The temporal and spatial development of vascularity in a healing displaced fracture

Masato Yuasa, Nicholas A. Mignemi, Joey V. Barnett, Justin M.M. Cates, Jeffry S. Nyman, Atsushi Okawa, Toshitaka Yoshi, Herbert S. Schwartz, Christopher M. Stutz, Jonathan G. Schoenecker

A R T I C L E   I N F O

Article history:
Received 17 January 2014
Revised 4 June 2014
Accepted 1 July 2014
Available online 10 July 2014

Edited by: Regis O’Keefe

Keywords:
Bone vascularity
Fracture healing
Angiogenesis
Angiography
Vascular endothelial growth factor
Non-union

A B S T R A C T

Underlying vascular disease is an important pathophysiological factor shared among many co-morbid conditions associated with poor fracture healing, such as diabetes, obesity, and age. Determining the temporal and spatial patterns of revascularization following a fracture is essential for devising therapeutic strategies to augment this critical reparative process. Seminal studies conducted in the last century have investigated the pattern of vascularity in bone following a fracture. The consensus model culminating from these classical studies depicts a combination of angiogenesis emanating from both the intact intramedullary and periosteal vasculature. Subsequent to the plethora of experimental fracture angiography in the early to mid-20th century there has been a paucity of reports describing the pattern of revascularization of a healing fracture. Consequently the classical model of revascularization of a displaced fracture has remained largely unchanged. Here, we have overcome the limitations of animal fracture models performed in the above described classical studies by combining novel techniques of bone angiography and a reproducible murine femur fracture model to demonstrate for the first time the complete temporal and spatial pattern of revascularization in a displaced/stabilized fracture. These studies were designed specifically to: i) validate the classical model of fracture revascularization of a displaced/stabilized fracture, ii) assess the association between intramedullary and periosteal angiogenesis and iii) elucidate the expression of VEGF/VEGF-R in relation to the classical model. From the studies, in conjunction with classic studies of angiogenesis during fracture repair, we propose a novel model (see abstract graphic) that defines the process of bone revascularization subsequent to injury to guide future approaches to enhance fracture healing. This new model validates and advances the classical model by providing evidence that during the process of revascularization of a displaced fracture 1) periosteal angiogenesis occurs in direct communication with the remaining intact intramedullary vasculature as a result of a vascular shunt and 2) vascular union occurs through an intricate interplay between intramembranous and endochondral VEGF/VEGF-R mediated angiogenesis.

© 2014 Elsevier Inc. All rights reserved.

I ntrod uction

Fracture is arguably the most common medical condition treated by orthopedic surgeons, with over 5 million fractures treated annually in the United States alone [1]. Between 2.5 and 10% of these fracture cases are complicated by delayed union or non-union [2–8]. The increasing incidence of comorbid conditions commonly associated with non-union, such as obesity, diabetes, and age suggests that the rate of non-union will only continue to rise [9–13]. In addition to substantial morbidity for patients, these complications impose a significant cost burden on the health care system. Thus, there are considerable ongoing efforts to develop novel methods of fracture fixation and/or application of bone-inducing biological agents to stimulate fracture healing. Given that angiogenesis is a requisite for fracture healing [14–19] and that vascular dysfunction is common to many comorbid conditions associated with poor fracture healing [20–24], it has been hypothesized that a primary cause of delayed union or non-union is...
impaired angiogenesis. A necessary step in developing approaches to augment angiogenesis during fracture repair is the identification of the temporal and spatial patterns of vascularization following an injury.

Seminal studies conducted in the last century have investigated the pattern of vascularity in bone following a fracture. Investigations by Kolodny [25], Teneff [26] and Göthman [27] suggest a model in which the dominant source of fracture revascularization is the periosteum and other extra-osseous vascular sources such as adjacent skeletal muscle. Conversely, Ladanyi and Trueta discovered patterns of revascularization that apparently contradicted this model by demonstrating the essential nature of the intramedullary vasculature in revascularization of a fracture [28,29]. In a series of papers published in the 1960s, this controversy was revisited by Rhinelander who showed that the mechanism of revascularization was dependent on the type of experimental fracture model employed [30,31]. He made a key observation that in non-displaced fractures, the intramedullary vascularity reunited relatively early, without development of a significant periosteal component (so-called primary bone healing through “trans-medullary revascularization”). However, a different pattern of revascularization was observed in fractures with significant displacement, even with subsequent reduction and stabilization. In the displaced fracture model, initial formation of new periosteal vasculature was followed by intramedullary revascularization (so-called secondary bone healing or “trans-periosteal revascularization”). Thus, Rhinelander’s work resolved the apparent discrepancy by showing that different mechanisms of revascularization are employed in different experimental models (displaced vs. non-displaced fractures). The consensus model of revascularization of a displaced fracture culminating from these classical studies depicts a combination of angiogenesis emanating from both the intact intramedullary and periosteal vasculature.

