.4ab</td>
<td>78.8 ± 4.3a</td>
<td>57.1 ± 5.8b</td>
<td>0.02</td>
</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>
</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>
</tr>
</tbody>
</table>

Values are means ± SE. Non-Rad-CTL, nonirradiated nondiabetic; Rad-CTL, irradiated nondiabetic; Rad-DM, irradiated type 2 diabetic; TG, triglycerides. Different superscripted letters (a, b) indicate significant difference between groups (P < 0.05).
TGFB1 in monkey muscle was measured by ELISA. Quantities in muscle lysates were determined using the human TGFB1 ELISA (Quantikine kit, R & D Systems) 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 TGFB1 provided by the manufacturer. Quantities were estimated based on a standard curve generated with recombinant TGFB1 provided by the manufacturer. Quantities were estimated based on a standard curve generated with recombinant TGFB1 provided by the manufacturer. Quantities were estimated based on a standard curve generated with recombinant TGFB1.

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 100 mM Tris-HCl, 10 mM EDTA, and 0.5% Triton X-100. Homogenates were centrifuged at 13,000 rpm for 20 min at 4°C. Supernatants (500 µg of protein) were incubated with gelatin-Sepharose beads for 2 h at 4°C to purify and concentrate gelatinases. After three washes, gelatin-Sepharose beads were resuspended in nonreducing Laemmli buffer and loaded onto 10% zymogram gels for electrophoresis. Conditioned medium from HT-1080 cells was used as a positive control. After 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 TGs. Muscle TG 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 PECA-1 (M-20) goat anti-mouse polyclonal antibody (Santa Cruz Biotechnology). Blinded assessors counted in duplicate the total number of cells that stained positively for **CD31** in 20 fields at ×20 magnification. Representative images are shown in Fig. 2, C–E.

Plasma nitrate. Nitrate (NO3) levels were measured in the plasma samples using an NOx analyzer (model eNO-20, Eicom) according to 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 (NO) synthase [endothelial NO synthase (eNOS)] mRNA in muscle tissue was measured by quantitative real-time polymerase chain reaction (qRT-PCR). Primer sequences for eNOS were as follows: 5′-gaagctggcatctggaa-3′ (forward) and 5′-cagacgcacctccc-3′ (reverse). Data are expressed in arbitrary units normalized by the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The primer sequences used for GAPDH were as follows: 5′-caccagtctgacaccc-3′ (forward) and 5′-tgctgctacaacctc-3′ (reverse).

Heat shock protein. Heat shock protein (HSP) 90 (HSP90) in muscle homogenate was quantified in duplicate using a sandwich ELISA with the HSP90 Alpha ELISA kit (StressMarq Biosciences, Victoria, BC, Canada) according to 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. Nonnormal parameters were log-transformed if required to achieve statistical assumptions of normality. Values are means ± SE. Group differences were analyzed using one-way ANOVA with α-level set at 0.05 for statistical significance and P = 0.10 for trends. We additionally examined for the main effects of radiation and diabetes by ANOVA. Outcome measures were covaried 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, Carlsbad, CA).

RESULTS

Demographic and metabolic characteristics of Non-Rad-CTL, Rad-CTL, and Rad-DM monkeys are shown in Table 1. There were no significant differences in age between groups. Among 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 yr, which is the human equivalent of ~20 yr, a significant period of life that 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 the outlier criterion.

Rad-DM monkeys were neither overweight nor obese, and their body weight was comparable to that of Non-Rad-CTL animals. Rad-CTL monkeys were significantly leaner (~40%) than the other groups. Waist circumference showed a pattern similar to body weight (Table 1), as did body fat percentage, which was previously documented 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. As expected, HbA1C and plasma TG levels were significantly higher in diabetic than nondiabetic monkeys. Circulating insulin levels were significantly lower in Rad-CTL than Rad-DM monkeys 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 test results were significantly worse in diabetic than nondiabetic animals. 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 IV deposition, as reflected by a significantly greater ratio of collagen IV to collagen I (Fig. 1a; P = 0.04) and a trend toward an increase in TGFB1 (Fig. 1B; P = 0.10). There were no differences between groups in ECM breakdown as measured by MMP9 activity (Table 2). Muscle TG content and absolute collagen IV deposition were greater in skeletal muscle of Rad-DM than nondiabetic monkeys (Table 2).

