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Amifostine Preserves Osteocyte Number and Osteoid Formation in Fracture Healing Following Radiotherapy

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Abstract

Purpose—Radiation is known to diminish osteocyte count and function leading to bone weakening. A treatment strategy to mitigate these consequences could have immense therapeutic ramifications. We have previously demonstrated significantly diminished osteocyte count and mineralization capacity in a rat model of fracture healing after radiotherapy. We hypothesize that amifostine (AMF) will preserve osteocyte number and function in this model.

Materials and Methods—Thirty-six rats were divided into three groups: fracture, radiated fracture, and radiated fracture with AMF. Radiated groups underwent human equivalent radiotherapy to the mandible prior to fixator placement and mandibular osteotomy. The AMF group received a subcutaneous injection prior to each dose of radiotherapy. After 40 days, mandibles were harvested for histologic processing. Quantification of osteocyte count (Oc), empty lacunae (EL) and osteoid ratio (OV/TV) was performed and the results were compared using ANOVA ($p < 0.05$).

Results—Radiated fractures demonstrated significantly diminished Oc, increased EL and diminished capacity to produce new osteoid at the fracture site as measured with OV/TV when compared to non-radiated fractures. In mandibles treated with amifostine, these metrics were not statistically different than control, indicating a preservation of osteocyte number and function.

Conclusions—Our results support the hypothesis that amifostine preserves osteocyte number and function, thereby preventing the pernicious effects of radiotherapy on the cellular environment of fracture healing. Based on these findings, we encourage future investigation of this promising therapy for use in the prevention of pathologic fractures and osteoradionecrosis.

Keywords

Osteoradionecrosis; Histology; Angiogenesis; Pathologic Fracture; Radiotherapy

Introduction

Despite marked advancements in the delivery of ionizing radiotherapy for the treatment of Head and Neck Cancer (HNC), osteoradionecrosis, pathologic fractures and associated non-

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unions still occur. Unfortunately, radiation-induced destruction to adjacent bone and surrounding tissues continues to be a driving factor in the development of these complex debilitating morbidities.¹ It is well established that radiation therapy imparts its detrimental effects on bone in part through the depletion of osteocytes and the diminution of their function.²⁻³ Conceivably, the use of therapeutic radioprotectants, such as amifostine, represents a plausible strategy to mitigate the effects of radiation on osteocyte survival and viability.

Amifostine (S-2-[3-aminopropylamino] ethylphosphothioic acid) exhibits its radioprotective effects through the stabilization of DNA and the scavenging of free radicals. Further, through the upregulation of p53 and the dependency of amifostine on activation by alkaline phosphatase, it selectively spares non-malignant tissues from the damaging effects of ionizing radiation.⁴⁻⁶

Its efficacy as a clinical therapeutic agent has been thoroughly examined and it is commonly utilized for the prevention of radiation-induced xerostomia in HNC.^{5,7-9} Additionally, the drug has a relatively low toxicity and its limited side effect profile consists of nausea, vomiting, hypotension and allergic reaction.⁷ While amifostine has been approved for xerostomia prevention, few studies have focused on the beneficial effects of amifostine with regards to the protection of bone.¹⁰⁻¹⁵

The purpose of this study was to examine the diminution of osteocytes within the radiated fracture site and the potential prevention of osteocyte depletion, both in number and function with the addition of amifostine. In order to do this, we examined the utility of amifostine in an established rat model of pathologic fracture healing in the setting of high dose radiotherapy.¹⁶ The selection of this particular model was based on our collective experience accumulating over 10 years of data related to the pathologic effects of radiation on fracture site healing in the rat mandible. Our previous investigations demonstrated quantifiably diminished metrics of vascular density, bone quality and biomechanical strength¹⁶⁻¹⁷. Here we investigate the effect of radiotherapy on the cellular environment of the fracture site. We hypothesize that the addition of amifostine will prevent osteocyte depletion and preserve osteocyte function, thereby allowing for significant remediation of osteogenesis and fracture healing in this setting.