Subsequent to the plethora of experimental fracture angiography in the early to mid-20th century there has been a paucity of reports describing the pattern of revascularization of a healing fracture. Consequently the classical model of revascularization of a displaced fracture has remained largely unchanged. Determination of the molecular elements responsible for and the pattern of revascularization following a fracture are essential for targeting and augmenting discrete events during fracture healing. Importantly, key elements of fracture revascularization were unclear following the development of this model. Specifically, the association of angiogenesis in the intramedullary and periosteal space was unknown. It was hypothesized that fracture angiogenesis in these anatomically distinct compartments occurred independently and only formed an anastomosis in the later stages of fracture healing [31]. It was proposed that intramedullary angiogenesis occurred as a result of direct vascular growth from the remaining intact intramedullary vascularity and periosteal angiogenesis occurred as a result of direct vascular growth from the surrounding musculature. Additionally, the factors directing vascular union were unknown as vascular endothelial growth factor (VEGF) and its receptors (VEGF-R) had yet to be discovered [32]. Thus, the molecular patterning leading to vascular union has not been incorporated into this classical model. In addition to these unanswered questions, the principle reason for the lack of advancement in this field has been critical methodological restraints prohibiting high throughput animal studies on fracture healing [25–26,30–33]. However, a different pattern of revascularization was observed following the development of this model. Consequently the classical model of revascularization of a displaced fracture 1) periosteal angiogenesis occurs in direct communication with the remaining intact intramedullary vasculature as a result of a vascular shunt and 2) vascular union occurs through an intricate interplay between intramembranous and endochondral VEGF/VEGF-R mediated angiogenesis.

Materials & methods

Murine fracture model and X-ray imaging

Protocols were approved by the Vanderbilt University IACUC. Open femur osteotomies were performed and fixed as previously described using a medial approach to the mid-shaft femur of 8 week old c57/6b mice. The fracture was fixed through the placement of a 23-G (0.6414 mm) retrograde intramedullary pin and the mice were examined from 7 to 42 days after fracture [44]. X-ray was performed as previously described [45]. Briefly, the mice were placed in the prone position and imaged for 4 s at 45 kV using a Faxitron LX 60. The mice were sacrificed at various time points (7–42 days) following a fracture.

Angiography

Perfusion with Microfil (MW-122 Flow Tech Inc., Carver, MA) vascular contrast was conducted as previously described [46–49]. Briefly, the mice were euthanized, positioned supine and a thoracotomy extending into a laparotomy was performed. The left ventricle of the heart was cannulated using a 25-G butterfly needle. The inferior vena cava (IVC) was transected proximal to the liver and the entire vasculature subsequently perfused with 9 ml of warm heparinized saline (100 units/ml in 0.9% saline) through the left ventricle cannula to exsanguinate and anticoagulate thus preventing erythrocyte aggregation and thrombosis thereby promoting consistent perfusion of subsequent infusion materials. Exsanguination and anticoagulation was deemed complete upon widespread hepatic blanching with clear fluid extravasating from the IVC. The mice were then perfused with 9 ml of 10% neutral buffered formalin followed by 3 ml of Microfil (Flow Tech Inc. Carver, Massachusetts) vascular contrast polymer. To best verify complete filling of the vascular contrast was conducted as previously described [45]. Briefly, the mice were placed in the prone position and imaged for 4 s at 45 kV using a Faxitron LX 60. The mice were excluded from the study if complete hepatic blanching prior to Microfil was not achieved, if contrast was not clearly or uniformly visible in the hepatic vasculature or if extra-vascular pooling occurred. Inspection of hepatic perfusion revealed no differences between manual and infusion pump perfusion set at a constant rate of 0.5 ml/min (Supplemental Fig. 2a). Manual filling was conducted at a goal rate approximately equal to the pump rate.

Generation of angiogram images

The mice perfused with Microfil were then stored overnight at 4 °C to allow the vascular contrast to polymerize. The femurs were dissected and fixed in 4% PFA for another 24 h. X-rays of the samples were then taken to visualize the femur and the vascular contrast (Supplemental Fig. 1b). The femur was placed in 0.5 M EDTA pH 8 for decalcification then imaged via X-ray (Supplemental Fig. 1c). The muscles surrounding the femur were removed, and the leg was photographed and X-rayed (Figs. 1d & e). Demineralized specimens were imaged by μCT (μCT40, Scanco-Medical-AG, Bassersdorf, Switzerland) with a 20 μm isotropic
voxel size. A Scanco-Medical evaluation script was applied to render the vasculature with colors corresponding to vessel diameter between 0 and 0.18 mm. These vascular images were integrated into the initial X-rays by matching size and orientation using Adobe Photoshop (San Jose, CA) (Supplemental Figs. 1f–h). Quantitation of liver vasculature also demonstrates no difference in vessel volume, number, or branching (Supplemental Fig. 2b) which in combination our gross observations indicates consistent filling of the vessels with angiogram contrast.