CD31+ cells were more abundant (Fig. 2A; P = 0.02) and plasma NO3 levels were higher (Fig. 2B; P = 0.04) in Rad-CTL than Rad-DM animals. Abundance of CD31+ cells was 150% greater and NO3 levels were 160% higher in Rad-CTL than Non-Rad-CTL animals. The NO3 results were repeated in an additional cohort of monkeys consistently demonstrating higher values in Rad-CTL than Non-Rad-CTL animals (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). HSP90 levels were significantly lower in muscle from irradiated monkeys (Fig. 3A). Both HSP90 and TNFR1A positively correlated with plasma NO3 \( [r = 0.44, P = 0.04 \text{ (Fig. 3B)}] \) and \( r = 0.44, P = 0.05 \text{ (Fig. 3C)} \).

The relationship between groups with respect to MMP9 activity and TNFR1A is similar to the group pattern for CD31+ endothelial cells and NO3. In Rad-CTL monkeys, mean MMP9 activity was 1.8-fold greater and mean TNFR1A level was 1.7-fold greater, which is similar to the 2- to 4-fold greater abundance of CD31+ cells and NO3 level, than in Rad-DM.

Evidence of an interaction between ECM and microvasculature-related indexes is demonstrated as TGFβ1 correlated negatively with both proangiogenic VEGF and the eNOS chaperone HSP90 \( [r = -0.42, P = 0.04 \text{ (Fig. 4A)}] \) and \( r = -0.51, P = 0.01 \text{ (Fig. 4B)} \). Additionally, greater muscle lipid content was associated with increased fibrilogenic signaling and increased collagen IV deposition \( [r = 0.48, P = 0.018 \text{ (Fig. 4C)}] \) and \( r = 0.49, P = 0.019 \text{ (Fig. 4D)} \).

**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 that very closely matches the pathogenesis of human metabolic disease (44). The preservation of microvascular abundance in nondiabetic monkeys exposed to radiation is consistent with previous studies showing a link between capillary density and metabolic disease (1, 11, 35). We also demonstrate that radiation is associated with changes in 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 the endothelial cell marker CD31 showed that microvascular abundance differentiated diabetic and nondiabetic irradiated animals, as Rad-DM had significantly fewer 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 that the response is dependent on dose. In mouse cardiac muscle, capillary density decreased following exposure to radiation at doses comparable to the level used in our study (8 Gy) (39). Conversely, Mathias et al. found an increase in CD31+ cells in mouse cardiac muscle following exposure to a low (2-Gy) dose of radiation (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 in this study. Capillary endothelial cells contain an enzyme, NO synthase (NOS), that 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, an NO metabolite, in Rad-DM monkeys. This may suggest decreased function of eNOS in Rad-DM animals, which is in agreement with the

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**Table 2. Effects of radiation and diabetes on mean abundance of collagen, TG levels, and MMP activity in skeletal muscle of Non-Rad-CTL, Rad-CTL, and Rad-DM rhesus macaques**

<table>
<thead>
<tr>
<th>Group</th>
<th>Non-Rad-CTL</th>
<th>Rad-CTL</th>
<th>Rad-DM</th>
<th>Overall</th>
<th>Radiation effect</th>
<th>Diabetes effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen IHC, %stained</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</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>Type III</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>Type IV</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>MMP9 activity, 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>Muscle TG, μg/mg protein</td>
<td>64.55 ± 12.7a</td>
<td>32.12 ± 3.54a</td>
<td>90.18 ± 25.19b</td>
<td>0.04</td>
<td>0.87</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE. MMP9, matrix metalloproteinase 9; AU, arbitrary units. Different superscripted letters (a and b) indicate significant difference between groups \( (P < 0.05) \).
finding of Kashyap et al. that NOS activity increased 2.5-fold in controls in response to insulin; however, insulin failed to stimulate NOS activity in humans with diabetes (23).

