Multiple Roles of Vascular Endothelial Growth Factor (VEGF) in Skeletal Development, Growth, and Repair

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Overview

Studies of bone morphogenesis have identified a large number of critical molecules and regulatory pathways. One of these molecules is vascular endothelial growth factor, VEGF. Several studies suggest that not only is this regulator of angiogenesis important in mediating interactions between the developing bone and the vasculature, but it also has a key role in regulating processes during bone development and growth which are not directly related to angiogenesis. Studies of the detailed mechanisms by which VEGF is involved in bone development promise therefore to shed new light on key steps in bone formation. This chapter summarizes findings with emphasis on the developmental roles of VEGF in skeletal morphogenesis. Furthermore, we speculate on the future directions of research in this area and describe some of the challenges in the field.
I. Introduction

In vertebrates, the skeleton is formed by mesenchymal cells that are derived from cranial neural crest, somites, and lateral plate mesoderm. At the sites of future bones, these cells condense and form the future skeletal elements. In the cranial vault, jaws, and part of the clavicle, the condensed mesenchymal cells differentiate into osteoblasts and generate bone in a process termed “intramembranous bone formation.” The remainder of the future skeleton develops by endochondral bone formation. During endochondral bone formation, the condensing mesenchymal cells differentiate into chondrocytes and form avascular cartilage models of the future bones (see Fig. 1). As development proceeds, chondrocytes in the centers of the cartilage models cease to proliferate and the post-mitotic cells differentiate to hypertrophy. The differentiation of chondrocytes to hypertrophy is followed by rapid invasion of blood vessels, osteoclasts, and other mesenchymal cells from the surrounding tissue (perichondrium) into the cartilage, which is progressively eroded and replaced by bone marrow and trabecular bone in the

Figure 1  Diagram of the developmental process leading to the formation of the mouse tibia. At early stages, differentiation of chondrocytes from mesenchymal cells results in the formation of an avascular cartilage model of the future bone. At ED14, chondrocytes in the center of the cartilage model mature to hypertrophy. One day later, the hypertrophic cartilage begins to be invaded by sprouting blood vessels, osteoclastic cells, and hematopoietic precursors; in the perichondrium, a bone collar is forming. At later stages, the marrow cavity expands by continued erosion of the hypertrophic cartilage in the growth plates at both ends (epiphyses) of the growing bone. As the hypertrophic cartilage is removed, it is replaced by trabecular bone in the regions beneath the growth plate cartilage (modified from Horton, 1990).
primary ossification center (Karsenty and Wagner, 2002; Kronenberg, 2003; Olsen et al., 2000).

The association of angiogenesis with endochondral bone formation was the trigger for initial studies of a possible involvement of VEGF in bone vascularization. As has happened many times in research, experiments aimed at answering one question opened the door to many unanticipated discoveries. In the case of VEGF, data from several laboratories now suggest that VEGF, in addition to being required for bone vascularization, has a key role in several other steps of skeletal development, including chondrocyte differentiation and survival, osteoclast recruitment, and osteoblast differentiation.

II. The Role of VEGF in Regulating Vascularization of Developing Bones

Vessel invasion into the primary ossification center and continued capillary sprouting as the center expands and growth plates are formed at both ends (epiphyses) are key steps in endochondral bone formation. Three papers by Trueta et al. (Trueta and Amato, 1960; Trueta and Buhr, 1963; Trueta and Trias, 1961) about 40 years ago firmly established the concept of a coupling between cartilage vascularization and endochondral bone formation. Although these studies were conducted with 6-week-old rabbits, the conclusions of the studies also apply to embryonic bone development. By interrupting the blood supply to the growth plate, Trueta and his colleagues observed a decrease in bone mineralization and an expansion of the hypertrophic zone in the growth plate. These findings led them to conclude that blood vessels are not only supplying oxygen and nutrition to forming (growing) bones, but play an active role in bone formation. Forty years later, Gerber et al. used injection of a soluble VEGF receptor to inhibit VEGF activity in 24-day-old mice and observed impaired angiogenesis, decreased trabecular bone formation, and expansion of the hypertrophic zone in growth plates (Gerber et al., 1999). These findings were similar to those reported by Trueta et al. and represented a first step toward a detailed understanding of the mechanism that couples cartilage vascularization and endochondral bone formation.