Histology

Histologic sections prepared from PFA-fixed, demineralized in 0.5 M EDTA (pH 7.2) and paraffin-embedded specimens were cut on to slides in 5 μm sections. The slides were then deparaffinized and hydrated for use in immunohistochemistry or histological staining (Safranin-O stain). The sections were stained with Safranin-O/Fast green (Safranin-O) as previously described[46]. Briefly the slides were placed in Weigert’s hematoxylin for 10 min, washed in running tap water and then stained with 0.1% fast green solution for 5 min. The slides were then rinsed for 5 s in 1% acetic acid and placed in 0.1% safranin and orange solution for 5 min. The slides were then dehydrated and coverslip for light microscopy.

Immunofluorescence microscopy

After, the slides were deparaffinized and hydrated. Sodium citrate antigen retrieval was performed using 0.1 M citric acid and 0.1 M sodium citrate. The slides were heated for 2 min in the microwave, cooled to room temperature and then washed gently with Tris buffer saline (TBS). The slides were blocked (5% BSA solution containing 10% goat serum) and immunostained with anti-mouse VEGF-A (1:200, Abcam 46154, Cambridge, MA), VEGFR2 goat anti-mouse (1:50, R&D systems AF644) or an rabbit anti-mouse VEGFR1 (1:100, Abcam 32152) antibody overnight at 4 °C. The slides were then washed and incubated with 10 μg/ml of Alexa Fluor 647-labeled anti-rabbit antibody (Life Technologies 792514, Grand Island, NY) in blocking buffer for 1 h. The slides were counterstained with DAPI and cover slipped using mounting solution (PolySciences Warrington, PA) and fluorescent images were taken (NIKON AZ100, Upright wide field microscope). The slides incubated without primary antibodies served as negative controls.

Quantification of soft and hard tissue callus size

Soft tissue callus delineated by safranin-O red staining was traced on 5 histological step-sections 200 μm apart as previously described with the following changes [50]. To determine the orientation of the 5 slides, the edge of the callus was visualized by identifying the first section with callus on both the medial and lateral femoral cortex and the middle of the callus was determined by the pin space. Each of the specimens’ 5 sections were measured by 4 blinded reviewers and results were then expressed as mm² the soft tissue callus area. Hard tissue callus size was measured from radiographs of fractured femurs as previously described [45]. Briefly the mice were placed in the prone position and an X-ray was taken. The total area of the mineralized callus was determined each week by tracing the area of the fracture callus on X-rays by 2 reviewers and results were then expressed as mm² the hard tissue callus. Image quantification for both the soft and hard tissue calluses was performed using the software program ImageJ (NIH, http://rsb.info.nih.gov/ij/). Trend lines were generated between the data points using a spline curve and GraphPad prism.

Fig. 1. Fracture and/or intramedullary reaming produce a segmental avascular diaphyseal segment. (a) Femoral angiography reveals larger unbranched vasculature within the diaphysis and smaller branched vasculature within the proximal and distal metaphysis. (b) Immediately following a fracture (yellow arrow) angiography demonstrates a segmental diaphyseal avascular segment approximately 1 mm proximal and distal to the injury without disruption of the metaphyseal vasculature. (c) Reaming and pin placement alone and (d) fracture with reaming and pin placement produce a similar diaphyseal avascular segment without disruption of the metaphyseal vasculature. (e) Two-dimensional slices from μCT demonstrate that the intramedullary pin does not prevent diaphyseal intramedullary vasculature (red arrows). Vessel diameter demarcated by color (0 and 0.18 mm) in all figures.
Quantification of vasculature

Vessel volume was determined by μCT on the fracture callus of the demineralized femurs perfused with angiogram contrast. A cylinder comprised of 250 slices with a 20 μm isotropic voxel size was placed over the fracture callus to define the volume of interest. A threshold of 122 (lower) and 1000 (upper) was applied for segmentation with Gauss sigma of 0.8 and a gauss support of 2 to reduce the noise. Vessel volume (TRI BV) was then evaluated using Scanco μCT standard algorithms and graphed. Spline trend lines were generated between the data points using a spline curve function in GraphPad Prism 6.

Sources of funding

Caitlin Lovejoy Fund, NIH (S10 RR027631), and the Musculoskeletal Transplantation Foundation.

