Prior Focal Radiation Causes Atrophic Nonunion Fracture in Mice

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Introduction

Unlike many other tissues that healing with a scar, bone is a unique one that can regenerate completely after injury. However, up to 5-10% of bone fractures have delayed or nonunion healing. Among them, atrophic nonunion is especially challenging for the physician and thus is a major clinical burden in skeletal trauma treatment. Patients treated with radiotherapy (e.g. cancer, heterotopic ossification) are less likely to regenerate bone and more prone to develop fracture nonunion within the irradiated area even after several years of treatment. To better understand the relationship between radiation and fracture nonuion, we investigated the fracture healing process in mouse long bones with prior focal radiation.

Methods

All procedures were approved by our institution's Animal Care and Use Committee.

Animals

Two-month-old male WT (C57BL/6) mice or Col2- Cre Rosa-Tomato mice in a C57BL/6J background received radiation (8 Gy twice, day 1 and day 3) at the midshaft of right tibiae (5 mm in diameter) from a focal irradiator (SARRP, Xstrahl). Two weeks later, closed transverse fractures were made within the irradiated area and the same area in the contralateral legs via a blunt guillotine with a pre-inserted intramedullary pin.

μCT- Bilateral tibiae were harvested at scheduled time points and scanned by vivaCT 40 (Scanco Medical AG) at a resolution of 10.5 μm for measuring callus volume (CV), bone volume (BV), and bone volume fraction (BV/CV).

Histology and Immunohistochemistry (IHC)

Tibiae were fixed in 4% PFA, decalcified in 10% EDTA, and processed for paraffin or frozen sections followed by Safranin- O/fast green staining or IHC. For EdU staining, mice received 1.6 mg/kg EdU at 3 h before sacrifice.

Mechanical testing

Tibiae harvested at 6 weeks after fracture were placed on a 3-point bending fixture and loaded with mechanical force at the previously fractured site using an Instron 5542. The force to failure curve was recorded for analyzing peak load, stiffness, and energy to failure.

Periosteal mesenchymal progenitor isolation

Mice long bones were dissected free of surrounding tissues and digested in 2 mg/mL collagenase A and 2.5 mg/mL trypsin. The first 5 min digested cells were discarded. Periosteal mesenchymal progenitors were released by a subsequent 20 min digest and cultured in 15% αMEM for standard osteogenic and chondrogenic differrentiation.

Statistics

Data are expressed as means±SEM and analyzed by paired, two-tailed Student's t-test.

Results

At two weeks after radiation and right before fracture, bone marrow hematopoietic components in the irradiated region had already recovered but the periosteal cellularity was significantly lower compared to non-irradiated bone. Three days after fracture, compared to that in control, the periosteum layer in irradiated bones expanded much less at the proximal side of fracture, the region close to the growth plate, and did not expand at all at the distal side of fracture, the region close to the ankle (Figure 1A). Consistently, EdU staining indicated less proliferation within the prior irradiated periosteum at the proximal site and almost no proliferation at the distal side compared to non-irradiated bone (Figure 1B). Consequently, at 1 and 2 weeks after fracture, CV and BV were drastically decreased in irradiated bones at the proximal side with virtually no bone detected distal to the fracture line (Figure 2). Histology uncovered that, while the irradiated bones attempted to heal through endochondral and intramembranous ossifications at the proximal side albeit at much less robust level compared to control, only cells with fibrotic morphology and type 1 collagen matrix were detected at the distal side (Figure 3). Those cells did not stain for osteogenic (osterix and osteocalcin) or chondrogenic (Sox9 and type 2 Collagen) markers (data not shown). They did not express VEGF, leading to no vessel infiltration (Figure 4) and no osteoclasts in the area (data not shown). Lineage tracing using Col2-Cre Rosa-Td Tomato

