Published in final edited form as:

Bone. 2013 November ; 57(1): 252–258. doi:10.1016/j.bone.2013.08.014.

Raman Spectroscopy Demonstrates Prolonged Alteration of Bone Chemical Composition Following Extremity Localized Irradiation

Bo Gong^a, Megan E. Oest^b, Kenneth A. Mann^b, Timothy A. Damron^b, and Michael D. Morris^{a,*}

^a Department of Chemistry; University of Michigan, Ann Arbor, MI 48109, USA

^b Department of Orthopedic Surgery, Upstate Medical University, Syracuse, NY 13210, USA

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 (4×5Gy) 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

© 2013 Elsevier Inc. All rights reserved.

* Corresponding author at: Department of Chemistry, University of Michigan, 930, N. University Avenue, Room 4811, Ann Arbor, MI 48109-1055, USA. Fax: +1 734 764 7360. mdmorris@umich.edu (M.D.Morris).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosures:

Michael D. Morris receives consulting income from Kaiser Optical Systems, Inc.; the rest of the authors have no conflicts of interest.

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 persist 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.

Keywords

Raman spectroscopy; Radiation; Tibia; Bone composition

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 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, 2, 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 20X/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 20X objective, the spatial dimensions were $130 \times 2.5 \mu\text{m}$ 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 $\sim 8 \text{ cm}^{-1}$. Raman scatter was collected on a 1024 $\times$ 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 were 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 ($\sim 958\text{ cm}^{-1}$) divided by the combined intensities of proline and hydroxyproline bands ($854+871\text{ cm}^{-1}$) while the carbonate to phosphate ratio was measured by the intensity ratio of the carbonate band ($\sim 1070\text{ cm}^{-1}$) to the phosphate ν_1 band ($\sim 958\text{ cm}^{-1}$). Mineral crystallinity is inversely proportional to full width at half maximum of the phosphate ν_1 band at $\sim 958\text{ cm}^{-1}$. The collagen cross-linking ratio was calculated by the intensity ratio of two sub-component bands ($\sim 1660/\sim 1683\text{ cm}^{-1}$) in the amide I region.

In polarized Raman experiments, the mouse tibia was placed on the microscope stage with the laser line focused parallel to the long axis of the diaphysis. Conventionally, the depolarization ratio (ρ) is used to obtain molecular structural information (e.g. orientation) since it strongly depends on molecular alignment in solid material. The depolarization ratio is defined by $\rho = I_{\perp} / I_{\parallel}$, where $I_{\perp}$ is the integrated band area of the perpendicular polarization component for the selected Raman band, and $I_{\parallel}$ is the integrated band area of the parallel polarization component for the same band. A lower ρ value reflects a higher degree of molecular alignment or orientation. In this study, band intensities of the phosphate ν_1 at $\sim 958\text{ cm}^{-1}$ and the amide I region at $\sim 1665\text{ cm}^{-1}$ were used to calculate the depolarization ratios of mineral ($\rho_{958} = I_{958\perp} / I_{958\parallel}$) and collagen ($\rho_{1660} = I_{1660\perp} / I_{1660\parallel}$) since both bands are strongly polarized along the long axis of the tibia diaphysis as described previously [27-29].

Data are presented as means $\pm$ standard deviation. Difference between irradiated specimens and control at each post-radiation time point was analyzed using one-way analysis of variance (ANOVA) with post Holm-Bonferroni test. Statistical significance was considered to be $p < 0.05$.

Results

Radiation effects on bone mineralization, mineral crystallinity, carbonate content, collagen cross-linking, mineral and collagen fibril orientation were investigated in a murine tibia model using Raman spectroscopy. The corresponding Raman parameters were calculated and listed in Table 2. Further statistical comparison of these Raman parameters of irradiated bones relative to controls at different post-radiation time points provided information on abnormal chemical composition and structure in irradiated bone during recovery (Table 3).

It should be pointed out that the sham animals' left tibiae and the irradiated animals' non-irradiated left tibiae were compared at post-radiation 1 week and 12 weeks. For all Raman parameters, no significant difference was observed between both groups (data not shown). Thus, we used non-irradiated contralateral tibiae of irradiated animal as controls in the present study.

A typical Raman spectrum of a mouse tibia along with band assignments is presented in Fig. 1. Most of the Raman bands are assigned to bone mineral and matrix collagen. The overall level of local bone mineralization can be estimated by the mineral to matrix ratio, which represents the amount of mineral normalized to the amount of organic matrix in the bone volume analyzed. In comparison to control, irradiated bone showed several significant changes in the mineral to matrix ratio (Fig. 2 (A) and Table 3). Transient hypermineralization was observed at 4 weeks, indicated by significant increase of mineral to matrix ratio ($p=0.048$). The ratio declined significantly at 12 weeks ($p<0.001$) and was not significantly different from controls at 1 ($p=0.949$), 8 ($p=0.608$) and 26 weeks ($p=0.729$).

