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Role of HIF-1α in skeletal development

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Abstract

Angiogenesis and osteogenesis are tightly coupled during bone development and regeneration. Mesenchymal cells in the developing stroma elicit angiogenic signals to recruit new blood vessels into bone. Reciprocal signals, likely emanating from the incoming vascular endothelium, stimulate mesenchymal cell specification through additional interactions with cells within the vascular stem cell niche. The hypoxia-inducible factor-1 alpha (HIF-1) pathway has been identified as a key component in this process. We demonstrated that overexpression of HIF-1 in mature osteoblasts through disruption of the von Hippel-Lindau protein profoundly increases angiogenesis and osteogenesis; these processes appear to be coupled by cell nonautonomous mechanisms involving the action of vascular endothelial growth factor (VEGF) on the endothelial cells. The same occurred in the model of injury-mediated bone regeneration (distraction osteogenesis). Surprisingly, manipulation of HIF-1 does not influence angiogenesis of the skull bones, where earlier activation of HIF-1 in the condensing mesenchyme upregulates osterix during cranial bone formation.

Keywords

knockout mice; osteoblasts; hypoxia-inducible factor; angiogenesis

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Conflicts of interest
The authors declare no conflicts of interest.

Introduction

The processes of angiogenesis and bone formation are coupled both temporally and spatially during skeletal development and repair. Blood vessels carry oxygen and nutrients to the bone but also play a role in bone formation and remodeling by mediating the interaction between osteoblasts, osteocytes, osteoclasts, and vascular cells. Ossification of the skeleton coincides with robust vessel in-growth. During endochondral bone formation, chondrocytes model the growth plate at the distal ends of the long bones and become hypertrophic and hypoxic. Blood vessel invasion from the metaphyseal region into the avascular cartilage coincides with the formation of bone on the cartilaginous template.[1] The nutrient artery arises from the systemic circulation, enters the diaphysis, and then branches into ascending and descending medullary arteries within the marrow cavity. These vessels are then further subdivided into arterioles, which penetrate the endosteal surface to form the primary supply of the diaphyseal cortex.[2] In the skull, parietal bones are supplied by one major branch of the meningeal artery. From each of these vessels, separate branches supply the dura wherein a fine-vessel network covers the developing bone. As the skull mineralizes, numerous fine vessels penetrate within the periosteum and dura and enter the cortical plates. At each of these skeletal sites, bone does not form unless blood vessels develop.

The critical relationship between angiogenesis and bone formation was demonstrated in early studies by Coolbaugh, who showed that surgical disruption of blood supply to bone produced marked alterations in bone density, tensile strength, and modulus of elasticity.[3] Subsequent work by Trueta and Harrison demonstrated that bone mineralization and the development of the hypertrophic zone in the growth plate were disturbed after the interruption of the blood supply to the growth plate.[4,5] Contemporary studies using chemical or physical approaches to block angiogenesis have provided more direct evidence that vascular invasion into cartilage of the growth plate is necessary for long-bone formation during endochondral ossification.[6]

Angiogenic-osteogenic coupling in bone development

The nature of the cellular and molecular mechanisms responsible for coupling angiogenesis and bone formation remain poorly understood, but a primary driving force is tissue hypoxia. During organogenesis, the orderly programs of differentiation and migration involve hypoxia-driven diffusion of oxygen in the embryo. In turn, molecular responses to oxygen gradients are responsible for the proper differentiation and maintenance of the developing vasculature.[7] Multiple cell types are involved in these processes, including endothelial cells, pericytes, smooth muscle cells, and leukocytes. In addition, an expanding list of factors has emerged that coordinate the angiogenic response.[8,9] Among these factors, VEGF is a well-characterized proangiogenic factor that is activated by hypoxia and plays a critical role in angiogenesis during the development of most tissues including bone.

As long bones form, VEGF is released by hypertrophic chondrocytes and functions to initiate blood vessel invasion into cartilage, a prerequisite for bone growth.[6,10,11] Administration of a soluble VEGF receptor 1 mFlt(1–3)-IgG completely blocked neoangiogenesis in the growth plates of 24-day-old mice and resulted in expansion of the hypertrophic zone and decreased bone mass. These findings suggested that growth plate vascularization is VEGF-dependent.[6] Zelzer and Olsen demonstrated that blood vessels were recruited to the perichondrium of the developing mouse tibia as early as embryonic day (E) 13.5–14.5 through the actions of VEGF produced in perichondrial cells.[12] This action was followed by vessel invasion into the hypertrophic cartilage at E14.5. Consistent with these observations, mice expressing only the soluble isoform of VEGF, $VEGF_{120}$, but lacking $VEGF_{164}$ and $VEGF_{188}$ isoforms, exhibited delayed blood-vessel penetration into the

