REVIEW

Skeletal Blood Flow in Bone Repair and Maintenance

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Bone is a highly vascularized tissue, although this aspect of bone is often overlooked. In this article, the importance of blood flow in bone repair and regeneration will be reviewed. First, the skeletal vascular anatomy, with an emphasis on long bones, the distinct mechanisms for vascularizing bone tissue, and methods for remodeling existing vasculature are discussed. Next, techniques for quantifying bone blood flow are briefly summarized. Finally, the body of experimental work that demonstrates the role of bone blood flow in fracture healing, distraction osteogenesis, osteoporosis, disuse osteopenia, and bone grafting is examined. These results illustrate that adequate bone blood flow is an important clinical consideration, particularly during bone regeneration and in at-risk patient groups.
Keywords: blood flow; angiogenesis; vascular remodeling; fracture; bone repair

Introduction

All biological tissues, including bone, require vascular support to survive. However, a frequently overlooked feature of bone is its extensive vascular network. Many studies have demonstrated that the blood vessels in bone are necessary for nearly all skeletal functions, including development, homeostasis, and repair (1). In addition, blood vessels lost due to trauma are regenerated, and new bone tissue formed in response to injury is vascularized. As a consequence of this environment, the blood vessels in bone are highly active, not simply a passive source for the delivery of nutrients (2). Readers are referred to recent publications for more information about the active processes of the vasculature not covered in this review, including the vascular niche and hypoxia-driven signaling pathways (3-4).

The rate at which blood vessels deliver oxygen, nutrients, growth factors, and circulating cells to bone is tightly regulated as a function of blood pressure and vascularization. Given that blood pressure is generally maintained, the number and size of blood vessels determines the local blood flow rate. These two factors are regulated through the processes of angiogenesis and vasomotor function, respectively. Although a variety of skeletal disorders are associated with general vascular dysfunction (5-7), this review will focus on the regulation of bone blood flow, through the number and size of vessels, during bone repair and regeneration. To introduce this topic, the skeletal vascular anatomy and techniques for measuring bone blood flow are briefly discussed.

Blood supply in bone

A dense vascular network delivers oxygen and nutrients to all 206 bones in the human body. In general, this requires a substantial portion of the total cardiac output. Experimental work in various animal species has demonstrated that a significant portion of the resting cardiac output is directed to the skeleton, likely between 10% and 15% (8-12).

For some time, the blood flow pattern in bones has been described as primarily centrifugal: blood is supplied to the cortical bone through the nutrient arteries in the

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marrow cavity (Figure 1), and returned by the periosteal veins (13). Particularly in long bones, the vascular anatomy is well characterized, with specific arterial inlets (the main nutrient artery, periosteal arteries, metaphyseal arteries, and epiphyseal arteries) and veinous outlets (14). However, the structure of the vascular network can vary greatly depending on the skeletal site. For example, arteries in the greater trochanter enter from the medial, lateral, and superior surfaces to supply a vascular network within the trochanter that is functionally separated from the blood supply for the femoral neck and shaft (15).

In addition, there is evidence that the blood flow direction can shift from centrifugal to centripetal, depending on the hemodynamic conditions in the bone (16). This hypothesis was tested experimentally in the ovine tibia by intramedullary reaming (17). After the destruction of the main nutrient artery in the marrow cavity, blood flow at the periosteum was rapidly increased to compensate for the loss, generating local centripetal

Figure 1 Femoral blood supply. The principal nutrient artery and its main medullary branches are shown in this angiograph of a rabbit femur (10).

blood flow. More recently, stress fracture healing was associated with a disruption of the normal centrifugal flow in the ulna, particularly the lacunocanalicular flow (18). Although the skeletal vascular network is generally robust, some bones are poorly perfused. For example, stress fractures in the fifth metatarsal typically heal slowly due to the poor vascular support in this area (19-20). Additionally, bone blood flow, particularly in the long bones, is known to decrease with age (21-23). In summary, the blood flow conditions in bone are site-specific and dynamically change in response to trauma, metabolic demands, and aging.