The reasons why band intensity ratios rather than individual component band intensities are reported in bone Raman spectroscopy (and in most other applications of Raman spectroscopy) have been discussed by Wopenka et al.[32]. The absolute intensities of individual bands are affected by the Raman scattering cross-section (i.e. probability of a photon causing a Raman scattering event), the volume of material excited by the laser, the sample orientation and light absorption. In the case of bone and other turbid solids, elastic light scattering is an important complication that makes it difficult to define the excitation volume. In the case of fresh tissue, which is used here, the difficulty in preparing a specimen that is optically flat (i.e. to within about 0.2 microns) and oriented exactly perpendicular to the laser beam exacerbates this problem.

Mineral crystallinity, which is a parameter that reflects the mineral crystal size [26], was significantly increased at 4 weeks ($p=0.045$) but decreased at 12 weeks ($p=0.02$) for irradiated bone (Fig. 2 (B) and Table 3). At 26 weeks, there is no significant difference between control and irradiated bone ($p=0.152$).

The carbonate to phosphate ratio corresponds to the extent of carbonate substitution for phosphate in the bone mineral crystals [26]. Relative to controls, the ratio was significantly decreased at 4 weeks ($p=0.004$) but dramatically increased at 12 weeks ($p<0.001$) (Fig. 2 (C) and Table 3). No significant difference was observed at 26 weeks ($p>0.05$).

Although the bone collagen cross-linking ratio has been used to assess the non-reducible to reducible collagen cross-linking ratio by both FTIR and Raman [26, 33], it really measures different collagen secondary structures. These can be influenced by other factors than presence or absence of normal cross-links. Perturbations may include mechanical damage and presence of non-enzymatic cross-links (advanced glycation end products) [34]. In this study, a significant increase in collagen cross-linking ratio of irradiated bone was observed at each post-radiation time point and interpreted as radiation-induced cross-links (Fig. 2 (D) and Table 3). With increasing post-radiation time, the cross-linking ratio of irradiated bone became closer to that of the control, showing partial return to a normal collagen secondary structure.

Figure 3 shows the representative polarized Raman (parallel and perpendicular) spectra of irradiated mouse tibia. It is evident that most of the mineral and matrix bands are polarized, including the phosphate ν_1 band at $\sim 958\text{ cm}^{-1}$ and the amide I region at $\sim 1665\text{ cm}^{-1}$. The perpendicular polarized Raman intensities for these Raman bands were found to be lower by 2 to 6 fold relative to the parallel polarized Raman intensities. The depolarization ratio of the phosphate ν_1 band (ν_{958}) for the irradiated group was significantly different from the control

at all time points but 8 weeks ($p=0.192$) and 26 weeks ($p=0.862$) (Fig. 4 (A) and Table 3). When the depolarization ratio of the amide I band (ν_{1665}) was considered, a significant difference was found between irradiated bone and control at each time point (Fig. 4 (B) and Table 3).

Discussion

Irradiated bone is at risk for sustaining severe damage. In the extremities, the damage can lead to catastrophic fractures with delayed or non-healing, and may require multiple procedures and even amputation. Previous biomechanical studies have shown that the irradiated bone is embrittled, but the cause is unclear and cannot be explained by morphological changes alone [25]. This suggests that there are crucial changes in irradiated bone material properties. These material properties, including amount of mineral relative to collagen, mineral crystallinity and carbonate content, the state of collagen cross-linking, and orientations of mineral and collagen fibril can be determined by Raman spectroscopy. In the present study, we used Raman spectroscopy and a murine tibia model to longitudinally study radiation effects on bone. This animal model has been used to study post-radiation growth plate pathophysiology and has been shown to recapitulate findings in human retrieval irradiated specimens [25]. The findings herein support our hypotheses that there are intrinsic biochemical changes, including those of both chemical composition and degree of molecular orientation, within the remaining irradiated bone that may contribute to the increased fragility, that these changes are time course-dependent, and that they are detectable by Raman spectroscopy in a localized extremity mouse irradiation model.

One of main compositional properties related to bone mechanical strength is mineral to matrix ratio (the amount of mineral relative to collagen). Both increased and decreased mineral content would degrade bone mechanical properties [35]. At 4 weeks post-radiation, there was a transient increase in the mineral content of irradiated bone (Fig. 2 (A) and Table 3). We recently reported similar hypermineralization in the irradiated rat mandible [36]. After radiation with 70 Gy in 5 fractions, rat mandible showed a significant increase in mineral content relative to the controls at 8 weeks post-radiation. The increased mineral content correlates with the increase of bone volume fraction (BVF), tissue mineral density (TMD) and bone mineral density (BMD) of the irradiated rat mandible [37]. Further study of biomechanical properties of irradiated rat mandible at 8 weeks showed an increase of elastic modulus. Similar transient increases were also found in our irradiated mouse tibia model, which showed a transiently increased bone volume and strength at 2 weeks post-radiation [25]. At 12 weeks, the mineral content of the irradiated bone had significantly decreased as measured by Raman spectroscopy at the periosteal surface. This contrasts with the Wernle results which show increased whole bone mineral content at 12 weeks as measured by microCT [25]. The discrepancy is likely due to the differences in measurement locations. At 12 weeks it is possible that new bone is forming on the periosteal surface and this local region may have lower mineral content. At 26 weeks in the current report, there was no significant difference in periosteal mineral content between irradiated bone and control, suggesting the mineral content of irradiated bone was almost fully recovered in this animal model.