Results

Fracture and/or reaming disrupt diaphyseal intramedullary vascularity

Consistent with previous reports [51], the vasculature of the femoral mid-diaphysis is composed primarily of larger intramedullary vessels.
with few branching points. In contrast, the proximal and distal metaphysis is composed of multiple intramedullary vessels significantly smaller and with many more branching points than the diaphysis (Fig. 1a). Upon fracture the diaphyseal vascularity is disrupted at the fracture site whereas the proximal and distal metaphyseal vascularity remain intact resulting in a segmental loss of intramedullary vascularity approximately 1 mm proximal and distal to the insult (Fig. 1b) without significant disruption of metaphyseal vascularity. A similar pattern of metaphyseal sparing segmental diaphyseal avascularity occurs following an intramedullary reaming and pin stabilization without (Fig. 1c) or with (Fig. 1d) fracture. μCT angiography confirms that the 23-G intramedullary pin utilized to stable the fracture permits revascularization through the intramedullary compartment of the diaphysis (Fig. 1e).

### Table 1: Vascular Development Relative to Soft Tissue and Hard Tissue Callus

<table>
<thead>
<tr>
<th>No Fracture</th>
<th>7 DPF</th>
<th>10 DPF</th>
<th>14 DPF</th>
<th>21 DPF</th>
<th>28 DPF</th>
<th>35 DPF</th>
<th>42 DPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soft Tissue Callus (Safranin-O)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hard Tissue Callus (X-ray)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascularity (Angiogram)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2 (continued).

Vascular development relative to soft tissue and hard tissue callus in a displaced fracture

Safranin-O-staining, radiographs, and angiograms of fractured femurs elucidates the temporal and spatial development of the fracture callus and associated vasculature (Fig. 2a). Seven days post-fracture (7-DPF), the diaphyseal intramedullary vasculature remains disrupted, resulting in an avascular femoral segment flanked proximally and distally by intact intramedullary vasculature. Radiographic and histopathologic examination shows formation of a cartilaginous soft tissue callus without evidence of osteoid formation within this avascular zone. The soft tissue callus rapidly enlarges to its maximal size by 10-DPF. Simultaneously, hard tissue callus is initially formed via intramembranous ossification at the extreme proximal and distal aspects.
of the fracture site, where the periosteum inserts on unaffected adjacent cortical bone. This process occurs in conjunction with the formation of small highly branching extramedullary vessels (10-DPF). As hard tissue callus replaces soft tissue callus (14-DPF), it is accompanied by an expansion of newly formed vasculature. Spatially, the regions of vascular expansion begin at the proximal and distal aspects of the fracture site and migrate centrally toward the soft tissue callus. Vascular ingrowth continues until anastomoses are developed, coinciding with complete dissolution of soft tissue callus and formation of bridging hard tissue callus (21-DPF). Following a vascular anastomosis and bridging of hard callus across the fracture site, the fracture callus remodels back to within the original cortices coinciding with the vasculature returning to larger vessels with reduced branching (28–42-DPF). Higher power views of the fracture callus are presented in Fig. 2b.

Quantitation of angiogenesis relative to soft tissue and hard tissue callus in a displaced fracture

Relative to hard tissue callus and vascularity, the soft tissue callus is the fastest developing matrix following a fracture, reaching its maximum size by 10-DPF and decreasing in size until it is undetectable at 28-DPF (Fig. 3a). Hard tissue callus develops later than the soft-tissue callus, reaching its maximum size between 14 and 21-DPF, and then steadily returns to its initial size by 42-DPF (Fig. 3b). In accordance with our findings that fracture and insertion of an intramedullary device (Fig. 1) produce an avascular segment of diaphyseal bone around the fracture site, the average vessel volume initially decreases immediately following a fracture (Fig. 3c). At the point of maximum soft tissue callus (hashed line) vessel volume begins to increases reaching maximum volume at the same time as maximum hard tissue callus (gray zone). Vessel volume subsequently decreases approaching, but not reaching, the pre-fracture vessel volume by 42-DPF. Data used for generation of the graphs are presented in Table 1.

Newly formed subperiosteal vasculature arises from intact intramedullary vasculature

High power magnification of angiograms reveal that by 10-DPF newly formed extramedullary vasculature arises from intact intramedullary blood vessels proximal and distal to the fracture site (Fig. 4a). These intramedullary–extramedullary vascular anastomoses are also demonstrated by 2D reconstruction of μCT images and close macroscopic inspection of the cut surfaces of contrast-injected and decalcified femur fracture specimens. These data demonstrate multiple communicating vessels apparently arising from intramedullary vasculature, traversing the cortical bone and branching into numerous small vessels supplying the newly formed hard tissue callus in the subperiosteal space (Figs. 4b–f). Such anastomoses were only observed at the proximal and distal ends of the fracture callus, indicating that they were not native nutrient vessels of the femur. Axial 2D reconstructions confirm that the 23-G pin used to stabilize the fracture did not completely occlude or completely disrupt the intramedullary vasculature.