Mechanisms of vascularization

The production of bone, whether during development or repair, entails the generation of new blood vessels to support the tissue. There are at least four distinct mechanisms for producing new vessels. Vasculogenesis, or neovascularization, is the generation of *de novo* blood vessels (24), generally observed during development. In contrast, angiogenesis is a broad term used to describe the generation of new vessels from existing vasculature (25). Angiogenesis can be thought of both in terms of sprouting angiogenesis, the process by which a new vessel branches off from an existing vessel, as well as intussusceptive angiogenesis, the process in which vessels split into two or more vessels (26). Finally, arteriogenesis is a separate process by which functional collateral arteries are generated between existing arteriolar anastomoses (27). Whereas angiogenesis is triggered by hypoxia through HIF-1α mediated gene transcription, arteriogenesis appears to occur as a response to physical forces, independent of hypoxia (28).

The structural morphology of blood vessels can be altered by a process known as vascular remodeling (29). In general, vascular remodeling is either outward (increased vessel diameter) or inward (reduced vessel diameter). Remodeling can be further characterized as hypertrophic (increased cross-sectional area), hypotrophic (reduced cross-sectional area), or eutrophic (no change in cross-sectional area) (30). Vascular remodeling has been studied extensively in hypertensive rodents and humans (31-33), and this may be a mechanism by which blood flow is stably altered in bone (34-36).

On the other hand, short-term, rapid expansion (vasodilation) or contraction (vasoconstriction) of blood vessels, known as vasomotor or vascular tone, has also been implicated as a mechanism for rapidly changing blood flow rate, mostly in response to injury (37-38). The regulation of this process is complex and multifactorial, with important contributions from the autonomic nervous sys-

tem and circulating hormones, as well as locally produced factors that include nitric oxide (NO) and endostatin (39-40).

Techniques for measuring bone blood flow

Like any fluid flow scenario, blood flow rate is quantified as volume per time and is determined as the product of the cross-sectional area of the vessel and the fluid velocity, which depends on pressure. This relationship is expressed in Poiseuille's law, a simplification of the Navier-Stokes equations for laminar flow of incompressible, Newtonian fluids, as:

$$Q = \frac{\pi r^4 \Delta P}{8 \mu L}$$

where flow rate (Q) is given as a function of the radius of the vessel (r), the difference in pressure (ΔP), the viscosity of the fluid (μ) and the length of the vessel (L). Although this equation is not appropriate for calculating absolute flow rates of blood, a non-Newtonian fluid, the general relationship illustrates that blood flow rate is weakly regulated by systemic blood pressure and strongly regulated by the local size of the blood vessel.

Vessel size and number can be robustly quantified *ex vivo* using histology or vascular casting (41). However, accurate quantification of *in vivo* blood flow, particularly in bone, is more difficult (42). Early attempts included the injection of red blood cells labeled using radioactive isotopes (43). As technology has improved, less onerous methods have been devised to provide more accurate results. The most important methods have been briefly summarized (Table 1).

Administration of labeled microspheres into the circulation by arterial catheterization or cardiac injection has been used for some time to quantify blood flow (44). With a sufficiently small diameter (<50μm), the labeled microsphere will get trapped in the microvasculature. While the quantification method depends on the method of labeling, the number of microspheres must be determined in the tissue of interest as well as a reference tissue, making this technique relatively invasive. Early on,

microspheres were labeled using radioactivity (45), but non-radioactive microspheres have also been used, including colored microspheres and fluorescent microspheres (46). With sufficient sampling, inherent error can be reduced to 20% or less (47). As a result, this technique has been used in a wide range of experimental models (48-50) and is generally accepted as the "gold standard" for blood flow measurements, even in bone (2).

Laser Doppler flowmetry (LDF) is quantitative technique developed to utilize the Doppler effect for determining the velocity of blood flow in a vessel (51-52). Real time blood flow rate can be measured continuously and non-invasively, but measurements can usually only be made in the most superficial vessels, typically in the skin, unless the procedure permits placing a probe inside the subject. Additionally, this technique does not provide anatomical data, so the region of interest must be determined externally and changes in vascular anatomy are not captured. Despite considerable limitations, this technique has been used successfully clinically and experimentally to quantify bone blood flow (53-54). Importantly, experimental work using LDF as well as simultaneous 85Sr-labeled microspheres found no statistically significant correlation between the two techniques in the femoral head or condyles, and suggested that LDF may be a superior choice for quantifying bone blood flow (55). Recent validation and experimentation in the mouse tibia using LDF confirms this opinion (56).