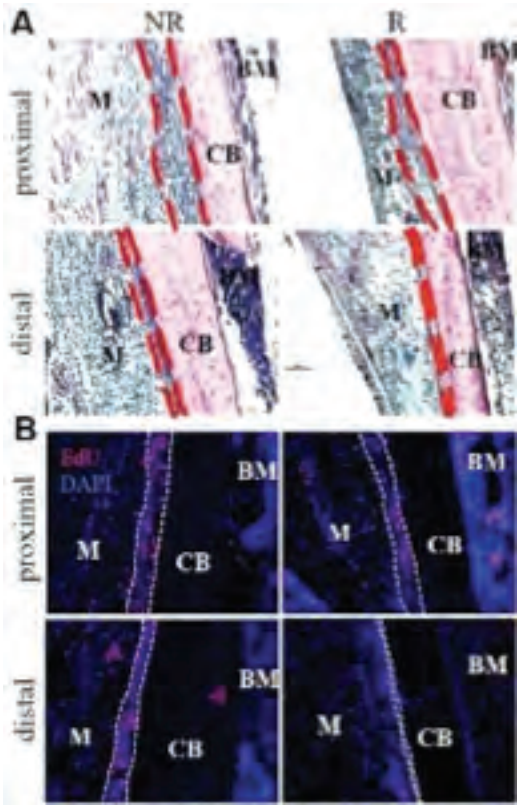

Figure 1. Prior radiation reduces periosteum responses toward fracture. HE **(A)** and EdU **(B)** stainings of fractured bones at 3 days post fracture. Dash lines depict periosteum. NR: non-irradiated; R: irradiated; CB: cortical bone; M: muscle; BM: bone marrow.

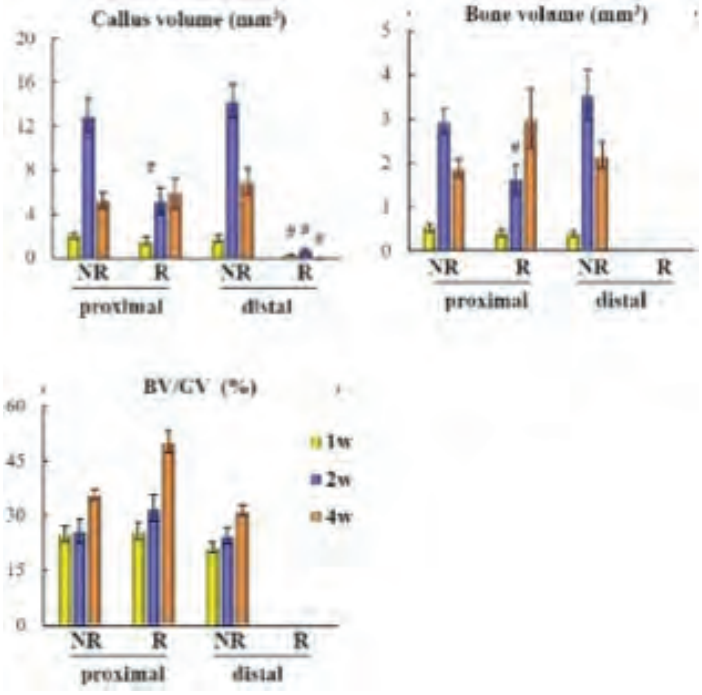

Figure 2. Prior radiation has distinct effects on callus formation at two ends of the fracture line. MicroCT analysis of callus volume (CV) and bone volume (BV) within the callus at 1, 2, and 4 weeks after fracture. n= 6 mice/group. #: $p<0.05$ R vs NR.

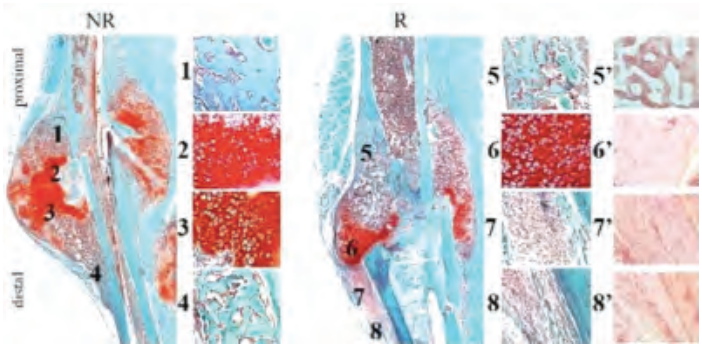

Figure 3. Fibrous tissue (images 7 and 8) is formed at the fracture distal end in prior irradiated bone at 2 weeks after fracture as shown by Safranin-O/fast green staining. Images 1-8 are magnified images for areas shown on the left panel. Images 5'-8' are Picro-Sirius red staining of the same sites as images 5-8.