Fracture angiogenesis co-localizes with the hard tissue callus and invades the soft tissue callus

Multiple lines of evidence illustrate that the extramedullary vasculature is associated with developing hard tissue callus and resorption of soft tissue callus. Sagittal sectioning of vascular contrast-filled fracture callus (10-DPF) reveals robust development of extramedullary vasculature in hard tissue callus at the proximal and distal aspects of the

---

**Table 1**

<table>
<thead>
<tr>
<th>Days post fracture</th>
<th>Soft tissue callus</th>
<th>Hard tissue callus</th>
<th>Vessel volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>N</td>
</tr>
<tr>
<td>Intact femur</td>
<td>4.14</td>
<td>1.70</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>4.84</td>
<td>0.96</td>
<td>7</td>
</tr>
<tr>
<td>10</td>
<td>2.87</td>
<td>1.61</td>
<td>11</td>
</tr>
<tr>
<td>21</td>
<td>0.37</td>
<td>0.33</td>
<td>11</td>
</tr>
<tr>
<td>28</td>
<td>0.00</td>
<td>0.00</td>
<td>11</td>
</tr>
<tr>
<td>35</td>
<td>0.00</td>
<td>0.00</td>
<td>11</td>
</tr>
<tr>
<td>42</td>
<td>0.00</td>
<td>0.00</td>
<td>11</td>
</tr>
</tbody>
</table>

N = number of samples, SD = standard deviation.

---

Fig. 3. Quantitation of vascular development relative to soft tissue and hard tissue callus in a displaced fracture. (a) Quantification of the soft tissue callus identified by Safranin-O staining reveals significant development of cartilage at 7–14-DPF relative to 42-DPF with maximum area at 10-DPF (hashed line). Soft tissue callus was last detected at 21-DPF. (b) Quantification of hard tissue callus identified by X-ray reveals the first significant new bone development which occurs at 10-DPF with maximum size between 14 and 21-DPF (gray zone). Hard tissue callus size subsequently remodels, decreasing in size, to its original pre-fracture size by 42-DPF. (c) Quantification of vessel volume identified by μCT of decalcified angiograms reveals an initial loss of vascularity with subsequent increase initiating at the time of maximum soft tissue callus and reaching maximum vessel volume between 14 and 21-DPF. Vessel volume then reduces from 21 to 42-DPF near the pre-fracture diaphyseal vessel volume. All samples were evaluated from 7 to 42 days following a fracture and trend lines were generated using a spline curve fit. Dotted line denotes the maximum soft tissue callus size and gray zone denotes the maximum hard tissue callus size. Statistical analyses of soft tissue callus, hard tissue callus, and vessel volume were performed using non-parametric ANOVA with a Dunn’s multiple comparison test. Significance (p value < 0.1) is denoted by asterisk and all error bars represent standard error of the mean. For statistical analysis of the soft tissue callus all values were compared to 21-DPF. For statistical analysis of the hard tissue callus all values were compared to 42-DPF. For statistical analysis of vessel volume all values were compared to un-fractured femora. See Table 1 for more details.
fracture callus (Figs. 5a, b) in contrast to the observed avascular fracture callus 7-DPF (data not shown). Histological examination shows a predominance of vascularity at the leading edge of the invading hard tissue callus as well as throughout the hard tissue callus. In agreement with macroscopic dissections and 2D-μCT reconstructions, no vessels were found within the avascular cartilaginous soft tissue callus (Fig. 5c). These findings were also observed 14-DPF however the soft tissue callus was greatly diminished in size while the hard tissue callus was much more predominate (data not shown).

Fracture callus (Figs. 5a, b) in contrast to the observed avascular fracture callus 7-DPF (data not shown). Histological examination shows a predominance of vascularity at the leading edge of the invading hard tissue callus in the transition zone between the hard and soft tissue calluses as well as throughout the hard tissue callus. In agreement with macroscopic dissections and 2D-μCT reconstructions, no vessels were found within the avascular cartilaginous soft tissue callus (Fig. 5c). These findings were also observed 14-DPF however the soft tissue callus was greatly diminished in size while the hard tissue callus was much more predominate (data not shown).

Spatial patterns of vascular endothelial growth factor and its type 1 and 2 receptors during fracture healing

Vascular endothelial growth factor-A (VEGF-A) and vascular endothelial growth factor receptor 1 (VEGFR1) and 2 (VEGFR2) expressions were investigated by immunofluorescence microscopy at distinct foci along the periosteal surface 10-DPF (Figs. 6a–b). Compared to unreactive normal periosteum, the immunoreactivity of VEGF-A staining was markedly increased along the periosteum at its junction with uninvolved cortical bone, which was also the site of initial intramembranous ossification.
during formation of the hard tissue callus (Fig. 6c, boxes 1 & 4). These areas align anatomically with the sites at which the intramedullary vasculature is disrupted (compare with Fig. 3). In contrast, an increase in immunoreactivity of VEGF-A staining was not observed in the periosteum overlying the soft tissue callus and disrupted intramedullary vasculature in the central region of fracture callus (Fig. 6c, boxes 2 & 3).