Positron emission tomography (PET) is another established *in vivo* technique for quantifying rates of flow and metabolism. The kinetics of an injected, short-lived radioactive tracer are quantified by detecting the positron ejected during radioactive decay. Although the technology underpinning PET imaging was developed in the 1960s and 1970s, the utility of this technique dramatically improved in the 1980s with the increased availability of radiopharmaceuticals, including 15O water typically used to evaluate blood flow (57). Since then, PET imaging has been used to evaluate blood flow in bone (58) as well as a variety of soft tissues (59-62). In addition to providing blood flow rates in absolute units (63), simultaneous PET/CT imaging can be registered to provide anatomic land-

Table 1 Comparison of blood flow measurement techniques

Factors	Microspheres	Laser Doppler Flowmetry	PET Imaging
Measurement Type	Static, relative to reference tissue	Continuous, quantitative	Continuous, absolute flow rates
Invasive?	Yes	No	No
Anatomical Data	Yes	No	Yes
Other Factors	Experimentally difficult, general gold standard	Limited depth of scan may require invasive probe placement	Limited by scan resolution and availability of radioisotopes

marks with high spatial resolution. A strong correlation between PET and microsphere injection was found when quantifying myocardial blood flow in rats ($P<0.000 1$) (64), but there has not been a similar study for bone blood flow comparison. The major limitation of PET imaging is resolution, though the most recent scanner technology is suitable for PET imaging in animals as small as rodents (65).

Blood flow during fracture

Skeletal fracture is typically associated with the disruption of the surrounding soft tissue as well as the marrow compartment of long bones. As a result, the vascular network in and around bone is compromised. Decreased perfusion concurrent with the increased metabolic demands of repair leads to hypoxia near the fracture site (4). As a result, restoration of blood flow through angiogenic mechanisms is a key component of fracture healing, a process of endochondral ossification that largely recapitulates skeletal development (66).

The relative importance of the main nutrient artery and the periosteal arteries during fracture healing has been debated for some time (67). Fixation of a canine tibial fracture with an intramedullary rod was shown to increase blood flow at the fracture site as compared to compression-plate fixation, suggesting that periosteal arteries are particularly responsive during fracture healing (68). This line of reasoning was bolstered by a careful histological examination of vasculature following fracture that nicely demonstrated the periosteal callus alone has extensive new vessel formation (69). Additionally, a body of experimental work has shown that the presence or absence of the periosteum significantly affects the healing response (70). Finally, recent work has demonstrated that invading blood vessels in the periosteal fracture callus are intimately associated with osteoblast precursors (71). This finding confirms the importance of periosteal vascularity for the restoration of perfusion and the influx of cells providing osteogeniccues (Figure 2).

Although new blood vessels are certainly required for vascularizing the fracture callus and reversing vascular damage from the injury (72), regulation of the preexisting vascular network also plays an important role in the early stages of repair (73). In a study of two patients using PET imaging, blood flow rate was 3 to 6 times greater in the fractured tibia than the contralateral tibia 24 hours after injury, suggesting significant vasodilation associated with the fracture (58). Using LDF to quantify blood flow rate, researchers demonstrated that the nitric oxide (NO) synthase inhibitor L-NAME only attenuated blood flow rate

in the first day after tibial fracture (74). Thus, the initial vascular response following fracture is vasodilation of the intact vascular network through NO signaling, but the subsequent vascular response does not appear to be affected by decreased NO. This conclusion is consistent with work showing NOS expression and gene regulation is temporally modulated during fracture healing (75-76). Other experimental work has shown that endothelial vasodilation through NO signaling in bone is impaired with aging (77), suggesting vasomotor control may be a contributing factor for poor fracture healing in the elderly. Other inflammatory cytokines, including TNF-α, IL-1, and IL-6, are expressed at the site of fracture and have been shown to affect repair, although their primary contribution is likely the migration and differentiation of progenitor cells, rather than the regulation of blood flow (78-80).