Vessel invasion into the primary ossification center of developing bones (at E14.5 in the mouse tibia) is preceded by recruitment of vessels to the surrounding perichondrium. At the time of this perichondrial recruitment (in the mouse tibia, this happens at E13.5–E14.5), VEGF is expressed by the perichondrial cells. This suggests that VEGF might be involved in the recruitment of blood vessels to the perichondrium. Support for this hypothesis has come from studies of bone development in mice that only express
one isoform of VEGF, VEGF120 (Zelzer et al., 2002). The murine VEGF gene encodes three isoforms: VEGF120, VEGF164, and VEGF188; all are products of alternative splicing of a single gene (Ferrara et al., 1992; Shima et al., 1996). In contrast to the other two isoforms, VEGF120 does not bind the extracellular matrix component heparan sulfate (Ferrara and Davis-Smyth, 1997; Park et al., 1993). Neuropilin-1 (NRP1) and neuropilin-2 (NRP2) are co-receptors for VEGF164 and can potentiate signaling through the VEGF receptor VEGFR2 (Flk-1), but they do not bind the VEGF120 isoform (Soker et al., 1998). In VEGF120 mice, the recruitment of vessels into the perichondrium is delayed. This delay suggests that the function of VEGF expressed in the perichondrium at E13.5 is to stimulate perichondrial angiogenesis (Zelzer et al., 2002).

Soon after perichondrial angiogenesis, vessels invade the hypertrophic cartilage from the perichondrium (Colnot et al., 2004), and the primary ossification center is established. This is preceded by upregulation of VEGF expression in the hypertrophic cartilage (Carlevaro et al., 2000; Colnot and Helms, 2001; Zelzer et al., 2001). As development proceeds and capillaries continue to invade the hypertrophic cartilage of the growth plate (see preceding text), VEGF expression in the hypertropic zone is maintained (Gerber et al., 1999). Vessel invasion into the primary ossification center is severely delayed in both VEGF120 mice and mice in which VEGF expression in chondrocytes is abolished. Vessel sprouting within the metaphyses is reduced as well. As a result of the reduction in vessel sprouting in the metaphysis, the erosion of the cartilage growth plate is reduced, and terminally differentiated hypertrophic chondrocytes accumulate (Haigh et al., 2000; Maes et al., 2002; Zelzer et al., 2002, 2004).

Expansion of the zone of hypertrophic chondrocytes in the growth plate is also observed following targeted inactivation of the genes encoding matrix metalloprotease-9 (MMP-9), the transcription factor Runx2, and connective tissue growth factor (Ctgf). Interestingly, all three genes have been reported to affect VEGF activity in the growth plate, although the suggested mechanisms by which they act are different (Ivkovic et al., 2003; Vu et al., 1998; Zelzer et al., 2001).

MMP-9 is a key molecule in bone formation (see Ortega et al., 2004). As has been mentioned, the growth plates of MMP-9 null mice have expanded hypertrophic zones. Moreover, MMP-9 null growth plates show a reduction in vascularization and ossification. These features are also seen in mice where VEGF activity is blocked. MMP-9, expressed by osteo(chondro)clasts in the growth plate, has been suggested to play a role in extracellular matrix degradation during vessel invasion. Since VEGF expression in MMP-9 null growth plates appears to be normal, it is not clear how the functions of these two genes are mechanistically connected (Blavier and Delaisse, 1995; Engsig et al., 2000; Vu et al., 1998). Possible mechanisms
will be discussed later when the role of VEGF in osteoclast biology will be described.

Runx2, a member of the runt-domain transcription factor family, has an essential role in osteoblast differentiation (Ducy et al., 1997). Runx2 null mice have an almost perfectly patterned skeleton composed of cartilage, but no bones (Komori et al., 1997; Otto et al., 1997). In addition, chondrocyte differentiation to hypertrophy is impaired, except in the distal appendicular skeleton (tibia–fibula, radius–ulna), where chondrocytes do differentiate to hypertrophy. Moreover, no blood vessel invasion into hypertrophic cartilage is apparent in Runx2 null mice (Inada et al., 1999; Kim et al., 1999).