Materials and Methods

Experimental Design

All studies were approved by the University of Michigan's Committee for the Utilization and Care of Animals. Thirty-six adult male Sprague-Dawley rats (400 g) were obtained through the University of Michigan's Unit for Laboratory Animal Medicine. All animals were paired in cages in a restricted, pathogen-free facility and maintained on a 12-hour light/dark schedule. Prior to handling and radiation, the rats had a 7-day acclimation period. These animals were assigned randomly to three experimental groups, each undergoing fracture repair. Group 1 (control, n=12) served as the standard fracture group (Fx), receiving only osteotomy surgery. Group 2 (radiation therapy, n=12) was radiated two weeks prior to the osteotomy (XFx). Group 3 (amifostine, n=12) was pretreated with amifostine (100 mg/kg; Ethiol, MedImmune, Gaithersburg, Md.) subcutaneously 45 minutes before radiotherapy, and subsequently underwent osteotomy surgery two weeks later (AMFXXFx). Our dosing and delivery of amifostine was based upon the established literature regarding the use of amifostine in rat models, and our previous experimentation with this therapy in this setting.^{13,18-21} All animal experimentation was conducted in accordance with the guidelines published in the *Guide for the Care and Use of Laboratory Animals: Eighth*

Edition, ISBN-10: 0-309-15396-4 and approved by the University of Michigan Animal Care and Use Committee.

Irradiation

Rats were given an oxygen-isoflurane mixture to achieve anesthesia. A lead shield was placed over the body, allowing only exposure to the region of interest (ROI) over the left hemimandible. Utilizing a Philips RT250 orthovoltage unit (250 kV, 15 mA) (Kimtron Medical, Woodbury, Conn.) we delivered a fractionated dose of external beam radiation of 7 Gy/day over the course of 5 days to this targeted ROI. The fractionated dosage delivery is based on our laboratory's previously established experimentation and is designed to deliver a human equivalent radiation dosage of 70 Gy in summation. This general radiation procedure has been used for several years in the Department of Radiation Oncology under protocols approved by the University of Michigan Unit for Laboratory Animal Medicine and the University Committee for the Utilization and Care of Animals.^{18-19,22-25} The animals received soft chow and water ad libitum for the two-week period between radiation and osteotomy surgery. During this time, rats exhibiting signs of discomfort or pain were administered subcutaneous injection of buprenorphine (0.15 mg/kg) with 5 mL of lactated Ringer's solution.

Surgical Procedure and Fixator Placement

Before undergoing surgery, the rats received subcutaneous injections of gentamicin (5 mg/kg), buprenorphine (0.03 mg/kg) and lactated Ringer's solution (25 mL/kg). Inhalational anesthesia was provided, and consisted of an oxygen-isoflurane mixture in preparation for surgery. After standard preparation and draping, a 2 cm midline incision was placed ventrally from the anterior submentum to the neck crease. A custom titanium fixator device was placed as previously described.²⁶ (Figure 1) After stabilization of the fixator, a vertical osteotomy was created using a reciprocating saw blade directly behind the third molar on the left hemimandible. After reduction of the osteotomy edges, wounds were irrigated, hemostasis verified and incisions were closed in layers. Four hours after surgery, a fixed 2.1-mm fracture gap was set by turning the fixator screw clockwise a total of 7 times. This gap ensures a grossly visible separation that can be later sectioned and analyzed histologically. (Figure 2) After a 40-day healing period, the animals were euthanized and left hemimandibles were harvested for histological analysis.

Histologic Processing

Left hemimandibles were harvested for histologic evaluation and underwent ethanol fixation (70%) at 4°C and subsequently rinsed in phosphate-buffered saline. Specimens were decalcified with Cal-ExII (Fisher Scientific, Fair Lawn, N.J.) (10.6% formic acid, 7.4% formaldehyde, and <1% methyl alcohol) and stored at 4°C. The decalcification solution was changed every 2 to 3 days and the specimens were followed radiographically by means of a self-contained Faxitron X-Ray device (MX20; Faxitron X-Ray, Lincolnshire, Ill.) until full decalcification was achieved. The specimens were vacuum processed (dehydration and paraffin infiltration) under a 48-hour program in a Hypercenter tissue processor (Hypercenter XP; Shandon, Inc., Pittsburgh, Pa.), then filtrated once more for 2 hours in a vacuum bath (Leica Embedding Center, model EG1160; Leica Microsystems, Wetzlar, Germany), followed by embedding in Paraplast Plus (McCormic Scientific, Richmond, Ill.). Peel-a-way embedding molds (22 × 40 mm) (Polysciences, Inc., Warrington, Pa.) were used and stored overnight at 4°C. Blocks were sectioned coronally from anterior to posterior into 7-μm thick sections taken on a Leica Reichert-Jung microtome (model 2030; Biocut, Bensheim, Germany) and mounted on glass slides (Fisherbrand Superfrost Plus; Fisher Scientific). Sections were surface-stained with Gomori's one-step trichrome.