Many studies have highlighted the importance of pro-angiogenic factors, such as VEGF, HIF-1, and IGF, in fracture healing (81-84). However, the regulation of blood flow is complex, involving a balance of both pro-angiogenic and anti-angiogenic factors. An endogenous anti-angiogenic factor, endostatin, was recently examined following fracture healing (85). Administration of exogeneous endostatin decreased vascularity and hard callus formation, but soft callus formation was increased. These results underscore that more study is required to understand how the angiogenic pathways affect the entire fracture repair process.

Unlike the acute trauma of a complete fracture, repetitive loading of a bone can generate fatigue damage, eventually resulting in an incomplete, non-displaced fracture or stress fracture (86-87). In rodent models, stress fracture healing has been shown to recapitulate the intramembranous portion of fracture repair (88), with the hallmark of stress fracture healing being the rapid formation of a hard periosteal callus of woven bone without a cartilaginous template (89). As shown in Figure 3, significant periosteal angiogenesis is associated with healing (90-91), and angiogenic inhibition attenuates the healing response (92). By quantifying gold microspheres, an immediate increase in blood flow rate was observed following the generation of a stress fracture in rats (18), consistent with reports that inflammatory markers, including IL-1, IL-6, and NOS2, are upregulated as early as 1 hour after the injury (93-94). Using *in vivo* PET imaging, recent work has demonstrated that an elevated blood flow rate is maintained at the site of a stress fracture by a two-step process of NOS-mediated vasodilation followed by angiogenesis, similar to a complete fracture (95).
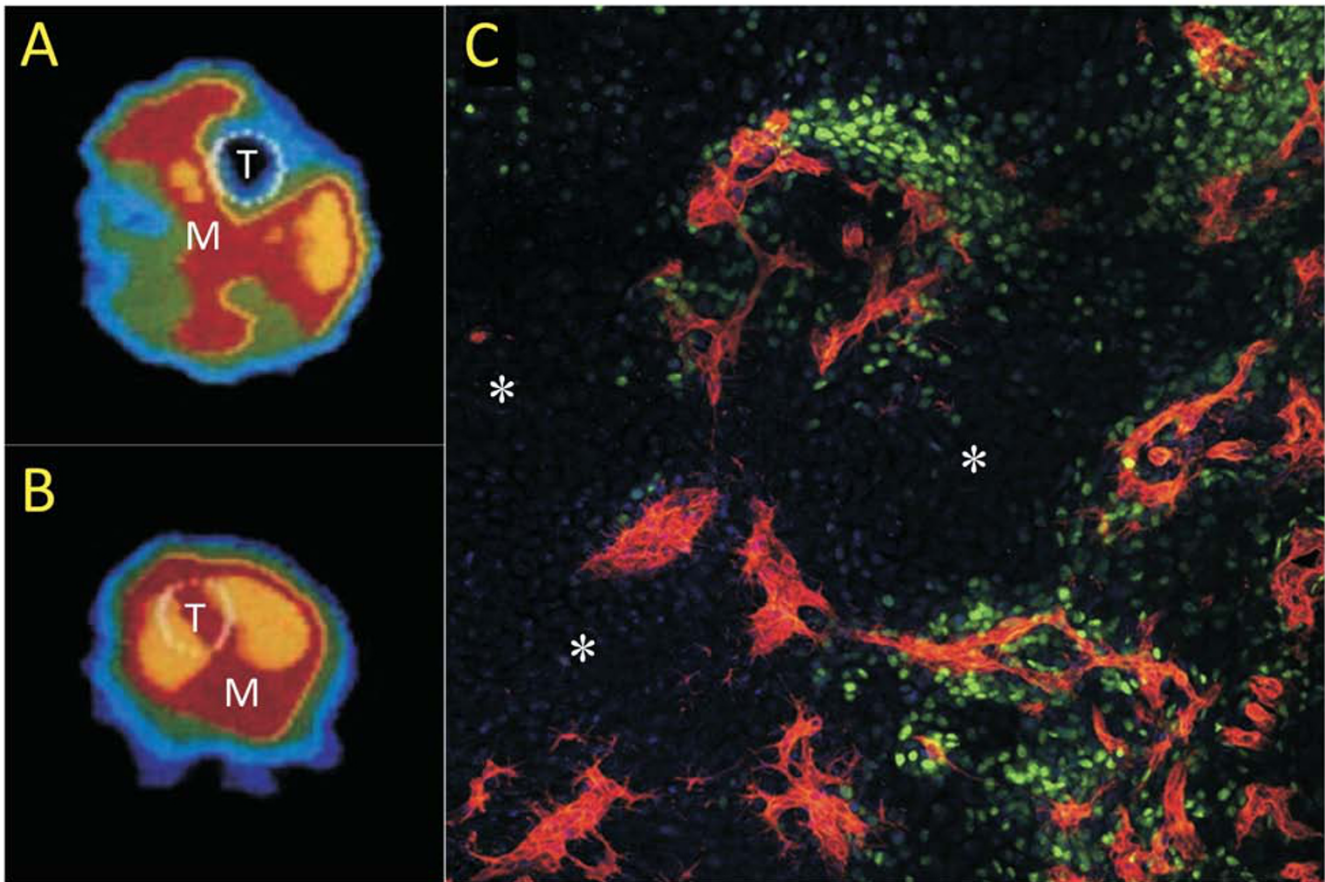
Figure 2 The vascular role in fracture. Blood flow rate was quantified using PET imaging after tibial fracture (58). A) In an uninjured limb, blood flow is highest in the muscle (M) near the tibia (T). B) In contrast, blood flow rate in and around the tibia (T) is markedly increased following fracture. This increase in blood flow rate is due in part to angiogenesis. Recent work has shown that these new blood vessels coinvade the cartilaginous template along with osteoblast precursors during endochondral fracture repair (71). C) In the periosteal fracture callus, Osx-expressing osteoprogenitors (green) are intimately associated with new vasculature (red) seven days after fracture. Pockets of avascular cartilage are labeled with a *. Figures used with permission (License Number: 3266071128303).