The lack of blood vessel invasion into hypertrophic cartilage in Runx2-deficient mice suggests a link between Runx2 and VEGF expression. Indeed, the upregulated expression of VEGF in hypertrophic zones in wild-type mice is absent in the hypertrophic cartilage of Runx2-deficient mice. This finding suggests that Runx2 is involved in the regulation of VEGF in hypertrophic chondrocytes (see Fig. 2). Whether Runx2 acts as a direct transcriptional regulator of the VEGF gene is not clear; however, the VEGF promoter contains Runx2 binding sites and overexpression of Runx2 in fibroblasts, transfected with a VEGF promoter-luciferase construct, results in induced expression of the luciferase reporter (Zelzer et al., 2001). The finding that Runx2, an essential regulator of osteoblastic differentiation, also regulates VEGF expression in hypertrophic cartilage, demonstrates the existence of a

Figure 2  Diagram indicating two major functions of VEGF during endochondral ossification. In hypertrophic chondrocytes, a high-level expression of VEGF, controlled by the transcription factor Runx2, is essential for vascularization of cartilage in the primary ossification center and continued capillary sprouting under the growth plates as the bone grows. Since the level of VEGF expression is reduced in Ctgf null growth plates, it is possible that Ctgf is a component of a pathway by which Runx2 stimulates VEGF expression. In epiphyseal chondrocytes, a moderate level of VEGF expression, controlled by HIF-1 and VHL, is necessary for chondrocyte survival.
tissue-specific genetic program that couples osteogenesis and vessel invasion into cartilage during endochondral bone formation.

Connective tissue growth factor (Ctgf), a member of the CCN family, is an important regulator of extracellular matrix production (Perbal, 2004). Gene-targeting experiments also suggest a role in bone development. Endochondral Ctgf null bones show a decrease in chondrocyte proliferation and expression of extracellular matrix molecules. Moreover, the hypertrophic zone of growth plates in Ctgf null bones is expanded, suggesting a reduction in angiogenesis and matrix erosion. Expression of VEGF in Ctgf null growth plates is also decreased. This decrease is not the result of reduced Runx2 expression, suggesting that Ctgf is either downstream of or acts in parallel with Runx2 in regulating VEGF expression (Fig. 2) (Ivkovic et al., 2003).

The upregulated expression of VEGF in hypertrophic chondrocytes results in the sprouting of endothelial cells in perichondrial blood vessels. Studies of the expression of VEGF receptors in these endothelial cells have

Figure 3  Diagram of sprouting capillaries under the zone of hypertrophic chondrocytes in the growth plate of an endochondral bone. Synthesis of VEGF by hypertrophic chondrocytes is likely to generate a concentration gradient of VEGF (indicated by a decrease in font size away from the chondrocytes) that serves to direct the migration of VEGF receptor-expressing endothelial cells. As described in the text, capillary sprouts close to the hypertrophic cells contain no basement membrane or pericytes; further away from the cartilage, basement membranes and pericytes are surrounding the endothelial tubes.
identified a potential mechanism for enhancing and refining the invasion of vessels into the cartilage and the continued capillary growth under the growth plates. The expression of VEGF receptors VEGFR1 (Flt-1) and VEGFR2 (Flk-1) in the perichondrial endothelium is upregulated as a result of VEGF expression in the hypertrophic cartilage (Colnot and Helms, 2001; Zelzer et al., 2001, 2002). This upregulation is maintained through all stages of bone development in the sprouting vessels under the growth plates (Gerber et al., 1999). A reduction in VEGF expression or activity in hypertrophic cartilage results in reduced VEGF receptor expression in perichondrial endothelial cells. This suggests that invasion of vessels into hypertrophic cartilage involves an active cross-talk between hypertrophic chondrocytes and endothelial cells (Fig. 3). Interestingly, sprouting growth plate capillaries contain no basement membranes or pericytes (Hunter and Arsenault, 1990). Further away from the hypertrophic cartilage, in the region where bone matrix is being synthesized, endothelial cells are surrounded by basement membranes and pericytes. It is tempting to speculate that high levels of VEGF signaling in the vicinity of hypertrophic chondrocytes maintain endothelial cells in a sprouting mode by inducing the expression of VEGF receptors and inhibiting the formation of basement membranes and pericyte recruitment (Fig. 3).

III. VEGF Regulates Osteoclast Activity

Vessel invasion into cartilage is a complex process involving the coordinated activities of both endothelial and osteo(chondro)clastic cells (referred to subsequently as osteoclasts). In addition to controlling endothelial cell activities, VEGF also regulates osteoclastic differentiation, migration, and activity. VEGF is, therefore, a key coordinator of the entire process.