At 4 weeks post-radiation, irradiated bone showed significantly higher crystallinity (Fig. 2 (B) and Table 3). The higher mineral crystallinity after irradiation was also found in our recent report on irradiated rat mandible and other studies [36, 38, 39]. This observation suggested the presence of mineral crystal of greater size crystallite and further indicated the growth of existing crystals may dominate at 4 weeks post-radiation rather than formation of new smaller crystals [39]. Increased bone mineral crystal size has been found in osteoporotic patients and was proposed to be associated with increased bone fragility [40]. Interestingly,

crystallinity of irradiated bone was significantly decreased at 12 weeks, suggesting more younger and smaller crystals might be formed. Because crystallinity is closely correlated to crystal size and shape [41, 42], alteration of crystallinity would induce local residue strains which may cause bone structure defects and increase the fracture susceptibility [43]. Thus, radiation-induced damage resulting in altered mineral crystallinity may also be associated with mechanical properties of the irradiated bone.

Abnormal carbonate content (carbonate to phosphate ratio) observed during radiation recovery probably reflects radiation-induced impaired mineralization and bone turnover (Fig. 2 (C) and Table 3). At 4 weeks post-radiation, a significant decrease in carbonate to phosphate ratio was observed in irradiated bone, which corresponds to the observed increase of crystallinity at the same time point. At 12 weeks, the carbonate content was significantly increased while crystallinity was decreased. These findings are in agreement with previous studies of synthetic carbonated apatites showing that carbonate content is usually closely associated with mineral crystallinity [44]. The higher crystallinity reflects reduced carbonate content, and vice versa. With accumulation of matured bone tissue, the carbonate to phosphate ratios were increased in both irradiated bone and control at 26 weeks, but no significant difference was found.

A significant correlation between the bone mineral properties and age-related impaired elastic deformability of cortical bone has been reported in a Raman spectroscopic study [43]. The increase of mineral to matrix ratio, crystallinity and carbonate to phosphate ratio in rat femur with age were found to be significantly correlated with decreasing elastic deformation capacity of bone. Similar changes of mineral properties were also found in the present study. Both irradiated bone and control showed increasing mineral to matrix ratio, crystallinity and carbonate to phosphate ratio at 26 weeks relative to 1 week, which should be associated with the age-related impaired bone mechanical properties too. Moreover, the alteration of bone mineral properties induced by radiation could play a critical role in the assessment of post-radiation fractures, although additional work is required for complete understanding of the mechanism by which the bone mineral properties change the bone mechanical function [43].

The effects of irradiation on bone mineralization remain incompletely understood. An earlier study proposed that irradiation causes hypoxic-hypovascular-hypocellular bone tissue in which the normal bone remodeling process was broken mainly due to radiation-induced loss of osteoblasts and osteoclasts and reduction of vascularity [45]. In the present longitudinal study, three phases of bone mineralization on the periosteal surface of mouse tibia have been defined. An initial early phase occurs during the first 4 weeks, represented by the increase of mineral to matrix ratio and mineral crystallinity, as well as the decrease of carbonate content. It has been reported that the hematopoietic osteoclast precursors are more radiosensitive than osteoblast progenitors (mesenchymal) [46]. Moreover, a recent study showed low dose radiation stimulated differentiation and mineralization of mouse calvarial osteoblasts [47]. Thus, the increased mineral to matrix ratio in the first phase may mainly reflect different radiation tolerance of osteoclasts and osteoblasts. This phase likely corresponds histologically to the nadir of osteoclasts. Then there is a second intermediate phase covering 4 to 12 weeks. The second phase may reflect partial recovery of vascularity with influx of the osteoclasts, which resulting in decrease of mineral to matrix ratio, increase of carbonate content and decrease of mineral crystallinity. Finally, a third phase, from 12 to 26 weeks, was found in this animal model. Continuous increase of mineral to matrix ratio, carbonate content and crystallinity during the third phase results in these complete return of the parameters to normal values at 26 week post-radiation. This observation may reflect better return of vascularity and recovery of osteoclasts and osteoblasts in mice compared to what might be seen in patients with post-radiation fractures.