Blood flow during distraction osteogenesis

Distraction osteogenesis (DO) is a regenerative procedure used to induce mostly non-endochrondral bone formation in long bones. This technique can be used clinically for leg lengthening (96) as well as the correction of pseudoarthroses (97), but has also been implemented in animal models to study the controlled formation of bone (98). The large increases in vascularity, associated with significant increases in pro-angiogenic gene transcription, were hypothesized to play an important role in bone formation during distraction (99). Treatment with the anti-angiogenic agent TNP-470 (100) as well as with antibodies against VEGF receptors (101) confirmed this theory–inhibition of angiogenesis blocks bone formation in the distraction gap. This result is in contrast to other osteogenic programs that are only impaired by angio-genic inhibition, a phenomenon due in part to the fact that the distraction gap is functionally avascular, having no preexisting vascular network. As a result, angiogenic inhibition completely blocks the migration of the osteo-progenitors that initiate bone formation. Consistent with this hypothesis, mice with overexpression of HIF-1α in the osteoblastic lineage had markedly increased bone regeneration following distraction due to significantly increased angiogenesis in the distraction gap (83).

While most research has been limited to the vascular network that is generated in the distraction gap follow-ing DO, recent work has shown that these vessels are part of a larger vascular network that extends from the muscles near the surgical site (102). In particular, arterio-genesis in the muscle compartment precedes angio-genesis that occurs in conjunction with bone formation. Importantly, the large vessels expressing smooth muscle
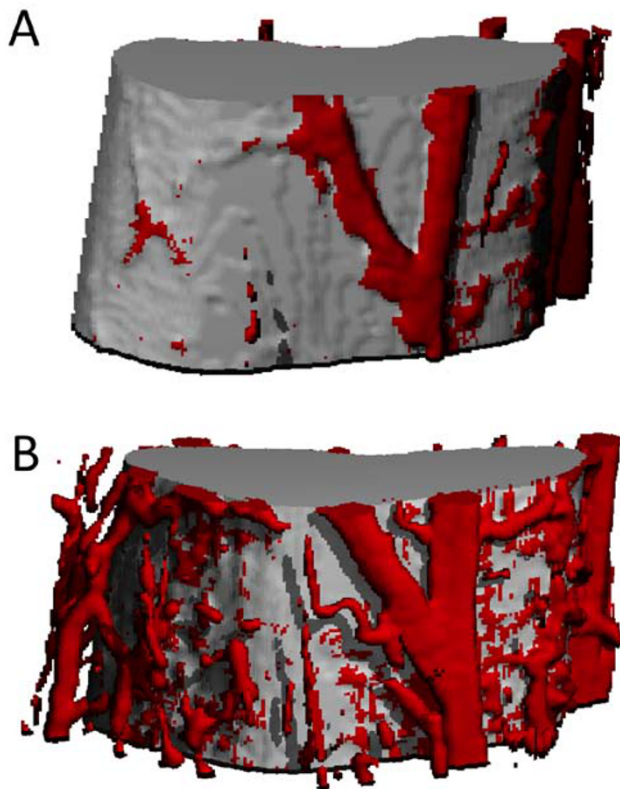
Figure 3 Rapid angiogenesis after bone injury. Damaging mechanical loading that culminates in an ulnar stress fracture is associated with significant angiogenesis to increase blood flow rate following injury (91). Microfil vascular perfusion was used to visualize periosteal vascularity in control (A) and fractured ulnae (B). Figures used with permission (License Number: 3260231064494).

α-actin were shown to be responsible for the expression of BMP2 (103), suggesting that larger vessels generated by arteriogenesis play an important role in driving osteo-genesis in this model.

Blood flow for maintenance of bone

Osteoporosis is not a bone injury as much as a metabolic disorder. Accordingly, bone blood flow has been hypo-thesized to play a role in its progression. In one study from 1986 to 1988, 2 401 women aged 65 or older were recruited to determine if reduced blood flow to the lower extremities was a contributing factor to decreased bone mineral density (BMD). The results from this study provided initial evidence that increased bone loss, particularly at the hip, may be a result of decreased vascular support (104). Because of the clinical correlation between vascular calcification and osteoporosis, some authors have suggested that preventive control of vas-