Osteoclasts, derived from monocytes, play an important role during cartilage vascularization (Tondravi et al., 1997). The discovery that monocytes express VEGFR1 (Flt-1) (Barleon et al., 1997) clearly raised the possibility that VEGF signaling may affect osteoclasts. Indeed, Niida et al. (1999) demonstrated that VEGF can stimulate osteoclastic bone resorption in vivo. Moreover, VEGF injection into osteopetrotic op/op mice (lacking the cytokine M-CSF) rescued the osteopetrotic phenotype by inducing recruitment and survival of osteoclasts. Bone marrow cell culture experiments demonstrate that VEGF can enhance osteoclastic activity by stimulating the differentiation of osteoclasts from monocytic precursor cells. In the process of differentiation of osteoclasts from monocytes, VEGF can substitute for M-CSF as a co-stimulator (with RANKL) (Niida et al., 1999). Furthermore, studies of bone resorption by mature osteoclasts suggest that VEGF is involved not only in osteoclastic recruitment and differentiation
but also in enhancing osteoclastic bone resorbing activity (Nakagawa et al., 2000). A possible pathway for regulating migration of osteoclasts by VEGF involves the extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Henriksen et al., 2003).

VEGF is necessary for osteoclastic activity both at the stage when the primary ossification center is established and later during bone growth. In VEGF120 mice, the number of osteoclasts is dramatically reduced in the perichondrium of developing bones at sites where vessels will invade the hypertrophic cartilage (Maes et al., 2002; Zelzer et al., 2002). Injection of a soluble chimeric VEGF receptor Flt-IgG protein into the circulation of mice blocks the recruitment of osteoclasts to the growth plates (Gerber et al., 1999). Addition of chimeric VEGF receptor protein to metatarsal organ cultures blocks recruitment of osteoclasts to the primary ossification center, whereas addition of VEGF induces osteoclast migration (Engsig et al., 2000).

As has been mentioned, it has been reported that a reduced expression of the osteoclastic protease MMP-9 affects angiogenesis-inducing activity in growth plates (Vu et al., 1998). MMP-9 null growth plates share several features with growth plates of mice in which VEGF activities are blocked, such as expansion of the hypertrophic zone and reduction in both vascularization and ossification (Vu et al., 1998). Since VEGF expression in hypertrophic chondrocytes of MMP-9 null growth plates is normal (Engsig et al., 2000), it has been suggested that MMP-9 regulates the availability of VEGF in the hypertrophic zone, perhaps by degrading extracellular matrix components and releasing VEGF from the matrix (Ortega et al., 2004). In our view, the simplest interpretation of the available data is that VEGF, produced by hypertrophic chondrocytes, induces osteoclastogenesis in the perichondrium and stimulates migration of the osteoclasts into hypertrophic cartilage by activating the appropriate migration-inducing signaling pathways. Osteoclasts are the major sources of MMP-9 and they use this protease to tunnel through the hypertrophic cartilage matrix (Blavier and Delaisse, 1995; Reponen et al., 1994; Vu et al., 1998). When MMP-9 expression is eliminated, as in MMP-9 null mice, osteoclast penetration into the cartilage is reduced, resulting in reduced angiogenesis. When VEGF signaling is reduced or blocked, osteoclast differentiation and invasion, as well as the level of the associated MMP-9, is reduced.

IV. VEGF is a Key Component of a Chondrocyte Survival Pathway

A number of studies have led to the identification of VEGF as a critical factor for survival of chondrocytes (Maes et al., 2004; Zelzer et al., 2004). One study discovered that the epiphyseal regions of some long bones in mice
expressing only the VEGF188 isoform contain areas of chondrocyte cell death. The cell death is first observed at E18.5 and becomes prominent at P5 (Maes et al., 2004). Since the VEGF188 isoform in these mice is ubiquitously expressed, the skeletal abnormalities are not necessarily caused by VEGF188 expression in skeletal tissues but may be indirect consequences of changes in surrounding nonskeletal tissues. However, blocking VEGF expression in chondrocytes confirms the importance of VEGF expression for chondrocyte survival. In VEGF null bones, regions of cell death are located in the central regions of skeletal elements at E16.5, starting at an articular surface, continuing through the resting to the proliferating zones of growth plate chondrocytes, and ending in a misshapen growth plate (Zelzer et al., 2004).

