Osteogenic Response to Damaging Mechanical Loading Is Diminished by Inhibition of Angiogenesis
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Introduction: Angiogenesis, the process by which new vessels extend from the existing vascular system, is strongly related to osteogenesis, the process of new bone tissue formation. In fact, it has been shown that angiogenesis is critical for many osteogenic processes, including fracture healing, distraction osteogenesis, and skeletal development, among others. Repetitive mechanical loading of the skeleton has been shown to be osteogenic, but the role of angiogenesis in this context is not well understood.

In the rat forelimb, a single bout of damaging, cyclic loading reproducibly generates woven bone formation at the ulnar mid-diaphysis. Woven bone formation is associated with upregulation of angiogenic genes (VEGF, PECAM-1), increased vascularity, and increased blood flow rates at the site of bone formation. These results suggest that angiogenesis may be a requirement for woven bone formation after mechanical loading. In this study, we inhibited blood vessel formation following osteogenic mechanical loading using αvβ3-targeted perflurocarbon nanoparticles that contain the anti-angiogenic agent fumagillin.

Methods: Male Fischer F344 rats were obtained at 13-14 weeks of age (Harlan) and housed under standard conditions until 18-22 weeks of age. All rats were mechanically loaded using a protocol that produces an abundant woven bone formation response at the mid-diaphysis of the ulna [1]. After loading, each rat was given up to 5 daily injections of αvβ3-targeted fumagillin nanoparticles (0.2 mg fumagillin/kg i.v.) or saline; the first injection was 1 hour prior to loading.

Rats were anesthetized with isoflurane gas (1-3%) for the loading procedure. A material testing system (Instron Electropuls 1000) was used to apply force to the rat forelimb and monitor displacement. 0.3 N of compressive pre-load was applied followed by a cyclic harmonic waveform of 18 N at 2 Hz until 65% of the displacement to fracture was achieved (1.3 mm, relative to the 10th cycle). The left forelimb was not mechanically loaded (control). Following the procedure, rats were given an intramuscular injection of analgesic (0.05 mg/kg buprenorphine).

Vessel morphology was quantified using an established vascular perfusion technique. After median sternotomy, an 18 gauge catheter was inserted into the aorta through the left ventricle and secured using adhesive. 10 mL of Heparin Lock Flush (100 U/mL) was injected to inhibit clotting, and the animal was euthanized by exsanguination. The vasculature was then irrigated with 100 mL of saline at 37 °C. Finally, 60 mL of silicone rubber (Microfil® MV-122, Flow Tech Inc.) was injected and allowed to cure overnight at 4 °C. After curing, the forelimb was harvested and fixed in formalin.

The central 12 mm of each ulna was imaged using a Scanco µCT 40 at 10 µm resolution (45 kV, 177 µA). Vasculature, newly formed woven bone, and original bone were manually segmented using Scanco imaging software. The length of bone along which new woven bone had formed (woven bone extent) was also quantified. After µCT imaging, ulnae were decalcified (14% EDTA) and embedded in paraffin. 5 µm sections were cut and stained with H&E or antibodies against CD105 (associated with angiogenic vessels) or αSMA (associated with mature blood vessels). Perfused vessels in the expanded periosteum were segmented from surrounding tissue, and vessel count and area were quantified.

All data is presented as mean ± standard deviation. Statistical significance was considered using Student’s t-test of unequal variance, where p < 0.05 was considered significant. All protocols were approved by our institution’s Animal Studies Committee.

Discussion: In summary, we used αvβ3-targeted fumagillin nanoparticles to inhibit angiogenesis after damaging mechanical loading known to produce woven bone formation. Nanoparticle treatment had no effect on vascularity (blood vessel count, area, or volume) on day 3, but fewer angiogenic (CD105 positive) blood vessels were present in treated limbs. Additionally, treatment significantly decreased vascularity 7 days after mechanical loading, and fewer mature vessels (αSMA positive) were present in treated limbs. These findings suggest that, although vessels are angiogenic on day 3, angiogenic inhibition does not affect overall vascularity until later (between 3 and 7 days after loading).

Results: Bone formation in treated (αvβ3-targeted fumagillin nanoparticles) and non-treated (saline) animals was quantified 7 days after mechanical loading (Figure 1). Treated limbs had significantly less woven bone volume (25%) and woven bone extent (32%) compared to non-treated controls. Vascularity was quantified 3 and 7 days after mechanical loading. At day 3, there were no differences between treated and non-treated limbs in vessel count, area, or volume. However, at day 7, vessel count (30% - Figure 2), vessel area (40%), and vessel volume (33%) were significantly less in treated limbs compared to non-treated limbs. Finally, blood vessel maturity was quantified with immunohistochemistry 3 and 7 days after mechanical loading (Figure 3). On day 3, significantly fewer (50%) blood vessels were CD105 positive in treated limbs compared to non-treated limbs. On day 7, significantly fewer (25%) blood vessels were αSMA positive in treated limbs compared to non-treated limbs.

Significance: The role of angiogenesis during mechanical loading represents a crucial area of study since it is required for many other osteogenic processes, including skeletal development, fracture healing, and distraction osteogenesis. In addition, damaging osteogenic mechanical loading is directly analogous to clinical stress fractures, and this research may inform treatment in patients with vascular complications.

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Figure 1 - Woven bone volume and extent are significantly less in limbs treated with nanoparticles (n = 7) compared to controls (n = 8). * p < 0.05 vs Control

Figure 2 - (A) H&E stained cross-section of a loaded ulna, with bone labeled B, muscle labeled M, perfused vessels black, and the expanded periosteum marked with a double-headed arrow. Scale bar is 250 µm. (B) Nanoparticle treatment (n = 7) results in significantly fewer vessels than control (n = 8) 7 days after loading. * p < 0.05 vs Control

Figure 3 - Immunohistochemistry staining for CD105 (A) and αSMA (C) is quantified (B, D) for treated (n = 4), non-treated (n = 4), and non-loaded control limbs (n = 4). Scale bar is 250 µm: a: p < 0.05 vs. non-loaded Control. b: p < 0.05 vs Treated.