HSP90 influences perfusion by associating with eNOS and augmenting the production of NO (9). HSP90 levels were significantly lower in muscle tissue of irradiated than nonirradiated monkeys and also significantly lower in Rad-DM monkeys than both groups without diabetes. In rats that developed fibrosing alveolitis following irradiation, HSP90 levels decreased >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 proinflammatory states such as obesity and aging (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 lack of direct measurement and comparison of microvascular responsiveness between groups.

**Extracellular Matrix**

TGFβ1 is widely recognized as a major regulating cytokine involved in 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, which suggests an ongoing initiation of fibrosis, 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 significantly greater collagen IV deposition in the muscle of diabetic than nondiabetic monkeys. 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 previous findings acutely with low doses of radiation in mouse cardiac muscle (29) and chronically in lung tissue following larger doses of radiation (31). Kang et al. 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 twofold in myofibroblasts differentiated from 32-mo-old rats compared with those from 3-mo-old rats (48). As age is the greatest risk factor for diabetes development, the parallels 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).

**Table 3. Effect of radiation and diabetes on mean perfusion-related parameters in skeletal muscle**

<table>
<thead>
<tr>
<th>Group</th>
<th>Non-Rad-CTL</th>
<th>Rad-CTL</th>
<th>Rad-DM</th>
<th>P Value Overall</th>
<th>Radiation effect</th>
<th>Diabetes effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF, pg/μg 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>TNFRI1A, pg/μg 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>

Values are means ± SE. VEGF, vascular endothelial growth factor; TNFRI1A, tumor necrosis factor-related ligand 1A; eNOS, endothelial nitric oxide synthase; ln, natural log.
thereby possibly further broadening the scope of relevance of our findings to the natural aging processes and associated age-related metabolic dysfunction. Illustrative of this accelerated aging is the average age of T2DM diagnosis of 13 yr in our cohort vs. 19 yr in other colonies (15).

High-dose therapeutic radiation is prevalent, and the persistent threat of malicious or accidental radiation exposure continues to exist; 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 that 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 that our model shares relevant pathogenesis, perhaps an exaggerated version, of radiation therapy-associated increased T2DM risk.

This study is particularly novel, in that it utilizes a nonhuman primate model of delayed adverse effects of radiation. This model is superior, in that it has more humanlike muscle architecture than 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 unavailability of a nonirradiated diabetic control group for comparison. The possibility of other subclinical disease, such as cardiac fibrosis, which has been described in animals within this cohort (6), exists in our irradiated monkeys. 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.

Fig. 3. Heat shock protein (HSP) 90 associates with endothelial nitric oxide synthase (eNOS) to improve its function. A: HSP90 levels were suppressed by radiation exposure \((P = 0.01\) for Non-Rad-CTL vs. Rad-CTL and RAD-DM), which were most reduced in T2DM monkeys \((P = 0.03\) for Non-Rad-CTL and Rad-CTL vs. Rad-DM). Values are means ± SE; \(n = 8\) in each group. Different superscript letters (a and b) denote significant difference between groups \((P < 0.05)\). B and C: HSP90 significantly correlated with plasma nitrate (NO3) and tumor necrosis factor-related ligand 1A (TNFR1A).

Fig. 4. A and B: greater fibrotic signaling, as indicated by TGFβ levels in skeletal muscle, correlated with less-abundant HSP90 and vascular endothelial growth factor (VEGF). C and D: increasing abundance of TGFβ1 and resultant collagen IV deposition in skeletal muscle were associated with greater muscle lipid, as measured by muscle triglyceride (TG) content.
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 on the noteworthy architectural differences in skeletal muscle microvasculature between groups within this study. Additionally, elucidation of potential therapeutic targets, such as HSP 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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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

D.H.W. and K.K. conceived and designed research; K.M.F. and K.K. analyzed data; K.M.F. and I.M.W. prepared figures; K.M.F. and I.M.W. wrote the first draft of the manuscript; D.H.W. edited and revised manuscript; K.M.F. and K.K. approved final version of manuscript; B.F., A.T.D., T.D.P., I.M.W., and K.K. performed experiments; K.K. interpreted results of experiments.

REFERENCES


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