Histomorphometric Evaluation

Using a light microscope interfaced with a digital camera connected to a computer, the point counting for osteocytes and empty lacunae was performed. We superimposed our ROI spanning 2.1 mm posterior to the third molar onto the digital image using the imaging analysis software program Bioquant NOVA Osteo version 7 (R&M Biometrics, Nashville, Tenn.). Using 16X magnification, nine high-power field (HPF) images were randomly selected per ROI. Three independent reviewers performed the point counting of osteocytes and empty lacunae.

Threshold Evaluation

Digital images of each slide were obtained and loaded onto the imaging analysis software Bioquant NOVA Osteo, version 7 (R&M Biometrics, Nashville, TN) for placement of a standard template over the ROI and color thresholding. Three independent reviewers each used the software to measure the tissue volume (TV) and osteoid volume (OV). TV included the entire volume of the ROI. While mature bone stained blue, non-mineralized, immature bone, including vacuoles, stained pink, thereby allowing for easy distinction during the thresholding process. The software calculated the OV/TV ratio, indicating the percentage of immature bone in the ROI.

Statistical Analysis

Values were analyzed with a one-way analysis of variance using PSAW for Windows version 20.0 (SPSS, Inc., Chicago, Ill.). Results were statistically significant at a value of $p < 0.05$.

Results

One animal in the XFx group exhibited oral mucositis following radiotherapy, prior to surgery. This interfered with his ability to eat a soft diet, and therefore buprenorphine was administered, as stated.

Histomorphometric Analysis

The mean number of osteocytes/HPF in the Fx group was 70.35 ± 10.78 and the mean number of empty lacunae was 2.35 ± 1.27 . The radiated group demonstrated a significant decrease in osteocyte count to 29.76 ± 10.83 ($p < 0.001$) and a corresponding significant increase in empty lacunae to 7.20 ± 3.86 ($p < 0.001$) when compared to the fracture group. With the addition of amifostine, the osteocyte count increased to 78.92 ± 13.40 , a significant boost when compared to the radiated group ($p < 0.001$). A difference in osteocyte number for the amifostine-treated group was not observed in comparison to the fracture group ($p = 0.189$). The amifostine-treated group also exhibited a significant difference in the number of empty lacunae per HPF, 3.55 ± 1.37 , that was lower than the radiated group ($p = 0.003$), but was also not different than the fracture group ($p = 0.471$). In summary, the fracture and amifostine-treated groups exhibited no differences in both number of osteocytes and empty lacunae, but the radiated group was significantly different from both groups in number of osteocytes and empty lacunae (Figures 3 and 4).

Threshold Analysis

The ratio of immature bone (OV/TV) in the ROI was found to be lower in the radiated group (0.159 ± 0.068) when compared to the fracture group (0.354 ± 0.141 , $p < 0.001$). When amifostine was used, no significant deviation occurred from the fracture group in OV/TV (0.277 ± 0.053 , $p = 0.139$). However, a difference was observed between the radiated group and amifostine-treated group in OV/TV ($p = 0.011$) (Figure 5). We found that normally

healing fractures (Figure 6) exhibited a mixed array of both mature mineral (blue) and immature mineral (pink). The radiated group (Figure 7) demonstrated a dramatic loss of immature mineral, or osteoid, which was not evident in the amifostine-treated group (Figure 8). In summary, the fracture and amifostine-treated groups again displayed no difference in OV/TV, whereas the radiated group differed from the former two groups.

Discussion

The purpose of this study was to investigate the therapeutic potential of amifostine to remediate radiation-induced damage to bone cellularity. We have previously investigated the effects of radiotherapy on the process of fracture healing. Utilizing this animal model, we have examined the degree by which radiotherapy impedes fracture site mineralization and vascularity as measured with μ CT imaging, and the limits of biomechanical integrity as quantified with biomechanical tension testing in the rat mandible.^{16-17,27-28}

While amifostine has been extensively investigated for the prevention of radiation-induced xerostomia, few studies have considered its utility for the prevention of severely debilitating bone pathologies such as osteoradionecrosis, pathologic fractures and associated non-unions. In fact, investigations regarding the use of amifostine in bone altogether have been limited to examining the potential prevention of growth plate restriction in the extremities, and the prevention of craniofacial bone growth inhibition for pediatric patients exposed to therapeutic levels of radiotherapy. In these regards, investigators have found promising results demonstrating quantifiable prophylactic effects on the rate of bone growth, bone mineralization, and the preservation of select histologic parameters.¹⁰⁻¹⁵

With the utility of amifostine in our radiated fracture model, our results consistently demonstrated a diminution of cellularity with the addition of radiotherapy that was not apparent with amifostine pretreatment. Both osteocyte count and thresholding analysis validate our hypothesis.