Evaluations of multiple anatomic locations representing distinct biological activities during fracture healing demonstrate the coordinated spatial expression of VEGF-A, VEGFR1 and VEGFR2 in the subperiosteal and extramedullary fracture callus 10-DPF (Figs. 7 & 8a). VEGFR1 & 2-positive cells are seen only in areas of intramembranous ossification or in hard tissue callus along the interface with soft tissue callus. These VEGFR1 & 2-positive non-chondrocytic cells are in close proximity to VEGF-A sources, either the periosteum at the proximal and distal aspects of the fracture callus or at the interface with soft tissue callus (Figs. 7 & 8b, box 1). Additionally, we observed that immature chondrocytes located deep in the soft tissue callus, and superficial to vascular deficient intramedullary bone did not express either VEGF-A, VEGFR1 or VEGFR2 (Figs. 7 & 8b, box 2). However, neighboring hypertrophic chondrocytes as well as chondrocytes situated at the transition between soft tissue and hard tissue callus expressed VEGF-A but not VEGFR1 or 2 (Figs. 7 & 8b, box 3). Juxtaposed to hypertrophic chondrocytes in the zone of transition, nondescript mesenchymal cells of the hard tissue callus invading the soft tissue callus were found to demonstrate immunoreactivity for VEGF-A, VEGFR1 and VEGFR2, demonstrating the dichotomous nature of the VEGFR-positive vascularized hard tissue callus from the avascular VEGFR1 & VEGFR2-negative soft tissue callus (Figs. 7 & 8b, box 4).

**Discussion**

The indispensable role of angiogenesis in fracture repair has been recognized for over a century [14,25,26]. However, elements of the temporal and spatial patterns of fracture revascularization have remained elusive. Employing a high-throughout, cost-efficient model of a displaced/stabilized fracture model with advanced imaging techniques; our data clearly supports the unifying theory of revascularization of a displaced fracture requiring both intramedullary and periosteal angiogenesis. These findings provide essential data needed to expand upon the current model of fracture revascularization developed by the pioneers of this field during the early to mid-20th century (graphical abstract, Fig. 9).

A critical aspect of fracture revascularization unanswered by the classical reports in this field was the association of intramedullary and periosteal angiogenesis. As stated above, it was thought that angiogenesis occurred in these anatomically distinct compartments independently. Trueta and Rhinelander demonstrated that angiogenesis initiated in the
intramedullary space directly from the remaining intact vasculature [28, 30, 31]. Less was known regarding the originating source of the periosteal vasculature and was conventionally believed to originate from the surrounding musculature as succinctly summarized by Kelly [31] in 1968:

... if the fracture is produced violently or is stabilized by intramedullary fixation, the needed new vessels originate outside the bone and the muscle vessels supply the periosteal system with these necessary new vessels.

To the contrary, our results suggest that intramedullary and periosteal angiogenesis occur in tandem, not independently. In support of this conclusion, our model is the first to demonstrate a reproducible disruption of the diaphyseal intramedullary vasculature following a fracture and or intramedullary nailing. We further show that the anatomic site of intramedullary vascular disruption corresponds to the region of initial periosteal/subperiosteal intramembranous ossification and revascularization. In 1960, Brookes showed that blood flows in a centrifugal manner from intramedullary vessels to the periosteal

Fig. 6. VEGF-A is primarily expressed in periosteum overlying intramembranous bone formation in hard tissue callus. (a, b) Radiograph and Safranin-O-stained section of a 10 days post fracture (10-DFP) transverse femur fracture and its fracture callus (yellow dashed line). VEGF-A staining was examined at various microanatomic periosteal sites, such as the area of initial intramembranous ossification (box 1), areas overlying the soft chondroid callus (boxes 2 and 3), and an area of unreactive normal periosteum (box 4). (c) Immunofluorescence demonstrates increased immunoreactivity of VEGF-A (red) in the periosteum (asterisk) overlying the area of hard callus formation (box 1) compared to minimal levels of immunoreactivity seen in the periosteum overlying the soft callus (boxes 2 and 3) and normal unreactive periosteum on intact cortical bone (box 4). Scale bars: 40 μm.
vasculature through cortical anastomoses. Thus, it is plausible that periosteal elevation results not only from traumatic hemorrhage and hematoma formation, but also from shunting the interrupted intramedullary blood flow to the regional periosteal vasculature. This theory is strongly supported by our application of novel, state-of-the-art molecular and imaging approaches which revealed enhanced vascular anastomosis between the intramedullary vasculature and the areas of periosteal vascular engorgement during the earlier phases of fracture healing. Although these findings do not disprove the conventional hypothesis that periosteal revascularization occurs via ingrowth of vessels from the surrounding soft tissues, it does provide the first evidence that periosteal vasculature is directly connected to the intact intramedullary vasculature. Thus, we posit that shunting blood flow from the intact intramedullary vasculature to the subperiosteal space adjacent to the fracture site is an initial step displaced fracture revascularization in our model.