A similar phenotype has been described in epiphyseal cartilage of mice in which the transcription factor HIF-1α is conditionally inactivated in chondrocytes (Schipani et al., 2001). This suggests that VEGF and HIF-1α are part of a chondrocyte survival pathway. HIF-1, in which HIF-1α is a subunit, regulates the transcription of a broad range of genes, including VEGF, that are involved in a variety of processes such as glucose metabolism, angiogenesis, and cell survival. The cellular level of HIF-1α protein is tightly regulated by the tumor-suppressor von Hippel-Lindau (VHL) protein, whereas the second subunit of HIF-1, HIF-1β, is constitutively expressed. Several factors, such as hypoxia, hormones, and growth factors, are known to induce stabilization of HIF-1α protein and, therefore, the HIF-1 heterodimeric transcription complex (Pugh and Ratcliffe, 2003; Semenza, 2003).

The existence of a chondrocyte survival pathway in the epiphysis of developing bones raises several questions regarding its regulation and the nature of the cellular survival mechanisms involved. VEGF shows a complex pattern of expression during bone development. A robust expression of VEGF is induced as chondrocytes differentiate to hypertrophy within cartilage models of the future bones (Carlevaro et al., 2000; Colnot and Helms, 2001; Zelzer et al., 2001). Later in development, as growth plates are established, a more moderate expression of VEGF is also detected in epiphyseal chondrocytes (Maes et al., 2004; Schipani et al., 2001; Zelzer et al., 2004). In the absence of VEGF expression, these epiphyseal chondrocytes undergo apoptosis. Interestingly, while VEGF expression in hypertrophic chondrocytes does not appear to be HIF-1α dependent, the moderate (and later) VEGF expression in epiphyseal cells depends on HIF-1α (Schipani et al., 2001; Zelzer et al., 2004). Thus, VEGF appears to be downstream of HIF-1α in the chondrocyte survival pathway (Fig. 2). VHL, a component of a ubiquitin ligase that regulates HIF-1α degradation, is likely a component of the pathway (Bruick and McKnight, 2001; Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Yu et al., 2001). Loss of VHL function in chondrocytes increases HIF-1α stabilization and leads to its accumulation. As a consequence of
HIF-1α accumulation, the expression of HIF-1 target genes, including VEGF, are upregulated in the growth plate (Fig. 2) (Pfander et al., 2004).

Understanding the detailed reasons for the time difference (E14.5 versus E16.5 in the distal femur/proximal tibia in the mouse) in the initial appearance of cell death in the HIF-1α and the VEGF conditional knockouts may shed light on some mechanistic aspects of the survival pathway. Several potential explanations for this time difference are possible. One possibility is that chondrocyte survival depends on different sets of genes at different stages of development. HIF-1α is known to regulate the expression of genes that have a role in cell survival (Pugh and Ratcliffe, 2003; Semenza, 2003). At early stages of development, when VEGF is not expressed by epiphyseal chondrocytes, one set of genes may be needed for cellular survival; later, when developmental needs are different, a different set, including VEGF, may be necessary. A second possibility is that chondrocytes, in response to increased need for oxygen and nutrients as the cartilage grows, initially make adjustments to increased metabolic stress by activating pathways that are controlled by HIF-1 without the need for VEGF. Examples of such adjustments could be an increase in glycolysis and increased levels of glucose transporters in the cell membrane. As growth continues, and the need for oxygen and nutrients increases as well, a better blood supply may become necessary. VEGF could then play a key role in regulating an angiogenic response. Such a two-step mechanism could explain the time difference in the initial appearance of cell death between the HIF-1α and the VEGF conditional knockouts. A third possibility is that the induction of expression of HIF-1α and its target genes is VEGF-independent, but that VEGF is required for maintaining expression of HIF-1α and/or its target genes.

Elucidation of these possibilities will require definitive identification of the signals that activate the chondrocyte survival pathway and insights into the nature of the downstream cellular processes that the pathway controls. The nonvascular environment in epiphyseal cartilage and the essential roles of HIF-1 and VEGF in hypoxic response mechanisms in many other tissues have led some investigators to propose that hypoxic conditions may activate the pathway. In fact, several lines of evidence favor this possibility, but there is also evidence to support the conclusion that hypoxia is not the main regulator of the pathway in chondrocytes. Among indications that hypoxia might be involved is the detection of EF-5 as a marker for bioreductive activity in the epiphyseal cartilage (Schipani et al., 2001). Further support comes from a study of mice that only express the VEGF188 isoform. In these mice, the number of blood vessels surrounding epiphyseal regions appears to be reduced, and the EF-5 signal is more broadly distributed within epiphyseal cartilage than in wild-type littermates (Maes et al., 2004). This suggests that the epiphyseal chondrocyte cell death in VEGF188 mice may well be caused by hypoxia. Moreover, chondrocytes
cultured under hypoxic conditions upregulate VEGF expression via HIF-1α (Pfander et al., 2003).