perichondrium.[11] This finding also suggests that $VEGF_{164}$ and $VEGF_{188}$ might specifically function to coordinate perichondrial angiogenesis. VEGF production in bone cells is regulated by hypoxia as well as a variety of signaling pathways including prostaglandins E1 and E2, transforming growth factor-β(TGF-β), bone morphogenetic proteins (BMPs), fibroblast growth factor 2 (FGF-2), insulin-like growth factor 1 (IGF-1), endothelin-1, and vitamin D_3.[12] Interestingly, each of these factors has been reported to be responsive to the hypoxia-inducible factor-α(HIF-α) pathway. Our recent studies, described below, showed that manipulation of the HIF-1 pathway in osteoblasts, with consequent overproduction of VEGF and other angiogenic factors, stimulated angiogenesis in the long bones that was associated with robust bone formation at the sites of vessel in-growth.

The hypoxia-inducible factor pathway

HIF is an αβ heterodimeric transcription factor that mediates the adaptation of many multicellular organisms to molecular oxygen.[13] The HIF family comprises three α subunits: HIF-1α, HIF-2α and HIF-3α. HIF-2α and HIF-3α have limited homology with HIF-1α, but all three subunits share the conserved pVHL-binding domain and are consequently regulated by hypoxia in the same way as HIF-1α.[14] HIF-1α and HIF-2α can at times function redundantly to promote the expression of the same set of target genes.[15] The HIF-1β subunit (also known as the aryl hydrocarbon receptor nuclear translocator, ARNT) is constitutively expressed in the nucleus in an oxygen-independent manner.[16] The abundance of the α subunits becomes elevated during hypoxia through a regulated proteolytic process. HIF-α subunits contain an oxygen-dependent degradation (ODD) domain, which contains prolyl residues that are recognized and hydroxylated by specific prolyl hydroxylase domain (PHD) enzymes.[17] Under normoxia, prolyl hydroxylation at residues 402 and 564 within the ODD domain mediates the binding of the E3 ubiquitin ligase pVHL, a component of the complex that targets HIF-α for proteasomal degradation.[18] Hydroxylation of HIF-α requires molecular oxygen and iron, and is inhibited by hypoxia. Under these conditions, the HIF-α subunit accumulates in the cytoplasm and then translocates to the nucleus, where it dimerizes with the HIF-1β subunit. This dimer then binds to a highly conserved hypoxia-response element (HRE) within promoters of hypoxia-responsive genes.[19] Genes containing functional HREs encode proteins involved in angiogenesis (VEGF, endothelin-1), maturation of red blood cells (erythropoietin, transferrin), energy metabolism (glucose transporter 1 and 3), and cell proliferation and viability (insulin-like growth factor 2, p21).[20]

In addition to hypoxia, proinflammatory cytokines, growth factors and biomechanical stimuli are also known to regulate the HIF pathway. For example, the proinflammatory mediator, nitric oxide (NO), has been shown to promote HIF-1α activation under normoxic conditions.[21] HIF-1α is highly expressed in cells treated with insulin-like growth factor 1 and 2, fibroblast growth factor 2, or epidermal growth factor in relationship to cell proliferation,[22] and HIF-1 DNA binding is stimulated by proinflammatory cytokines or insulin.[23,24] Mechanical stretch induces HIF-1α accumulation in myocytes through activation of the PI3K/AKT/mTOR pathway in rat myocardium. Similarly, HIF-1α is upregulated in smooth muscle following experimental distension of rat aorta[25] and in vascular smooth muscle cells subjected to cyclical stretch.[26] Both biomechanical and proinflammatory signals are generated in skeletal tissue after injury and therefore may contribute to the acute induction of HIF-1α during bone regeneration.

Developmental functions of HIFα depend on the skeletal context

Because of the established role of HIF-1 in sensing and responding to oxygen and nutrient demands in a variety of cell types, we hypothesized that HIF-1 functions during bone development to promote skeletogenesis. We tested this idea using a genetic approach to

determine the cellular and molecular effects of gain or loss of HIF-1 function by conditional mutagenesis in mouse osteoblasts.[27,28] Mice overexpressing HIFs by disrupting VHL (referred to herein as ΔVHL) showed striking and progressive increases in bone volume, whereas the diameter of the ΔHIF-1 mutant bones was reduced relative to the controls. Importantly, the amount of bone in the axial skeleton of these two mutants was directly proportional to the amount of skeletal vasculature. These observations suggested the possibility that loss of pVHL with consequent upregulation of HIFs in osteoblasts increased the production of angiogenic factors which promoted bone formation secondarily to increasing angiogenesis. Consistent with this idea, the expression of VEGF mRNA was upregulated in the trabecular bone of ΔVHL femurs.