Formation of new bone during healing of a displaced fracture is achieved through both intramembranous and endochondral ossification. During intramembranous ossification, new bone is produced by direct extension of osteoblasts and endothelial cells. Previous investigations have demonstrated that this process is tightly regulated by chemotactic and proliferative responses of endothelial cells and osteoblasts to VEGF-A through VEGF receptors including VEGFR1. In our studies, we found that the initial site of intramembranous ossification occurs at the periosteal region with the most robust expression of VEGF-A and VEGFR1-positive cells morphologically consistent with osteoblasts and endothelial cells. Based on these observations, we propose the following revisions to the current model of fracture repair: 1) the initial site of intramembranous ossification and revascularization occurs at the proximal and distal aspects of the fracture site, where periosteum inserts on adjacent uninvolved cortical bone because this is the

**Fig. 7.** Differential expressions of VEGF-A and VEGFR1 in the subperiosteal space in fracture callus. (a) Safranin-O staining of a fracture callus 10 days post fracture (10-DFP) shows a zone of intramembranous bone formation (box 1), a zone of primitive mesenchymal-appearing cells in the soft tissue callus (box 2), and an area of mature hyaline-type cartilage in the soft tissue callus (box 3). The interface between the soft tissue callus and the hard tissue callus is denoted in box 4. (b) High power photomicrographs of the 4 regions of interest stained with Safranin-O and immunofluorescence for VEGF-A (red) or VEGFR1 (yellow). (box 1) Hard tissue callus consisting of intramembranous ossification shows abundant immunoreactivity of VEGF-A primarily in the overlying peristeum and numerous VEGFR1-positive cells within the sub-periosteal space. (box 2) Safranin-O staining shows a cellular zone of primitive spindle cells without evidence of matrix deposition, in which VEGF-A or VEGFR1 is not detectable. (box 3) VEGF-A appears to be limited to occasional enlarged, hypertrophic chondrocytes within this area of hyaline cartilage. No VEGFR1 immunostaining is observed. (box 4) Abundant VEGF-A immunoreactivity is present in the stromal at the interface of the soft and hard tissue calluses, in addition to hypertrophic chondrocytes at the periphery of the avascular soft tissue callus. VEGFR1-positive cells are also present within the hard tissue callus at the transition zone. Scale bars: 20 μm.
site where intramedullary blood flow is shunted to the periosteum.

2) Although the initiating signal for VEGF release is not clear, the immunolocalization of VEGF-A in the periosteum at this site is likely instrumental for the recruitment and/or activation of VEGFR1 & 2-positive mesenchymal cells, endothelial cells, and osteoblasts to the subperiosteal space to support intramembranous angiogenesis and bone formation.

In contrast to intramembranous ossification, bone is formed on a pre-existing cartilaginous template during endochondral ossification [59]. In the central area of the fracture callus that is far from the disrupted regional vasculature, the initial mesenchymal cell proliferation differentiates along the chondrocyte lineage, likely due to the low oxygen tension in this avascular environment. This is supported by our angiographic and histologic data showing that the soft tissue callus is composed of avascular hyaline cartilage and primitive fibrocartilage. In addition, there was minimal detectable periosteal VEGF-A in this location and no VEGF-A positive cells were seen within the central soft callus. Thus, during the initial phases of fracture healing the central, avascular cartilaginous soft tissue callus abuts areas of richly vascular intramembranous ossification composed of VEGFR1-positive osteoblasts and endothelial cells, thereby recapitulating the primary spongiosum of the physis. Therefore, we propose that the fundamental event during fracture repair determining whether bone heals through a vascular intramembranous or an avascular endochondral pathway is its proximity to intact intramedullary vascular system and the presence of VEGFR1-positive cells.

In the active physis, expression of VEGF-A by hypertrophic chondrocytes recruits VEGFR1-positive endothelial cells and osteoblasts