Good arguments can also be made against hypoxia as a major regulator. The strongest argument is that chondrocytes in culture do survive under hypoxic conditions. Moreover, elimination of the expression of both VEGF and HIF-1α in such cultures does not affect cell survival (Pfander et al., 2003; Zelzer, unpublished results). It is possible, therefore, that the HIF-1/VEGF pathway in epiphyseal chondrocytes is an essential component of a genetic program induced by an as yet unknown regulator, turned on to support chondrocyte survival in a specific area of cartilage and at a specific developmental time. Although HIF-1α is best known as a key component in mechanisms that mediate cell responses to hypoxic stress, a growing body of evidence suggests that HIF-1α is a key factor in mediating the induction of many genes, including VEGF, in response to hormones and growth factors (Zelzer et al., 1998).

The nature of the cellular events regulated by the HIF-1/VEGF pathway in epiphyseal chondrocytes is largely unknown. The reduced numbers of blood vessels surrounding epiphyseal cartilage in the VEGF188 mice (Maes et al., 2004) indicate that VEGF may affect peri-epiphyseal angiogenesis. However, this local reduction in vessel numbers around epiphyses could also be the consequence of abnormalities in the tissue surrounding the cartilage since the expression of the VEGF188 isoform is not restricted to chondrocytes in these mice. Histomorphometric examination of vessels surrounding epiphyseal cartilage in mice with a targeted deletion of VEGF in chondrocytes should help clarify this tissue. It is also possible that VEGF has a direct effect on chondrocytes. In VEGF188 limbs cultured under hypoxic stress, some chondrocytes in the resting zone of growth plates became apoptotic. Exogenous VEGF164 was able to rescue this cell death (Maes et al., 2004). However, to firmly establish whether VEGF has a direct effect on chondrocytes and to understand the mechanisms of action of the HIF-1/VEGF pathway, it will be essential to identify the receptors that mediate the VEGF effects on chondrocytes. The finding of NRP1 and NRP2 expression in epiphyseal chondrocytes makes these receptors potential candidates for mediating actions of VEGF in chondrocytes (Maes et al., 2004; Zelzer et al., 2004).

V. A Role for VEGF in Control of Osteoblastic Activity

Several factors with important roles in regulating bone formation also induce the expression of VEGF by osteoblasts. Prostaglandins E1 and E2, BMP-4, BMP-6, BMP-7, FGF-2, TGF-β, endothelin-1, IGF-1, and vitamin D3 can all induce VEGF expression in osteoblasts by activating a variety of signaling pathways (Akeno et al., 2002; Deckers et al., 2002; Harada et al.,
This raises the possibility that VEGF itself may be involved in regulating osteoblastic activity. VEGF could play a role in osteoblast biology in several different ways. First, VEGF, expressed by osteoblasts, could couple angiogenesis to bone formation by adjusting the angiogenic response to osteoblastic activity. Second, VEGF could serve as a messenger in bilateral regulation. By expressing VEGF, osteoblasts could induce cells in the vicinity to express factor(s) that, in turn, regulate osteoblastic activity. Third, VEGF could be an autocrine regulator of osteoblastic differentiation and activity. There is current evidence in support of all three possibilities.

The first study describing a role for VEGF in coupling angiogenesis to bone formation was the study of Gerber et al. (1999) in which inhibition of VEGF in 24-day-old mice resulted in impaired angiogenesis and impaired trabecular bone formation. Our study of skeletogenesis in VEGF120 mice extended this to embryonic bone development (Zelzer et al., 2002). This study also demonstrated that VEGF has a direct effect on osteoblastic activity. The ossification of membranous bones was reduced and osteoblastic differentiation was altered in VEGF120 mice. Since membranous bones are formed directly in mesenchyme without cartilage intermediates and without the need for invasion of blood vessels into cartilage, as in endochondral ossification, the identification of VEGF effects on osteoblastic activities in membranous bones of VEGF120 mice permits a distinction to be made between direct and indirect effects. Consistent with direct effects, VEGF is initially expressed in craniofacial mesenchyme at E13.5; at E14.5, the expression gets stronger and more restricted to the region where mesenchymal cells are differentiating into osteoblasts.

