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Raman spectroscopy demonstrates prolonged alteration of bone chemical composition following extremity localized irradiation

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ABSTRACT

Introduction: Radiotherapy to the appendicular skeleton can cause an increased risk of developing catastrophic fractures with delayed bone healing or non-union, and may subsequently require multiple procedures and amputation. Biomechanical studies suggest that irradiated bone is more brittle, but the cause is unclear and cannot be explained by changes to bone structure or quantity, suggesting that there are crucial changes in irradiated bone material properties. Raman spectroscopy provides a means to assess the chemical properties of the mineral and matrix constituents of bone, which could help explain post-radiation embrittlement. In this study we use a murine tibial model with focal irradiation and perform Raman spectroscopy to test the hypothesis that changes in bone chemistry following irradiation is consistent with reduced bone quality and persists in the long term after irradiation.

Methods: Female BALB/F mice aged 12 weeks were subjected to unilateral, localized hindlimb irradiation in 4 daily 5 Gy fractions (4 × 5 Gy) totaling 20 Gy, and were euthanized at 1, 4, 8, 12, and 26 weeks post-irradiation (n = 6/group). The irradiated (right) and non-irradiated contralateral control (left) tibiae were explanted and assessed by non-polarized and polarized Raman spectroscopy over the proximal cortical bone surface. Raman parameters used included the mineral/matrix ratio, mineral crystallinity, carbonate/phosphate ratio, collagen cross-link ratio, and depolarization ratio.

Results: Significantly increased collagen cross-link ratio and decreased depolarization ratio of matrix were evident at 1 week after irradiation and this persisted through 26 weeks. A similar significant decrease was observed for depolarization ratio of mineral at all time points except 8 and 26 weeks. At 4 weeks after irradiation there was a significantly increased mineral/matrix ratio, increased mineral crystallinity, and decreased carbonate/phosphate ratio compared to controls. However, at 12 weeks after irradiation these parameters had moved in the opposite direction, resulting in a significantly decreased mineral/matrix ratio, decreased crystallinity and increased carbonate/phosphate ratio compared to controls. At 26 weeks, mineral/matrix, crystallinity and carbonate/phosphate ratios had returned to normal.

Discussion: In this mouse model, Raman spectroscopy reports both bone mineral and collagen cross-link radiation-induced abnormalities that are evident as early as one week after irradiation and persists for 26 weeks. The picture is one of extensive damage, after which there is an attempt at remodeling. We hypothesize that pathological cross-links formed by radiation damage to collagen are poorly resorbed during the altered remodeling process, so that new tissue is formed on a defective scaffold, resulting in increased bone brittleness.

Introduction

Despite advances in radiation therapy techniques, post-radiation fragility fractures remain "a significant public health issue that deserves attention." [1–3]. Post-radiation fractures have been reported with frequency in numerous types of cancers. Fractures of the pelvis predominate following radiotherapy for anorectal and gynecologic malignancies, rib fractures after radiotherapy for breast cancer, and long bone fractures after adjunctive radiation for extremity sarcomas [3–15]. Recent reports show fracture incidence as high as 22% in patients with breast cancer and 24% in some series of patients with soft-tissue sarcoma [1,16,17].

In patients with carcinomas suffering post-radiation fractures, healing times are commonly 6 months or longer. In sarcoma patients with post-radiation fractures, frequently the long bones of the lower limb are involved, and healing times in excess of a year along with non-union rates of 45 to 67% have been reported [9,18]. In the latter group, operative treatment is a necessity, and in many cases major bone resections are required to salvage the limb [5]. Although risk factors have been identified and nomograms developed, these fractures

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remain difficult to predict [19,20]. Clinical studies have also shown that irradiation does not routinely decrease bone density, eliminating DXA as a viable assessment tool [21]. Further, preventative measures other than radiotherapy dose modification remain elusive, and there is no consensus on treatment for high risk patients or for patients after fracture [19,20]. Internal fixation is frequently considered for prophylaxis of femur fractures, but this is often criticized for its potential to cause further disruption of the blood supply within the irradiated field [20].

The limiting factor preventing progress in the prevention and treatment of post-radiation fragility fractures is the poor understanding of the pathophysiology. Some description of the cellular and vascular response to radiotherapy has emerged, but much of that has been in total body irradiation models, which are less applicable to the medical experience [22,23]. That work, combined with the limited work that has been done utilizing a clinically relevant focal irradiation field, suggests that an early increase in osteoclasts may explain the decreased bone trabecular scaffold seen in retrieval specimens [22–24]. However, loss of trabecular bone and resultant changes in the structural properties of the remaining bone fail to explain the biomechanical weakening of the bone [25]. The aforementioned clinical study showing that irradiation does not routinely decrease bone density further suggests an intrinsic material abnormality in the irradiated bone [21,25].

Raman spectroscopy offers the clinician and the basic scientist a powerful new tool for measuring bone compositional information in mineral/matrix ratio, mineral crystallinity, carbonate content, collagen cross-linking ratio and depolarization ratios of mineral and collagen fibril (orientation markers) [26–29], which are unavailable using established histological and imaging techniques. It provides chemical composition and chemical structural information that is indicative of the health of the patient and of the progress of therapeutic interventions. Neither ionizing radiation nor exogenous labels are required for Raman spectroscopy. Raman spectroscopy is especially useful for bone disorders, because it provides information about both bone mineral and matrix composition, which in turn are directly related to the quality and mechanical competence of the bone tissue. Laboratory and clinical investigations underway in the Morris lab [30,31] at the University of Michigan are investigating the use of non-invasive spectroscopy as an assessment tool for osteoradionecrosis of the jaw, and this same technology could potentially be applied to the radiotherapy of skeletal extremities.