---

**Fig. 8.** Differential expressions of VEGF-A and VEGFR2 in the subperiosteal space in fracture callus. (a) Safranin-O staining of a fracture callus 10 days post fracture (10-DPF) shows a zone of intramembranous bone formation (box 1), a zone of primitive mesenchymal-appearing cells in the soft tissue callus (box 2), and an area of mature hyaline-type cartilage in the soft tissue callus (box 3). The interface between the soft tissue callus and the hard tissue callus is denoted in box 4. (b) High power photomicrographs of the 4 regions of interest stained with Safranin-O and immunofluorescence for VEGF-A (red) or VEGFR2 (green). (box 1) Hard callus consisting of intramembranous ossification shows abundant VEGF-A immunoreactivity primarily in the overlying periosteum and numerous VEGFR2-positive cells within the sub-periosteal space. (box 2) Safranin-O staining shows a cellular zone of primitive spindle cells without evidence of matrix deposition, in which VEGF-A or VEGFR2 is not detectable. (box 3) VEGF-A immunoreactivity appears to be limited to enlarged, hypertrophic chondrocytes within this area of hyaline cartilage. No VEGFR2 immunostaining is observed. (box 4) Abundant VEGF-A immunoreactivity is present in the stromal at the interface of the soft and hard tissue calluses, in addition to hypertrophic chondrocytes at the periphery of the avascular soft tissue callus. VEGFR2-positive cells are also present within the hard tissue callus at the transition zone. Scale bars: 20 μm.
to the zone of hypertrophy, where osteoid deposition on the cartilage matrix results in formation of the primary bone spongiosum. Indeed, the interface between soft and hard tissue calluses in a fracture site closely resembles the primary spongiosa of the physi[s 17,18,59–61]. In support of this, we have shown that hypertrophic VEGF-A-positive chondrocytes are present in areas of transition from avascular soft tissue callus to vascularized hard tissue callus, which are rich in VEGFR-positive cells. The essential nature of VEGF-A during fracture repair is supported by evidence that inhibiting VEGFR1 & 2 or VEGF-A impairs ossification and results in a persistent avascular soft tissue callus composed of hypertrophic chondrocytes [15,19,62]. Hence, these results indicate that 1) the VEGF-A/VEGFR system is an essential component of the transition from soft-tissue callus to hard tissue callus resulting in vascular and bone union and 2) chondrocyte hypertrophy and release of VEGF-A directs polarized bone formation through the recruitment of the vasculature and mesenchymal osteoprogenitor cells at the periphery of callus.

Our results further suggest that after union of the fracture, angiogenesis and vascular remodeling continue. Specifically, we found that fracture remodeling begins around the time at which the intramedullary vasculature is re-established. From these observations we hypothesize that fracture remodeling occurs as a result of the restoration of intramedullary vascular continuity and as normal intramedullary blood flow is re-established, periosteal shunting is reduced and the periosteal callus becomes relatively hypo-perfused. In support of this theory, Rhinelander observed that when fracture fragments are approximated such that the intramedullary vasculature directly communicates with periosteal vessels, and does not occur until re-establishment of intramedullary blood flow [31,36].

In conclusion, we have developed a model that indicates that there is an intricate interplay between intramembranous and endochondral ossification during fracture healing in young growing male mice. Periosteal intramembranous bone formation is critical in providing a richly vascularized network of osteoblastic potential that is necessary for subsequent invasion and replacement of soft tissue callus. Hypertrophic chondrocytes releasing VEGF-A in the avascular soft tissue callus seem to be required for recruiting the developing vascular network and osteoblastic mesenchymal cells to replace the soft tissue callus and form an ossified callus across the fracture site. By quantification of the phases of fracture healing over time (Fig. 3 & Table 1), we discovered that fracture revascularization is inversely proportional to the size of the soft tissue callus and directly proportional to the size of the hard tissue callus. Thus, the temporal and spatial development of fracture vascularity is controlled by the precise orchestration of intramembranous and endochondral processes.

The diseases most commonly associated with delayed fracture healing or fracture non-union all share a component of vascular dysfunction. Given this association, several investigations have proposed that addressing vascular dysfunction may significantly reduce fracture healing complications in certain patient populations [63–67]. This model of revascularization during healing of displaced/stabilized fractures identifies the specific temporal and spatial events that will provide future insight as to the causes of and potential means to prevent and treat delayed fracture healing and non-union. Additionally, while other investigations have revealed vasculature emanating from muscle surrounding a fracture [52,68], our model clearly demonstrate communication between the intramedullary vasculature and the fracture callus. Future studies are required to determine the relative significance of the contribution of intramedullary as opposed to muscle derived angiogenesis on fracture revascularization. Additionally, future studies designed to determine if the results regarding the temporal and spatial revascularization of a healing fracture are altered by the mode of fracture or in conditions known to affect bone and vascular biology such as age, sex, and chronic medical conditions such as diabetes or obesity. If so, these findings may provide insight into novel surgical and medical treatments intended to restore the normal process of bone revascularization following a fracture.
Supplementary data to this article can be found online at dx.doi.org/10.1016/j.bone.2014.07.002.

Acknowledgments

The authors would like to thank Craig Duvall for teaching the angiography technique used in this manuscript and David A. Haynes, Cesar Molina-Andrade, Adam Gailani, Lynda O'Rear and Marissa Mencio for their assistance with performing, imaging and attending to the mice utilized in this study. The authors would also like to thank Perry Schoenecker, Gregory Mencio, William Obremskey, Kevin O’Neill, Megan Mignemi, Richard Jacobson and Melanie Phillips for their critical review of this manuscript. This work was presented in part at the 2013 Annual Salter Lecture in Toronto, Canada, Emyry Orthopae&cacute;tics Grand Rounds, Atlanta, Georgia and the 2014 POSNA annual meeting.

References