While a previous study found no effect of irradiation (70 Gy) on solubility of both enamel and synthetic hydroxyapatite [48], another study showed irradiation (72 Gy) decreased solubility of enamel at pH 5 *in vitro* [49]. Because we find that mineral to matrix ratio of the irradiated bone did not significantly differ from that of the control at 1 week post-radiation, our results are in agreement with those of ref. 48. However, we cannot fully exclude the possibility that the solubility of bone mineral at acidic pH has been affected by 20 Gy irradiation.

Collagen cross-linking is another important factor in determining bone structure and biomechanical properties. X-ray irradiation of bone tissue results in the formation of intermolecular and interfibrillar cross-links of collagen. The random cross-links are likely induced by the free radicals formed during radiation. However, the nature of radiation-induced cross-links in bone is still unclear. In this study, the collagen cross-linking ratio of irradiated bone was significantly higher than that of the control at each post-radiation time point (Fig. 2 (D) and Table 3). The collagen damage was observable even at post-radiation 26 weeks, implicating pathological cross-links formed by radiation damage to collagen were poorly resorbed although continuous recovery was observed. An elevated collagen cross-link ratio has been reported for osteoporotic patients and was proposed to contribute to bone fragility, although the role of these abnormal cross-links in osteoporosis remains unclear [50]. A simulation study using a finite element analysis model suggested that the number of cross-links and the density of mineral affects the strength of microfibrils and is associated with bone fracture [51]. In addition, studies *in vivo* and *in vitro* show that increases in cross-linking are related to increased bone strength and stiffness, but decreased energy absorption through microdamage formation [52-54]. Thus, the radiation-induced abnormal cross-links can degrade bone mechanical properties since the formation of new tissue occurs on a defective scaffold.

Raman scattering is strongly dependent on both bone composition and ultrastructure. Polarization anisotropy of phosphate 1 band ($\sim 958\text{ cm}^{-1}$) and the amide I band (1665 cm^{-1}) can be used to elucidate the orientation of bone mineral and collagen fibrils, respectively [27, 28]. In this study, the irradiated bone had significantly reduced depolarization ratios of mineral and collagen (Figure 4 and Table 3). The lower ratio indicates that irradiation results in bone with more ordered collagen fibrils and mineral crystallites along the diaphysis compared to controls. The alteration of mineral and collagen fibril alignment in irradiated bone is likely due to the formation of radiation-induced pathological cross-links between intermolecular and interfibrillar collagen molecules within collagen fibril bundles.

The study does have several limitations. The mice used in this study were 12 weeks of age when they underwent radiation. While this is considered skeletally mature, as with all murine models, growth plates remain open in both the irradiated animals and in the controls. Since the growth plate is adversely affected and functions markedly abnormally in the irradiated group while there is continued normal, albeit minimal, growth plate function in the control group, this may contribute to some of the differences between controls and irradiated limbs. Hence, the current longitudinal results on irradiated bone may be the combined result of both direct damage due to radiation and impaired bone development. It is unknown if similar results would be obtained with skeletally mature animals in which the growth plates are fused as they are in adult humans. In addition, the current study examined only the periosteal surface of the bone. Transverse sectioning of irradiated bone from periosteal to endosteal surface could provide more insights into the underlying pathophysiology of radiation-induced bone damages.

In conclusion, we longitudinally studied radiation effects on bone tissue using a murine tibia model by Raman spectroscopy. This technique was shown to be capable of detecting radiation-induced changes in both bone chemical composition and structure in this animal model. Radiation damage was demonstrated in both bone mineral and collagen. Some of the damage, such as mineral crystallinity, cross-links and collagen fibril orientation, were still present even at 26 weeks postradiation. These changes would theoretically compromise bone biomechanics, potentially leading to increased brittleness of irradiated bone as has been previously shown biomechanically. With a noninvasive fiber optic probe, this technique holds the potential of use in humans as a valuable diagnostic test and could be a complementary approach to conventional CT and DXA clinical scans which have not been shown to be associated with fracture risk.

Acknowledgments

Research reported in this publication was supported in part by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number RO1 AR055222 (MDM) and grants from the David G. Murray Endowment (TAD) and the Carol Baldwin Breast Cancer Foundation (MEO).