Hence, the primary hypothesis of this manuscript is that there are intrinsic biochemical changes, including both chemical composition and degree of molecular orientation, within the remaining irradiated bone that may contribute to the increased fragility. We further hypothesize that these changes are time course-dependent and are detectable by Raman spectroscopy in a localized extremity mouse irradiation model.

Materials and methods

Sample preparation for Raman spectroscopy

Female BALB/F mice aged 12 weeks (Jackson Labs, Bar Harbor, ME) were anesthetized (ketamine/xylazine, 75/5 mg/kg IP) for unilateral localized hind limb irradiation. Mice were placed on a polycarbonate platform with their right hind limbs secured in an extended position and lead shielding was placed over the body and non-irradiated (control) hind limb. Mice were exposed to a total of 20 Gy delivered as four consecutive daily 5-Gy doses using an orthovoltage source (Philips RT-250, Andover, MA) at a dose rate of 2.3 Gy/min (300 kV, 10 mA). Sham animals were anesthetized but not irradiated. All methods were approved by the Upstate Medical University Committee for the Humane Use of Animals. At 1, 4, 8, 12, and 26 weeks post-irradiation, mice were euthanized with pentobarbital ($n = 6$ per group at each time point). Six tibias for each group were collected, stripped of soft tissues, and wrapped in saline-soaked gauze for storage at −20 °C for Raman analysis. Specimens were prepared for Raman spectroscopy at SUNY Upstate Medical University and then shipped on dry ice to the Morris Laboratory at the University of Michigan.

Raman spectroscopy

Raman measurements were performed using a locally constructed Raman microprobe. The system consists of a Nikon E600 microscope (Melville, New York) fitted with a 20×/0.75 NA objective (S Fluor, Nikon Instruments, Inc., Melville, New York) operated in epi-illumination and collection. A linearly polarized 785 nm excitation diode laser (Innovative, Kaiser Optical System, Inc., Ann Arbor, Michigan) was modified for line focusing. With the 20× objective, the spatial dimensions were 130 × 2.5 μm at the bone surface. An axial transmissive imaging spectrograph (Holospec f/1.8, Kaiser Optical Systems, Inc., Ann Arbor, Michigan) with 50 μm entrance slit provided a spectral resolution ~8 cm^{-1}. Raman scatter was collected on a 1024 × 256 pixel deep depletion charge-coupled device (CCD) detector (DU 401-BR-DD, Andor Technology, South Windsor, Connecticut). For most measurements the plane of polarization was parallel to the long axis of the specimen diaphysis. For polarized Raman measurements, a half-wave plate (WPMH05M-780, Thorlabs, Inc., Newton, NJ) positioned in front of the laser was used to select the polarization direction of the laser beam to be parallel or perpendicular to the long axis of the diaphysis. A polarization analyzer (LPNIR050, Thorlabs, Inc.) was placed after the high pass dichroic filter (Semrock Inc., Rochester, NY) to select the parallel or perpendicular polarization components. The specimen orientation and analyzer position were kept stationary during measurement, but the half-wave plate was rotated. A wedge depolarizer was placed after the polarization analyzer to minimize intensity artifacts from dependence of the grating transmission efficiency on polarization.

Using 45 mW laser power on the specimen and 120 s exposure time, fifteen Raman spectra were acquired at different locations on the irradiated region of each proximal tibia. Polarized Raman spectra were obtained by positioning the half-wave plate to collect the parallel or perpendicular component of the Raman scattered light at each location. During the Raman measurement, specimens were kept hydrated with phosphate buffered saline (PBS) solution.

Data was preprocessed with MATLAB (The Mathworks, Inc., Natick, MA) using locally written scripts. The standard procedure included removal of CCD noise spikes, dark current subtraction, correction for slit image curvature, and wavenumber calibration. Then the average spectrum for each collection was calculated, baselined, and normalized to the phosphate ν_1 band at 958 cm^{-1}. The final spectrum was subsequently imported into GRAMS/AI (Thermo Galactic, Madison, WI). Band fitting was performed for mineral and matrix bands using mixed Gaussian and Lorentzian polynomials and integrated area of selected bands was measured for band intensity. Raman parameters shown in Table 1 were calculated using the same software. Briefly, the mineral to matrix ratio was determined by intensity of phosphate ν_1 band (~958 cm^{-1}) divided by the combined intensities of proline and hydroxyproline bands (854 + 871 cm^{-1}) while the carbonate to phosphate ratio was measured by the intensity ratio of the carbonate band (~1070 cm^{-1}) to the phosphate ν_1 band (~958 cm^{-1}). Mineral

Table 1
Raman spectroscopic parameters used in this study.

Raman parameters	Quantification of the parameters
Mineral/matrix ratio	[a]958/(854 + 871) cm^{-1}
Carbonate/phosphate ratio	1070/958 cm^{-1}
Mineral crystallinity	Inversely proportional to full width at half maximum of phosphate 958 cm^{-1} band
Collagen cross-linking ratio	1660/1683 cm^{-1}
Depolarization ratio of mineral[b] (ρ_{958})	$I_{958(\perp)}/I_{958(\parallel)}$
Depolarization ratio of collagen (ρ_{1660})	$I_{1660(\perp)}/I_{1665(\parallel)}$

[a] Band assignments see Fig. 1.
[b] Depolarization ratio was defined in the section of Materials and methods.