The Temporal and Spatial Development of Vascularity in a Healing Displaced Fracture

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Introduction: As fracture angiogenesis is an indispensable process in fracture healing and that the most common shared pathophysiology in comorbid diseases associated with poor fracture healing is vascular disease[1], our overarching hypothesis is that the primary cause of delayed or nonunion is impairment of fracture angiogenesis. Despite incremental advances in our understanding of the bone specific cells and growth factors responsible for the development of new bone during fracture healing, there have been fewer advances in our comprehension of angiogenesis during fracture repair. As a result, our capacity to augment fracture associated vascularity is limited. In an effort to define the key stages of fracture angiogenesis, we adapted advanced vascular imaging techniques with our murine model of displaced/stabilized femur fractures to allow for resolution of the temporal and spatial development of vascularity in healing fractures relative to key biological points of intramembranous and endochondral fracture healing. From these data, in conjunction with classical studies of fracture angiogenesis[2-7], we propose a novel model defining the process of bone revascularization subsequent to injury which should provide insight into means of enhancing fracture healing altogether.

Methods: All animal protocols were reviewed and approved by the IACUC. Mice (n=158) were male and 8 weeks of age C57BL/6 at the time of fracture. Mid-shaft femoral osteotomies were fixed by a 23-gauge needle. Fracture healing was followed with the Faxitron X-ray system. Mice were sacrificed at 7-42 days after surgery and samples were processed for Microfil perfusion, μCT and histology. Mice were positioned supine and the heart was exposed through a midline sternotomy incision and the left ventricle was accessed using a 25- gauge butterfly needle. The Intra vena cava (IVC) was then cut above the liver for drainage, and the vessels were subsequently perfused with 9mL of warm heparinized saline. Exsanguination was complete upon blanching of the liver and clear fluid exiting the IVC. Mice were then perfused with 9mL of 10% neutral buffered formalin. Microfil vascular contrast polymer was then injected via syringe into the vessels until the contrast was clearly visible at the IVC, the liver, and within the tail and ear veins. X-rays of the samples were then taken to visualize the femur and the vascular contrast. The muscles surrounding the femur were then dissected away, and the leg was photographed and X-rayed (Figure 1). The femurs then underwent μCT imaging to visualize the vessels (Figure 1), and a Scanco evaluation script was used for the color-coded visualization of the scan. Vessels were Color-coded based on their diameter. Finally, the 3D image was overlaid onto the non-decalcified x-ray of the femur using Adobe Photoshop (Figure 1). Slides were immunostained for rabbit anti-mouse VEGF using a 1:200 dilution of the primary antibody in the blocking solution. The slides were immunostained for rabbit anti-mouse VEGF-R1 using a 1:100 dilution of the primary antibody in the blocking solution.

Results: Based on our findings (Figure 2), and structured from of the classical literature[2-7], we propose a new model (Figure 3) of the temporal and spatial revascularization of a displaced fractured. In this model, in agreement with Rhinelander and Kelly[5], fractures with significant injury to the intramedullary vasculature (Figure-2 7days) revascularize initially through the development of a trans-periosteal vascular network (Figure-2 10-14days). As demonstrated by Tennef[7], revascularization of displaced fractures initially occurs in the periosteal/subperiosteal space at the periphery of the zone of injury (Figure-2 10-14days). However, we found that this vasculature develops as a result of increased flow diverted toward the periosteal vasculature/subperiosteal space as a result of interruption of downstream medullary vascularity, not from increased flow from muscle; a findings strongly supported by our demonstration of enhanced vascular anastomosis developed between the medullary vasculature and the areas of periosteal vascular engorgement. Following the initial phases of fracture revascularization there exists centrally, an avascular cartilaginous matrix predominated by VEGF-A/VEGFR-1 negative cells surrounded by peripherally, a richly vascular new bone matrix predominated by endothelial cells and osteoblasts expressing high levels of VEGF-A/VEGFR-1 (Figure-1 and 2 10-14days). Our histological data revealed hypertrophic VEGF-A producing chondrocytes in all areas of transition from avascular/soft tissue to vascular hard tissue callus. From this data, it is postulated that a primary role of chondrocytes is, through late expression of VEGF-A, to recruit VEGFR expressing endothelial cells formed in areas of intramembranous angiogenesis/bone formation in an effort to reach vascular and bone union. Hence, these results indicate that the VEGF-A/VEGFR system is an essential component of transition from soft-tissue callus to hard tissue callus resulting in vascular and bone union. As the chondrocytes continue to hypertrophy and release VEGF-A in a manner which directs the polarized bone formation together, the periosteal vasculature and bone eventually unite (Figure-2 21days). Following union our results reveal that angiogenesis and vascular remodeling continue (Figure-2 21-42days). Specifically, we found that medullary vascularity united immediately following vascular union of the periosteum. In addition we observed the development
of many anastomoses between the medullary and periosteal vascular system. Interestingly, upon medullary vascular union and medullary periosteal vascular anastomosis, fracture remodeling ensued (Figure 2 21-42days).

**Discussion:** The data presented here demonstrate that the first step of angiogenesis within the peripheral sub-periosteal space observed in healing of a displaced fracture occurs as a result of vascular shunting from the remaining intact medullary vasculature in a centrifugal pattern. An essential function of intramembranous bone formation is to sustain and expand this shunted vascularity through VEGF/VEGFR thus providing a rich vasculature network required for subsequent invasion of soft tissue callus produced by endochondral ossification. Contrastingly, an essential function of the chondrocytes within the soft tissue callus formed during endochondral ossification is to draw the newly formed vessels at the periphery of the fracture into the avascular environment to the fracture gap ultimately resulting in vascular and bone anastomosis. Further, from these observations we propose that fracture remodeling occurs as a result of the restoration of the normal vascular anatomy of bone. As the medullary vascularity and branches to the cortex re-establishes, the need for shunting through periosteal vascular networks lessens resulting in atrophy of these vessels and surrounding bone. Thus, the temporal and spatial development of fracture vascularity is controlled by the precise orchestration of intramembranous and endochondral processes (Figure 3).

**Significance:** As the most commonly associated diseases associated with fracture delayed or non-union all impose vascular disease. It is proposed that addressing the vascular impairment in these patients will significantly reduce fracture healing complications. As such, it is our hope that this novel model of fracture revascularization of displaced/stabilized fractures will provide insight as to the cause, and potential means to restore, fracture delay or non-union.

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**References:**
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