References

1. Song Y, Wang S, Chan M, Chandra B, Dhawan A, Song Y. Femoral fracture risk assessment after intensity modulated radiation therapy (IMRT) for the treatment of soft tissue sarcoma using a novel mathematical model. *Conf Proc IEEE Eng Med Biol Soc.* 2006; 1:95–8. [PubMed: 17946381]
2. Small W Jr, Kachnic L. Postradiotherapy pelvic fractures: cause for concern or opportunity for future research? *JAMA.* 2005; 294:2635–7. [PubMed: 16304079]
3. Park SH, Kim JC, Lee JE, Park IK. Pelvic insufficiency fracture after radiotherapy in patients with cervical cancer in the era of PET/CT. *Radiat Oncol J.* 2011; 29:269–76. [PubMed: 22984680]
4. Baxter NN, Rothenberger DA, Morris AM, Bullard KM. Adjuvant radiation for rectal cancer: do we measure up to the standard of care? An epidemiologic analysis of trends over 25 years in the United States. *Dis Colon Rectum.* 2005; 48:9–15. [PubMed: 15690651]
5. Cannon CP, Lin PP, Lewis VO, Yasko AW. Management of radiation-associated fractures. *J Am Acad Orthop Surg.* 2008; 16:541–9. [PubMed: 18768711]
6. Dickie CI, Parent AL, Griffin AM, Fung S, Chung PW, Catton CN, Ferguson PC, Wunder JS, Bell RS, Sharpe MB, O'Sullivan B. Bone fractures following external beam radiotherapy and limb-preservation surgery for lower extremity soft tissue sarcoma: relationship to irradiated bone length, volume, tumor location and dose. *Int J Radiat Oncol Biol Phys.* 2009; 75:1119–24. [PubMed: 19362782]
7. Fontanesi J, Mott MP, Kraut MJ, Lucas DR, Miller PR. A unique radiation scheme for the treatment of high-grade non-metastatic soft tissue sarcoma: the detroit medical center experience. *Sarcoma.* 2005; 9:141–5. [PubMed: 18521422]
8. Grigsby PW, Roberts HL, Perez CA. Femoral neck fracture following groin irradiation. *Int J Radiat Oncol Biol Phys.* 1995; 32:63–7. [PubMed: 7721641]
9. Helmstedter CS, Goebel M, Zlotecki R, Scarborough MT. Pathologic fractures after surgery and radiation for soft tissue tumors. *Clin Orthop Relat Res.* 2001:165–72. [PubMed: 11501806]
10. Holt GE, Griffin AM, Pintilie M, Wunder JS, Catton C, O'Sullivan B, Bell RS. Fractures following radiotherapy and limb-salvage surgery for lower extremity soft-tissue sarcomas. A comparison of high-dose and low-dose radiotherapy. *J Bone Joint Surg Am.* 2005; 87:315–9. [PubMed: 15687153]
11. Huh SJ, Kim B, Kang MK, Lee JE, Lim DH, Park W, Shin SS, Ahn YC. Pelvic insufficiency fracture after pelvic irradiation in uterine cervix cancer. *Gynecol Oncol.* 2002; 86:264–8. [PubMed: 12217746]
12. Ikushima H, Osaki K, Furutani S, Yamashita K, Kishida Y, Kudoh T, Nishitani H. Pelvic bone complications following radiation therapy of gynecologic malignancies: clinical evaluation of