Further support for a regulatory role of VEGF in osteoblastic differentiation has been obtained by cell culture and bone explant studies. Human VEGF165 protein binds to osteoblasts in culture and is capable of inducing migration and alkaline phosphatase activity (Midy and Plouet, 1994). Moreover, exogenous VEGF stimulates mineralization of osteoblast cultures. Adding VEGF or its neutralizing receptor to the medium of calvarial explant cultures enhances or blocks bone growth (Zelzer et al., 2002). Finally, VEGF receptors VEGFR-1, VEGFR-2, and neuropilin are all expressed by osteoblasts (Harper et al., 2001; Street et al., 2002).

VI. VEGF is Involved in Bone Repair

Bone fractures can heal in two different ways, similar to the two ways of forming bone during embryonic development. Stabilized fractures will heal by intramembranous ossification; unstable fractures undergo endochondral
ossification. The similarity between the embryonic bone development and repair of fractured bones, coupled with the finding that VEGF is expressed at sites of bone fracture, suggests that VEGF is involved in bone repair as it is in bone development (Ferguson et al., 1999; Le et al., 2001; Probst and Spiegel, 1997; Vortkamp et al., 1998).

A study by Street et al. (2001) demonstrates that VEGF is indeed a key regulator of bone repair. Inhibition of VEGF by administration of a soluble chimeric VEGF receptor Flt-IgG protein to mice during fracture repair impairs bone regeneration. Blocking VEGF affects cartilage formation (soft callus) and its replacement by bone (hard callus). Moreover, the healing of cortical bone defects is also affected. Whereas the blocking of endogenous VEGF attenuates bone repair, exogenous VEGF accelerates bone repair. The role of VEGF in the healing process involves regulation of the vascularity of the healing fracture as well as a direct effect of VEGF on osteoblastic differentiation and activity. As the fractured site is believed to be under hypoxic tension, the finding that osteoblastic cultures under hypoxic conditions produce VEGF suggests the possibility of an autocrine loop that regulates osteoblastic activity at the repair site. Another possible source of VEGF at the fracture site is the soft callus and fracture hematoma (Street et al., 2001).

Support for the role of VEGF in the process of soft callus ossification comes from a study by Colnot et al. (2003). This study also underscores the similarity between the genetic control of bone development and regeneration following bone fracture. During endochondral ossification of MMP-9 null bones, there is a reduction in metaphyseal angiogenesis, the zone of hypertrophic chondrocytes is expanded, and there is decreased ossification. In the nonstabilized model for bone fracture, MMP-9 null bones exhibit a delay in hypertrophic callus remodeling and bone formation (Colnot et al., 2003); exogenous VEGF can rescue this delay.

VII. Future Directions and Questions

Based on studies reviewed here, it is clear that VEGF is a major regulator of bone morphogenesis. In retrospect, the connection between VEGF and angiogenesis during endochondral bone formation may not seem very surprising based on the well-documented involvement of VEGF in angiogenesis generally, but the finding that VEGF is critically important for several other aspects of bone morphogenesis is surprising and exciting. In this chapter, we have described evidence for a role of VEGF in a variety of cellular processes in skeletal tissues, including migration, differentiation, and survival (Fig. 4).
Most of the current understanding of the different roles of VEGF in bone development is derived from experiments in which VEGF activity was manipulated. Since VEGF is a secreted cytokine that binds to different receptors on various target cells, it is difficult to extrapolate from these experiments to the precise mechanism of action by which VEGF regulates cellular processes in skeletal tissues. More specifically, most of the current data do not allow a distinction to be made between autonomous and nonautonomous cell functions of VEGF. The best way to address this problem in future studies will be to identify and manipulate the activity of the responsible receptors that mediate the effects of VEGF on the target cells. This approach will help identify both the target cells and the cellular events regulated by VEGF in these cells, and should help provide answers to four important questions.

How does VEGF orchestrate the processes of endothelial and osteoclastic cell migration into hypertrophic cartilage during endochondral ossification?

What are the conditions/signaling events that induce the chondrocyte survival pathway, and what are the cellular responses in chondrocytes controlled by this pathway?

At what timepoint in osteoblastic differentiation is VEGF critical, and what are the cellular activities promoted by VEGF in osteoblasts?

To what extent do the different isoforms of VEGF contribute to the different receptor-mediated responses in target cells?

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References


6. Multiple Roles of VEGF