cular risk factors, in addition to standard therapy, would reduce the risk of osteoporotic fracture (105). In a recent study of 103 postmenopausal women, patients with osteoporosis were found to have increased arterial stiff-ness compared to patients with normal BMD (106). In addition, a trend for decreased spine BMD with increased carotid stiffness was observed. However, the relationship between the multifactorial disease states of osteoporosis and vascular dysfunction remains elusive, with recent evidence to suggest that osteoporosis may actually be a risk factor for cardiovascular disease (107).

Similarly, chronic unloading of the skeleton results in significant decreases in bone mass (21, 108). Importantly, even a short period of skeletal unloading has been asso-ciated with significant decreases in bone blood flow (109). In a recent study, rats were subjected to 7 or 14 days of hindlimb unloading, then blood flow and vas-cular resistance were quantified following reloading (35). Decreased blood flow and increased vascular resistan-ce were associated with unloading, suggesting that skeletal loading is required to maintain vascular function in bone. This response appears to have been mediated through arterial remodeling, a powerful and largely unexplored mechanism in bone (Figure 4).

The response to hindlimb unloading is similar to what has been observed in streptozotocin-diabetic rats (110). In this scenario, bone blood flow is significantly decreased while bone turnover is almost totally suppressed. In con-trast, the significant increase in bone turnover in ovari-ectomized rodents has been associated with significant decreases in bone blood flow in a variety of studies using multiple techniques (111-114). However, this vascular effect has been shown to strongly depend on genetic background (56), potentially explaining controversial reports of increased bone blood flow following rodent ovariectomy by quantifying microspheres (115-116). Finally, other rodent models of rapidly induced osteo-penia, such as paraplegia (117) and orchidectomy (118), have increased bone blood flow in the face of rapid bone loss. Determining the relationship between bone blood flow and formation/resorption is highly de-sirable, but the signaling that regulates these mechani-sms is not well understood (1). Additional work on the interaction and regulation of the bone and vascular compartments, particularly during acute and chronic bone loss, is necessary.