- radiation-induced pelvic insufficiency fractures. *Gynecol Oncol.* 2006; 103:1100–4. [PubMed: 16919711]
13. Kwon JW, Huh SJ, Yoon YC, Choi SH, Jung JY, Oh D, Choe BK. Pelvic bone complications after radiation therapy of uterine cervical cancer: evaluation with MRI. *AJR Am J Roentgenol.* 2008; 191:987–94. [PubMed: 18806132]
 14. Livi L, Santoni R, Paiar F, Bastiani P, Beltrami G, Caldora P, Capanna R, De Biase P, Detti B, Fondelli S, Meldolesi E, Pertici M, Polli C, Simontacchi G, Biti G. Late treatment-related complications in 214 patients with extremity soft-tissue sarcoma treated by surgery and postoperative radiation therapy. *Am J Surg.* 2006; 191:230–4. [PubMed: 16442951]
 15. Rimmer A, Brennan MF, Zhang Z, Singer S, Alektiar KM. Influence of compartmental involvement on the patterns of morbidity in soft tissue sarcoma of the thigh. *Cancer.* 2009; 115:149–57. [PubMed: 19090010]
 16. Kelly J, Damron T, Grant W, Anker C, Holdridge S, Shaw S, Horton J, Cherrick I, Spadaro J. Cross-sectional study of bone mineral density in adult survivors of solid pediatric cancers. *J Pediatr Hematol Oncol.* 2005; 27:248–53. [PubMed: 15891558]
 17. Dieckmann KP, Pichlmeier U. Is risk of testicular cancer related to body size? *Eur Urol.* 2002; 42:564–9. [PubMed: 12477651]
 18. Lin PP, Schupak KD, Boland PJ, Brennan MF, Healey JH. Pathologic femoral fracture after periosteal excision and radiation for the treatment of soft tissue sarcoma. *Cancer.* 1998; 82:2356–65. [PubMed: 9635528]
 19. Taremi M, Hope A, Lindsay P, Dahele M, Fung S, Purdie TG, Jaffray D, Dawson L, Bezjak A. Predictors of radiotherapy induced bone injury (RIBI) after stereotactic lung radiotherapy. *Radiat Oncol.* 2012; 7:159. [PubMed: 22985910]
 20. Gortzak Y, Lockwood GA, Mahendra A, Wang Y, Chung PW, Catton CN, O'Sullivan B, Dehesi BM, Wunder JS, Ferguson PC. Prediction of pathologic fracture risk of the femur after combined modality treatment of soft tissue sarcoma of the thigh. *Cancer.* 2010; 116:1553–9. [PubMed: 20108335]
 21. Dhakal S, Chen J, McCance S, Rosier R, O'Keefe R, Constine LS. Bone density changes after radiation for extremity sarcomas: exploring the etiology of pathologic fractures. *Int J Radiat Oncol Biol Phys.* 2011; 80:1158–63. [PubMed: 20888134]
 22. Bandstra ER, Thompson RW, Nelson GA, Willey JS, Judex S, Cairns MA, Benton ER, Vazquez ME, Carson JA, Bateman TA. Musculoskeletal changes in mice from 20–50 cGy of simulated galactic cosmic rays. *Radiat Res.* 2009; 172:21–9. [PubMed: 19580504]
 23. Kondo H, Searby ND, Mojarrab R, Phillips J, Alwood J, Yumoto K, Almeida EA, Limoli CL, Globus RK. Total-body irradiation of postpubertal mice with (137)Cs acutely compromises the microarchitecture of cancellous bone and increases osteoclasts. *Radiat Res.* 2009; 171:283–9. [PubMed: 19267555]
 24. Cao X, Wu X, Frassica D, Yu B, Pang L, Xian L, Wan M, Lei W, Armour M, Tryggestad E, Wong J, Wen CY, Lu WW, Frassica FJ. Irradiation induces bone injury by damaging bone marrow microenvironment for stem cells. *Proc Natl Acad Sci U S A.* 2011; 108:1609–14. [PubMed: 21220327]
 25. Wernle JD, Damron TA, Allen MJ, Mann KA. Local irradiation alters bone morphology and increases bone fragility in a mouse model. *J Biomech.* 2010; 43:2738–46. [PubMed: 20655052]
 26. Morris MD, Mandair GS. Raman assessment of bone quality. *Clin Orthop Relat Res.* 2011; 469:2160–9. [PubMed: 21116756]
 27. Raghavan M, Sahar ND, Wilson RH, Mycek MA, Pleshko N, Kohn DH, Morris MD. Quantitative polarized Raman spectroscopy in highly turbid bone tissue. *J Biomed Opt.* 2010; 15:037001. [PubMed: 20615030]
 28. Kazanci M, Roschger P, Paschalis EP, Klaushofer K, Fratzl P. Bone osteonal tissues by Raman spectral mapping: orientation-composition. *J Struct Biol.* 2006; 156:489–96. [PubMed: 16931054]
 29. Kazanci M, Wagner HD, Manjubala NI, Gupta HS, Paschalis E, Roschger P, Fratzl P. Raman imaging of two orthogonal planes within cortical bone. *Bone.* 2007; 41:456–61. [PubMed: 17602910]

