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Review

Raman spectroscopy monitors adverse bone sequelae of cancer radiotherapy

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ABSTRACT

Raman spectroscopy provides information on bone chemical composition and structure *via* widely used metrics including mineral to matrix ratio, mineral crystallinity and carbonate content, collagen cross-linking ratio and depolarization ratios. These metrics are correlated with bone material properties, such as hardness, plasticity and Young's modulus. We review application of Raman spectroscopy to two important irradiated animal models: the mouse tibia, a model for damage to cortical bone sites including the rib (breast cancer) and to healthy tissue adjacent to extremity sarcomas, and the rat mandible, a model for radiation damage in head and neck cancer radiotherapy. Longitudinal studies of irradiated mouse tibia demonstrate that radiation-induced matrix abnormalities can persist even 26 weeks post-radiation. Polarized Raman spectroscopy shows formation of more ordered orientation of both mineral and collagen. At 8 weeks post-radiation, irradiated rat hemimandible exhibits transient hypermineralization, increased collagen cross-linking and decreased depolarization ratios of mineral and collagen. A standard radioprotectant, amifostine, mitigates rat mandible radiation damage, with none remaining detectable 18 weeks post-radiation. Already a powerful tool to monitor radiation damage, Raman spectroscopy may be important in development of new radiotherapy protocols and radioprotective agents. Further *in vivo* studies of radiation effects on the rodent models are underway, as are development of methodologies for eventual use in human subjects.

1. Introduction

Adjuvant radiotherapy for cancer patients degrades the biomechanical properties of adjacent healthy bone tissue, which results in increase of susceptibility to bone fracture in the affected region and even osteoradionecrosis. For example, it is estimated that 5%–15% of patients with head and neck cancer being treated by radiotherapy will develop symptoms of osteoradionecrosis (ORN) of the jaw and 3% of patients finally have ORN of the jaw [1,2]. Recent reports also show post-radiation fracture incidence as high as 22% in patients with breast cancer and 24% in some populations of patients with soft-tissue sarcoma [3–5].

The underlying pathophysiology of radiation-induced bone damages remains poorly understood. "Three H", hypovascular, hypocelluar and hypoxic, has been used to describe the cellular and vascular response to radiotherapy [6], which results in suppression of the remodeling and healing capacity of bone. However, radiation damage to murine bone cannot be completely understood as depletion and recovery of cell populations. This problem suggests the likely involvement of "cell-independent, physicochemical erosion enabled by radiation damage to the organic and inorganic constituents of the matrix." [7]. In addition, clinical studies showing that irradiation does not routinely decrease bone density further suggest intrinsic material abnormalities in the irradiated bone [8,9]. These abnormalities, such as pathological collagen cross-links, altered mineral crystallinity and carbonation, are not detectable by conventional histology and computed tomography (CT) or dual-energy X-ray absorptiometry (DXA) and require application of other techniques.

Over the past 12–15 years there has been increasing interest in the application of Raman spectroscopy to bone studies [10]. In comparison to Fourier transform infrared (FTIR) spectroscopy, Raman spectroscopy provides experimental advantages including spectroscopic microscopy with <1 μm lateral spatial resolution, applicability to fresh tissue and *in vivo* measurement capability. Because Raman provides both chemical and structural signatures

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of bone mineral and matrix, it can be used to quantify relevant mineral and matrix chemical composition and structural properties of bone, including bone mineral crystallinity, carbonate content, amount of mineral relative to matrix, the state of collagen cross-linking and the orientation of mineral crystallite and collagen fibrils. Alteration of these parameters can be easily monitored by Raman spectroscopy and could help explain radiotherapy-induced changes in bone material behavior.

In collaboration with other medical, dental and biomedical engineering research groups, the Morris laboratory at the University of Michigan has applied Raman spectroscopy to investigate a range of problems in bone tissue, such as age-related effects in biomechanical competence [11], composition problems in the Brtl mouse model of osteogenesis imperfecta [12], correlation between bone composition and osteoporotic fracture outcomes [13], early detection of osteomyelitis [14], kinetics of early mineral deposition [15], heterotopic ossification [16,17] and spatial detection of collagen cross-linking differences due to relative tissue age and effects of cross-link inhibition [18]. In this mini review, we will focus on our recent studies on radiation-induced bone damage and the effect of adjuvant therapy with radioprotectants such as amifostine (AMF) [19–21]. In an irradiated mouse tibial model, Raman spectroscopy was used to follow changes in both bone chemistry and structure during 26 weeks post-radiation recovery. We have also studied irradiated rat hemimandibles to elucidate radiation-induced chemical and structural differences and efficacy of amifostine adjuvant therapy at 8 and 18 weeks post-radiation. It should be noted that most previous studies on irradiated bone used either very high dose (>10 kGy) ionizing irradiation, employed for sterilization of allograft material [22–24] or low dose irradiation on excised bones or in total body irradiation models [25–27]. We used focal irradiation on the clinically relevant body regions of living animals with clinically relevant irradiation doses. Our studies are more applicable to the conditions of cancer radiotherapy than is earlier work. The results summarized in this review provide more insights into radiation-induced degradation of bone biomechanical properties and show that Raman spectroscopy can investigate and monitor both the adverse effects of radiation and the efficacy of therapeutic remediation of radioprotectant drugs on bone material quality.