Blood flow in bone grafts

Sufficient bone blood flow must be maintained for the successful repair of large skeletal defects using bone grafts (119-120). Since the late 1970s, vascularized fibular
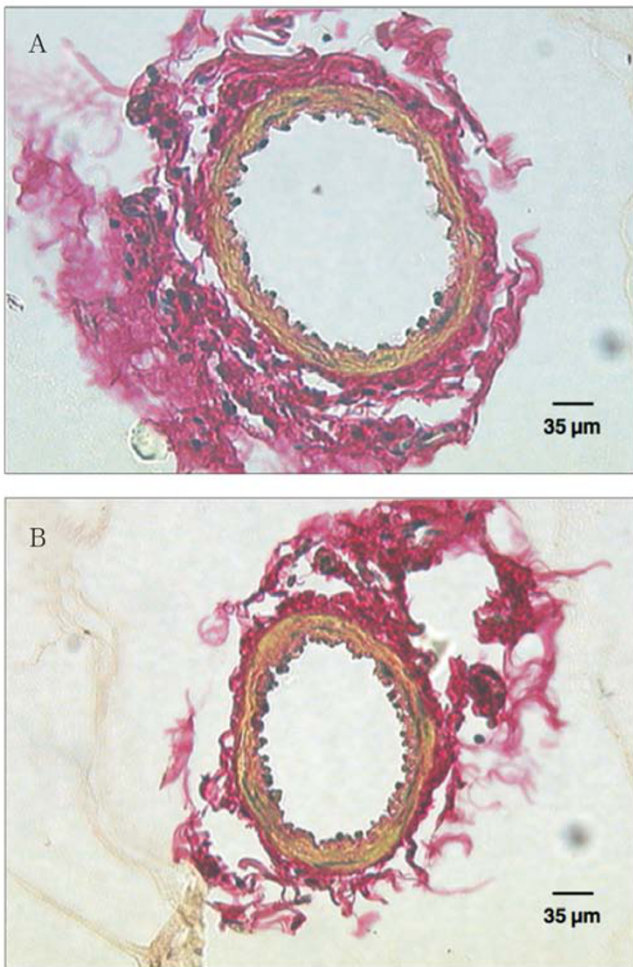
Figure 4 Vascular remodeling after unloading. Following cannulation with micropipettes, the diameter of the primary nutrient artery was measured in A) control and B) 14 day hindlimb unloaded animals (35). These results demonstrate that blood flow is decreased following chronic skeletal unloading by vascular remodeling. Figures used with permission (License Number: 3260231186916).

bone (VFB) grafts have been used to treat osteonecrosis of the femoral head (121). In this procedure, a portion of the fibula is removed intact with the attached vasculature and inserted into a cavity created in the osteonecrotic femoral head (122). In a recent review, VFB grafts were found to be superior to other grafting techniques, with better clinical outcomes and fewer patients that progress to total joint replacement (123). However, VFB grafts are generated from the patient's own fibula, resulting in additional morbidity associated with the procedure. As a result, current tissue engineering efforts focus on developing synthetic bone grafts with preformed, functional vascular networks (124-125). Before implantation, mineral deposition in these grafts is spatially asso-

ciated with the vascular network, consistent with the hypothesis that vascularized bone grafts improve subsequent bone formation (126). Improvements in this important area of regenerative medicine will rely on previous research of bone blood flow, as researchers hunt for the right combination of scaffold, cell source, and growth factors.

Clinical relevance

Because bone blood flow affects bone regeneration following injury, patient populations with poor vascular function may have a slower recovery of fracture resistance than expected. This extra recovery time creates an additional risk of reinjury following skeletal damage. This issue is particularly problematic in cancer patients, since fracture risk is already elevated (127-128) and anti-angiogenic therapy is a widespread therapy for many cancers (129-130). Additionally, patients with diabetes (131-132), COPD (133), and sickle cell disease (134) as well as smokers (135) have vascular dysfunction that has been associated with skeletal defects. As a result, the maintenance of adequate vascular function during recovery from skeletal injury should receive additional consideration in these patient groups. Finally, patients should be advised that a healthy cardiovascular system is crucial for the long-term prevention of osteoporosis.