30. Okagbare PI, Esmonde-White FW, Goldstein SA, Morris MD. Development of noninvasive Raman spectroscopy for in vivo evaluation of bone graft osseointegration in a rat model. *Analyst*. 2010; 135:3142–6. [PubMed: 20924520]
31. Schulmerich MV, Cole JH, Dooley KA, Morris MD, Kreider JM, Goldstein SA, Srinivasan S, Pogue BW. Noninvasive Raman tomographic imaging of canine bone tissue. *J Biomed Opt*. 2008; 13:020506. [PubMed: 18465948]
32. Wopenka B, Kent A, Pasteris JD, Yoon Y, Thomopoulos S. The tendon-to-bone transition of the rotator cuff: a preliminary Raman spectroscopic study documenting the gradual mineralization across the insertion in rat tissue samples. *Appl Spectrosc*. 2008; 62:1285–94. [PubMed: 19094386]
33. Paschalis EP, Verdelis K, Doty SB, Boskey AL, Mendelsohn R, Yamauchi M. Spectroscopic characterization of collagen cross-links in bone. *J Bone Miner Res*. 2001; 16:1821–8. [PubMed: 11585346]
34. Morris MD, Finney WF, Rajachar RM, Kohn DH. Bone tissue ultrastructural response to elastic deformation probed by Raman spectroscopy. *Faraday Discuss*. 2004; 126:159–68. discussion 169–83. [PubMed: 14992405]
35. Ling Y, Rios HF, Myers ER, Lu Y, Feng JQ, Boskey AL. DMP1 depletion decreases bone mineralization in vivo: an FTIR imaging analysis. *J Bone Miner Res*. 2005; 20:2169–77. [PubMed: 16294270]
36. Tchanque-Fossuo CN, Gong B, Poushanchi B, Donneys A, Sarhaddi D, Gallagher KK, Deshpande SS, Goldstein SA, Morris MD, Buchman SR. Raman spectroscopy demonstrates Amifostine induced preservation of bone mineralization patterns in the irradiated murine mandible. *Bone*. 2013; 52:712–7. [PubMed: 22885239]
37. Tchanque-Fossuo CN, Donneys A, Deshpande SS, Nelson NS, Boguslawski MJ, Gallagher KK, Sarhaddi D, Poushanchi B, Goldstein SA, Buchman SR. Amifostine remedies the degenerative effects of radiation on the mineralization capacity of the murine mandible. *Plast Reconstr Surg*. 2012; 129:646e–55e.
38. Lakshmi RJ, Alexander M, Kurien J, Mahato KK, Kartha VB. Osteoradionecrosis (ORN) of the mandible: a laser Raman spectroscopic study. *Appl Spectrosc*. 2003; 57:1100–16. [PubMed: 14611040]
39. Hong SI, Hong SK, Wallace JM, Kohn DH. Ultrastructural observation of electron irradiation damage of lamellar bone. *J Mater Sci Mater Med*. 2009; 20:959–65. [PubMed: 19034616]
40. Gourion-Arsiquaud S, Faibish D, Myers E, Spevak L, Compston J, Hodsman A, Shane E, Recker RR, Boskey ER, Boskey AL. Use of FTIR spectroscopic imaging to identify parameters associated with fragility fracture. *J Bone Miner Res*. 2009; 24:1565–71. [PubMed: 19419303]
41. Baig AA, Fox JL, Young RA, Wang Z, Hsu J, Higuchi WI, Chhetry A, Zhuang H, Otsuka M. Relationships among carbonated apatite solubility, crystallite size, and microstrain parameters. *Calcif Tissue Int*. 1999; 64:437–49. [PubMed: 10203421]
42. Handschin RG, Stern WB. Crystallographic and chemical analysis of human bone apatite (Crista Iliaca). *Clin Rheumatol*. 1994; 13(Suppl 1):75–90. [PubMed: 7750247]
43. Akkus O, Adar F, Schaffler MB. Age-related changes in physicochemical properties of mineral crystals are related to impaired mechanical function of cortical bone. *Bone*. 2004; 34:443–53. [PubMed: 15003792]
44. Awonusi A, Morris MD, Tecklenburg MM. Carbonate assignment and calibration in the Raman spectrum of apatite. *Calcif Tissue Int*. 2007; 81:46–52. [PubMed: 17551767]
45. Marx RE. Osteoradionecrosis: a new concept of its pathophysiology. *J Oral Maxillofac Surg*. 1983; 41:283–8. [PubMed: 6572704]
46. Scheven BA, Wassenaar AM, Kawilarang-de Haas EW, Nijweide PJ. Comparison of direct and indirect radiation effects on osteoclast formation from progenitor cells derived from different hemopoietic sources. *Radiat Res*. 1987; 111:107–18. [PubMed: 3602348]
47. Park SS, Kim KA, Lee SY, Lim SS, Jeon YM, Lee JC. X-ray radiation at low doses stimulates differentiation and mineralization of mouse calvarial osteoblasts. *BMB Rep*. 2012; 45:571–6. [PubMed: 23101511]

48. Jansma J, Buskes JA, Vissink A, Mehta DM, Gravenmade EJ. The effect of X-ray irradiation on the demineralization of bovine dental enamel. A constant composition study. *Caries Res.* 1988; 22:199–203. [PubMed: 3165710]
49. Pioch T, Moller D, Staehle HJ, Hoppe W. [Solubility of enamel and synthetic hydroxylapatite on irradiation]. *Dtsch Zahnarztl Z.* 1991; 46:413–5. [PubMed: 1817062]
50. Paschalis EP, Shane E, Lyrithis G, Skarantavos G, Mendelsohn R, Boskey AL. Bone fragility and collagen cross-links. *J Bone Miner Res.* 2004; 19:2000–4. [PubMed: 15537443]
51. Barkaoui A, Hambli R. Finite element 3D modeling of mechanical behavior of mineralized collagen microfibrils. *J Appl Biomater Biomech.* 2011; 9:199–205. [PubMed: 22139755]
52. Viguet-Carrin S, Roux JP, Arlot ME, Merabet Z, Leeming DJ, Byrjalsen I, Delmas PD, Boussein ML. Contribution of the advanced glycation end product pentosidine and of maturation of type I collagen to compressive biomechanical properties of human lumbar vertebrae. *Bone.* 2006; 39:1073–9. [PubMed: 16829221]
53. Allen MR, Gineyts E, Leeming DJ, Burr DB, Delmas PD. Bisphosphonates alter trabecular bone collagen cross-linking and isomerization in beagle dog vertebra. *Osteoporos Int.* 2008; 19:329–37. [PubMed: 18094911]
54. Tang SY, Vashishth D. Non-enzymatic glycation alters microdamage formation in human cancellous bone. *Bone.* 2010; 46:148–54. [PubMed: 19747573]

Highlights

- We irradiated murine tibiae with 4x5 Gy doses, approximating dosage for human cancer radiotherapy.
- Using Raman, we observed both bone mineral and collagen abnormalities induced by radiation.
- Initial increases in collagen cross-linking return to near-normal values over 26 wks. Collagen and mineral orientation along diaphysis followed same pattern.
- Other Raman parameters (mineral/matrix, crystallinity, carbonate content) also showed initial deviation, with return to normal over 26 wks.
- Results are consistent with initial radiation damage to mineral, matrix and slow remodeling of damaged tissue.