2. Raman metrics for bone quality assessment

Bone is a heterogeneous and hierarchical material, which contains a mineral component, an organic matrix and water. The mineral is a carbonated hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). The organic matrix mainly includes type I collagen with ~10% contributions from other proteins. Water accounts for ~20% weight of fresh bone tissue [28]. Both inorganic and organic constituents have specific Raman signatures that can be used to assess bone material quality. Fig. 1 shows a typical Raman spectrum of bone. The main bands due to mineral and matrix are labeled. Among them, the phosphate ν_1 band at ~958 cm^{-1} and the B-type carbonate band at ~1070 cm^{-1} are often used to evaluate bone mineral properties. The amide I envelope at ~1660 cm^{-1} is an important indicator of protein secondary structural changes. The Raman metrics developed for analyzing bone material properties have been described in detail previously [10]. Briefly, the mineral to matrix ratio is determined by intensity of phosphate ν_1 band at ~958 cm^{-1} divided by the collagen protein band(s) (*e.g.* phenylalanine band or amide I band or proline and hydroxyproline bands). This ratio represents the relative amount of mineral in the examined region of bone tissue. Mineral crystallinity is inversely proportional to the full width at half maximum (FWHM) of the phosphate ν_1 band (~958 cm^{-1}) and provides the information on

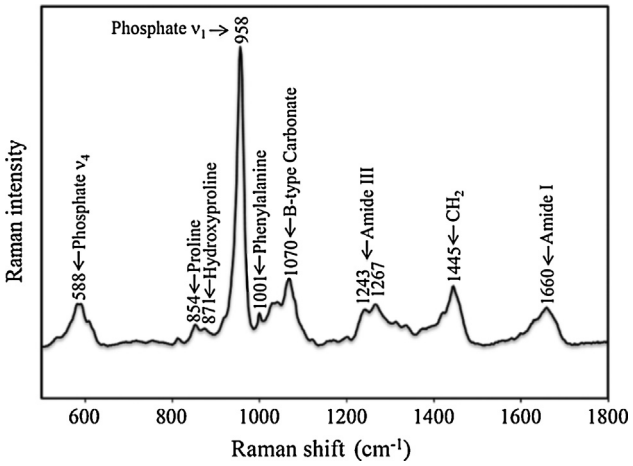

Fig. 1. Raman spectrum of bone, with band assignments.

bone mineral structure and crystallite size. The carbonate to phosphate ratio is measured by the intensity ratio of the B-type carbonate band (~1070 cm^{-1}) to the phosphate ν_1 band (~958 cm^{-1}) and represents the relative carbonate content in bone mineral. The collagen cross-linking ratio is calculated by the intensity ratio of two sub-component bands (~1660/~1690 cm^{-1}) in the amide I envelope. This ratio was first reported to indirectly measure the non-reducible/reducible collagen cross-link ratio in bone and reflects the secondary structural perturbation of collagen protein [29].

Bone structural organization affects bone quality. Polarized Raman spectroscopy provides structural information (*e.g.* orientation) of bone mineral and collagen fibrils [30,31]. Most Raman bands of bone (Fig. 1) are sensitive to molecular orientation and to the polarization direction of incident laser light [19]. The intensities of the phosphate ν_1 at ~958 cm^{-1} and the amide I region at ~1660 cm^{-1} are used to calculate the depolarization ratios of bone mineral and matrix because both bands are strongly polarized [30,32]. The depolarization ratio is defined by $\rho = I(\perp)/I(//)$, where $I(\perp)$ is the intensity of the perpendicular polarization component for the selected Raman band, and $I(//)$ is the intensity of the parallel polarization component for the same band. A lower ρ value reflects a higher degree of molecular alignment or orientation.

3. Prolonged chemical and structural damage to irradiated mouse tibia

A longitudinal study has been performed in a mouse tibial model to evaluate radiation-induced bone damage during long term recovery [19]. Right tibiae of female BALB/F mice aged 12 weeks underwent a localized irradiation by 4 daily 5 Gy fractions. Mice were euthanized at 1, 4, 8, 12 and 26 weeks post-irradiation and both irradiated right tibiae and non-irradiated left tibiae (control) were harvested for Raman analysis. The detailed experimental conditions are given in [19]. Raman bone metrics, including mineral to matrix ratio, mineral crystallinity, carbonate to phosphate ratio, collagen cross-linking ratio, depolarization ratios of mineral and collagen, were calculated from Raman measurements performed on the proximal cortical bone surface of tibiae. Compared to control, irradiated bone showed several significant changes in these Raman metrics (Fig. 2). Hyperminerialized bone tissue was observed at 4 weeks post-radiation, 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 deviations