Conclusion

Although the knowledge of vascular anatomy in the skeleton has greatly increased since the microscopists of the 16th and 17th centuries first revealed blood vessels in calcified tissue (2), the role that blood flow plays to regulate important physiological processes of bone regeneration and maintenance is still being discovered. In general, bone blood flow is robust and centripetal. Following a skeletal injury, blood flow is increased by angiogenesis. If metabolic demand is decreased, blood flow is decreased by vascular remodeling (Figure 5). However, the processes that disrupt and reverse bone blood flow have not been well characterized, particularly in regards to skeletal healing. The importance of an intact vascular network following injury is beginning to be realized, with experimental results demonstrating that inflammation-mediated vasodilation provides a critical source of additional blood flow before revascularization begins. Similarly, the contribution of muscle to bone blood flow during healing may be important, since the blood vessel network that vascularizes bone during regeneration has been observed to be an extension of the blood vessel network in adjacent muscle. The reports of blood

flow regulation in osteoporotic patients, disuse osteopenia, and models of rapid bone loss underscore the idea that bone blood flow is rapidly and dynamically regulated. In summary, more study is required to understand the important role of bone blood flow as researchers generate new therapies for skeletal regeneration and maintenance.
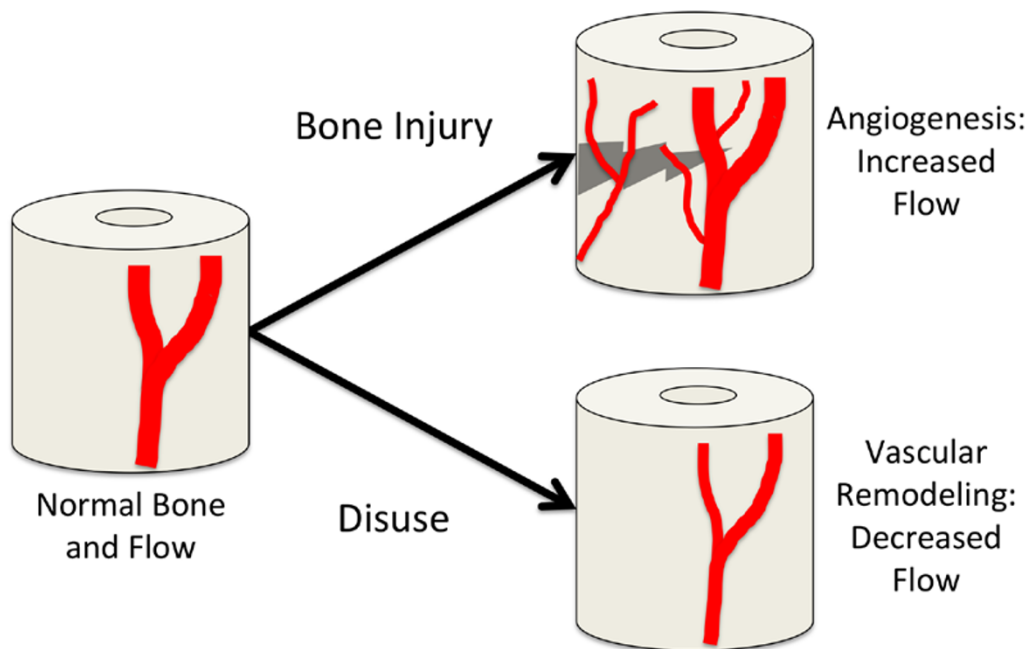
Acknowledgments

Figure 5 Vascular responses in bone. In this schematic, normal bone blood flow is represented on the left. In response to bone injury, such as a fracture, robust angiogenesis occurs to relieve oxygen tension and transport osteoprogenitor cells for repair. In contrast, bone disuse can result in decreased blood flow by vascular remodeling.

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