Fig. 1. Mouse tibia Raman spectrum and band assignments of bone mineral carbonated apatite and bone matrix collagen, with minor contributions from other proteins, lipids, GAGs.

Fig. 2. Comparisons of Raman parameters between irradiated tibiae and controls. (A) Mineral/matrix ratio; (B) Mineral crystallinity; (C) Carbonate/phosphate ratio and (D) Collagen cross-linking ratio. Asterisk (*) represents p<0.05.

Fig. 3. Representative Raman spectra of mouse tibia when measured with parallel polarized ($\theta=0^\circ$, upper trace) and perpendicular polarized ($\theta=90^\circ$, lower trace) laser beam. Band assignments see Fig. 1.

Fig. 4. Comparisons of depolarization ratios of (A) mineral and (B) collagen matrix between irradiated tibiae and controls. Asterisk (*) represents $p < 0.05$.

Table 1

Raman spectroscopic parameters used in this study

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

^aBand assignments see Fig. 1.^bDepolarization ratio was defined in the section of Materials and Methods.

Table 2

Comparisons of Raman parameters: Irradiated bone (Irrad.) vs. Control (Con.)

Raman parameters		Results				
		1 week	4 weeks	8 weeks	12 weeks	26 weeks
Mineral/matrix	Irrad.	14.63 ± 2.55 ^a	16.99 ± 2.71	15.98 ± 4.90	12.83 ± 2.75	17.73 ± 1.92
	Con.	14.67 ± 3.11	15.21 ± 3.16	15.48 ± 3.47	16.22 ± 4.42	18.09 ± 4.09
Crystallinity (×10 ⁻²)	Irrad.	5.70 ± 0.04	5.74 ± 0.07	5.76 ± 0.05	5.71 ± 0.05	5.79 ± 0.03
	Con.	5.72 ± 0.06	5.71 ± 0.05	5.74 ± 0.05	5.74 ± 0.05	5.76 ± 0.03
Carbonate/mineral	Irrad.	0.254 ± 0.019	0.230 ± 0.025	0.255 ± 0.016	0.264 ± 0.015	0.272 ± 0.010
	Con.	0.246 ± 0.022	0.252 ± 0.024	0.247 ± 0.013	0.246 ± 0.018	0.269 ± 0.009
Collagen cross-linking ratio	Irrad.	3.81 ± 0.28	3.76 ± 0.41	3.40 ± 0.20	3.31 ± 0.23	3.01 ± 0.16
	Con.	2.29 ± 0.44	2.35 ± 0.18	2.47 ± 0.16	2.55 ± 0.22	2.77 ± 0.24
Depolarization ratio of mineral	Irrad.	0.145 ± 0.017	0.166 ± 0.029	0.173 ± 0.034	0.171 ± 0.053	0.172 ± 0.030
	Con.	0.218 ± 0.047	0.220 ± 0.067	0.206 ± 0.052	0.199 ± 0.028	0.181 ± 0.019
Depolarization ratio of collagen	Irrad.	0.424 ± 0.026	0.449 ± 0.039	0.535 ± 0.047	0.526 ± 0.034	0.519 ± 0.030
	Con.	0.556 ± 0.041	0.543 ± 0.054	0.453 ± 0.041	0.459 ± 0.035	0.478 ± 0.048

^aData are presented as means ± standard deviation.

Table 3

Statistical analysis of Raman parameters: Irradiated bone vs. Control

Raman parameters	p values with significant changes				
	1 week	4 weeks	8 weeks	12 weeks	26 weeks
Mineral/matrix	0.949 ^a (-)	0.048 ^b ()	0.608 (-)	<0.001 ^c ()	0.729 (-)
Crystallinity	0.344 (-)	0.045 ()	0.824 (-)	0.02 ()	0.152 (-)
Carbonate/mineral	0.486 (-)	0.004 ()	0.216 (-)	<0.001 ()	>0.05 (-)
Collagen cross-linking ratio	<0.001()	<0.001()	<0.001()	<0.001()	0.005()
Depolarization ratio of mineral	<0.001 ()	0.009 ()	0.192 (-)	0.046 ()	0.862 (-)
Depolarization ratio of collagen	<0.001 ()	<0.001 ()	<0.001()	<0.001 ()	0.02 ()

^a(-): No significant difference was found in irradiated bone relative to control (p 0.05)

^b(): Raman parameter was significantly increased in irradiated bone relative to control

^c(): Raman parameter was significantly decreased in irradiated bone relative to control.