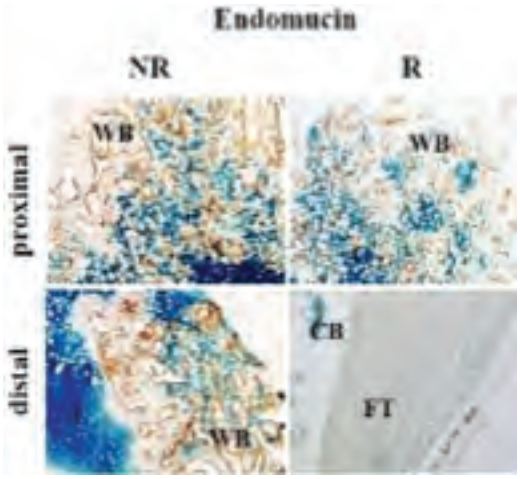

Figure 4. Fibrous tissues formed at 2 weeks after fracture at the distal end in prior irradiated bone are devoid of vessel invasion as shown by IHC of endomucin (a marker for endothelial cells, brown). CB: cortical bone, C: cartilage, WB: woven bone, FT: fibrous tissue.

mice that specifically label bone mesenchymal lineage cells, including periosteal progenitors [1], revealed that these fibrotic cells are not originated from periosteal progenitors (Figure 5). At 4 and 6 weeks after fracture, the bony callus at the proximal side appeared to drape over the fibrous tissue of the distal side but without consolidation (Figure 6, arrows). This resulted in a nonunion in the entire irradiated cohort (n = 11 mice). Mechanical testing confirmed a drastically decreased peak load (−86%), stiffness (−75%), and energy to failure (−73%). Culturing periosteal mesenchymal progenitors under hypoxia conditions (0.1% oxygen) showed that radiation suppresses cell proliferation and inhibits osteogenic differentiation but not chondrogenic differentiation (data not shown).

Discussion

This location-dependent healing in prior irradiated bones demonstrates that both periosteum insult and a lack of surrounding vasculature are critical elements leading to fracture nonunion.This partially explains why fracture healing is difficult to achieve in patients who have been treated with radiotherapy. In our animal model, fibrous tissue instead of

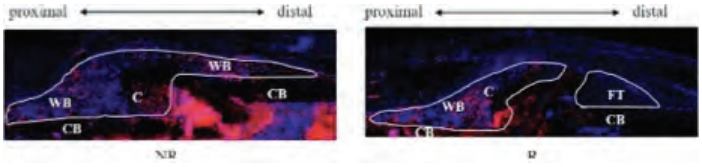

Figure 5. The fibrous tissue at 2 weeks post fracture in prior irradiated Col2-Cre Rosa-Tomato mice contains no Tomato positive cells. CB: cortical bone, C: cartilage, WB: woven bone, FT: fibrous tissue.

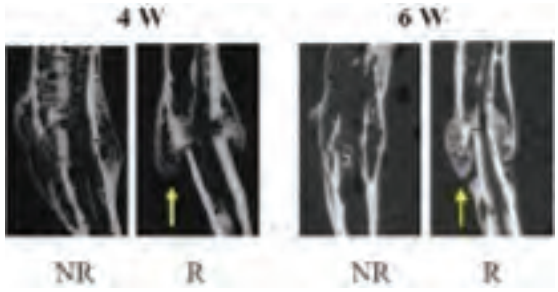

Figure 6. Representative microCT images of nonirradiated and irradiated fracture callus at 4 and 6 weeks post fracture.

bone/cartilage is formed at the distal end of fracture after radiation, and these fibrous cells lack chondrogenic and osteogenic differentiation ability. These changes mimic clinical atrophic nonunion in which primarily fibrous tissue is detected at the fracture ends. Although the origin of this fibrous tissue is currently unknown, our data indicated that they do not come from periosteum. One possible source could be the surrounding muscle resident cells.

Significance

We establish a highly reliable, nonsurgical, and clinically relevant atrophic nonunion fracture model in mice for future investigations that will be relevant to patients undergoing radiotherapy but more broadly for those with atrophic nonunion. Particularly, identifying the source and characteristics of fibrous tissue may pave a way to resolve this clinically challenging disease.

References

1. Chandra, A., *et al.,* *J Bone Miner Res*, 2017. 32(2): p. 360-372.