Osteocyte count is a metric describing the survival of osteocytes within bone tissue. Similarly, empty lacunae can be viewed as an inverse, yet corroborating metric indicating osteocyte death.²⁹⁻³⁰ As expected, following radiation therapy, we observed a decrease in osteocyte count to half the level of our normal fracture group. This diminution was not observed in the amifostine treated group, indicating a preservation of the osteocytes. Similarly, while the radiated group demonstrated a three-fold increase in empty lacunae count from fracture levels, this was not observed in the amifostine treated group. This further validates the efficacy of amifostine in preventing cellular diminution and preserving the cellular viability of bone tissue.

In addition to investigating osteocyte viability, we also examined the mineralization distribution at the fracture site by quantifying the amount of osteoid or immature mineral within the total ROI. While the normal fracture exhibited a rich combination of both mature and immature mineralization, the amount of osteoid was significantly diminished after radiation, presumably indicating a limitation of the capacity of osteocytes to produce new non-mineralized bone. This was not observed in mandibles treated with amifostine, as the normal fracture distribution of osteoid was preserved.

The results of this study must be placed in context with certain limitations regarding the investigation of human pathologies in animal models. While the development of osteoradionecrosis, pathologic fracture and associated non-unions usually occur years after radiotherapy, subjecting animals to prolonged experimentation would be impractical. We have therefore previously developed a protocol designed to replicate the effects of high dose radiotherapy on mandibular fracture repair in an accelerated manner. Furthermore, to

establish a reproducible and controlled model, we used a surgically created osteotomy instead of the natural mechanisms leading to closed mandibular fracture. Therefore, although our model is not clinically identical to the cause of radiation-induced fractures, it does simulate the clinically analogous circumstances in which they are treated by open reduction. In both cases, bone healing is impaired by radiotherapy. Based on these assertions, if amifostine can prevent radiotherapy-impaired bone healing in our model, it may be able to provide a clinical solution to an otherwise damaged area to avoid the deleterious outcomes of non-unions.

A second limitation is our selection of a single time point, chosen based on the time when normal fracture healing would occur. Future directions will entail truncated time points to examine the effects of radiation on fracture healing at earlier stages. Nonetheless, our findings demonstrate the prevention of osteocyte depletion and the preservation of mineralization in a rigorous model of radiotherapy-induced detriments to the process of mandibular fracture healing. Based on these findings, we encourage future investigation of this promising therapy for potential use in the prevention of osteoradionecrosis, pathologic fractures and associated non-unions.

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Figure 1.
Titanium fixator device with directional markers indicated.

Figure 2.
X-ray of left hemi-mandibular osteotomy with 2.1 mm fracture gap indicated.

Figure 3. Osteocyte count per high power field (#/HPF) means and standard deviations for the fracture group (Fx), radiated group (XFx) and amifostine-treated group (AMFXFx). * denotes significance at $p < 0.05$ between means.

Figure 4.

Empty Lacunae count per high power field (#/HPF) means and standard deviations for the fracture group (Fx), radiated group (XFx) and amifostine-treated group (AMFXFx). * denotes significance at $p < 0.05$ between means.

Figure 5.

Ratio of non-mineralized, immature bone in the ROI (OV/TV) means and standard deviations for the fracture group (Fx), radiation group (XFx) and amifostine-treated group (AMFXFx). * denotes significance at $p < 0.05$ between means.

Figure 6. Representative image of histologic section (stained with Gomori's trichrome) of the fracture group, depicting the heterogenous mixture of osteoid production (pink) and mature, mineralized bone (blue).

Figure 7. Representative image of histologic section (stained with Gomori's trichrome) of the radiated fracture group, depicting the depletion of osteoid (pink) in comparison to the non-radiated group.

Figure 8. Representative image of histologic sections (stained with Gomori's trichrome) of the amifostine-treated group depicting the preservation of osteocytes and osteoid production (pink) in response to amifostine treatment prior to radiotherapy.