Surprisingly, manipulation of HIF-1 levels in osteoblasts using the OC-Cre mouse did not influence the formation of the flat bones of the skull. As reviewed above, the calvarial bones are formed through an intramembranous process involving condensing mesenchymal cells derived from the neural crest. These precursor cells appear to be able to differentiate directly into osteoblasts without first forming a cartilaginous intermediate.[29] This difference in the development programs and embryologic origin of the bone precursor cells responsible for endochondral and intramembranous bone might explain the site-specific skeletal phenotype of the VHL deficient mice.

To test this idea further we created a mouse lacking HIF-1 in early mesenchymal cells using a dermo-1 promoter-driven Cre transgenic mouse in which Cre was expressed in the condensing mesenchyme.[30] These mice had skeletal defects in the spine, shortened ribs and limbs, but no missing bones. Endochondral bone development was impaired, as evidenced by the shortening of E14.5 tibias. By E18.5, there was further disorganization of the growth plates accompanied by complete failure of chondrocyte hypertrophy. Striking defects in intramembranous bone were also observed. The mutant mice had smaller, less-mineralized skulls compared to controls with diminished parietal bone mineralization and widened sutures. These defects were first noted at day E15.5 and persisted in the newborn mice. Close inspection of skulls from the dermo1-$Cre^{+/-}$, HIF-1 fl/fl mutants showed only a modest difference in vascularization compared to control littermates. By contrast, the mutant mice exhibited a pronounced defect (or delay) in the mineralization of the parietal bones of the cranial vault. *In situ* hybridization of candidate genes expressed by developing osteoblasts revealed a decrease in osterix, Runx2, Col-1 and osteocalcin mRNA compared to that observed in control littermates. Analysis of Runx2 and osterix promoters revealed two consensus hypoxia-responsive elements (HRE binding motifs) in the proximal osterix promoter, whereas none were evident in the Runx2 promoter. To determine whether osterix is a direct target for HIF-1, C2C12 cells were co-transfected with pHAHIF-1α plasmid and osterix promoter luciferase reporter excluding (OSX-71) or including (OSX-1269) HIF-1α binding sites or pGL3 vector as control, and then incubated for 24 h under normoxic and hypoxic conditions. Under these conditions, the activity of OSX-1269 was significantly increased under both normoxic and hypoxic conditions. Chromatin immunoprecipitation (ChIP) assays using lysates from primary osteoblasts were cultured under either normoxic or hypoxic conditions for 12 h. Interaction of HIF-1 with the osterix promoter was confirmed by RT-PCR and quantified by real-time PCR. These data strongly suggest that HIF-1 influences mesenchymal cells to differentiate along the osteoblast pathway, in part though its ability to activate osterix gene expression. When considered together with the effects of HIF manipulations in the long bones described above, it appears that HIF-1 is essential for both endochondral and intramembranous bone formation, but that it functions differently at different stages of skeletal development as suggested by previous studies.[31–33]

Conclusion

The studies summarized above support a conceptual model in which HIF-1 is a key molecule that couples angiogenesis to bone formation.[34] In this model, mesenchymal cells at the surface of developing long bones sense reduced oxygen and or nutrient levels and upregulate HIF subunits. HIF-1 targets such as VEGF and other angiogenic mediators are produced in close proximity with the stroma. In this context, it is also possible that HIF-1 influences the development of the osteoblast-vascular niche.[35] Thus, reciprocal signals generated by the vascular endothelial cells, or alternatively from a distinct subendothelial cell population such as CD146 positive cells,[36] likely have an impact on resident pre-osteoblasts that causes them to mature and divide. This process is exponential, with ever-increasing numbers of new blood vessels inducing more osteoblast progenitors, which then mature and function to form more individual bone formation units (niches). Other stimuli might mediate angiogenic-osteogenic coupling after bone injury, a tissue setting replete with inflammatory mediators. At other skeletal sites such as the calvaria, the relationship of angiogenesis and osteogenesis appears to be different. In this setting, it appears that HIF-1 acts to directly stimulate bone progenitor cell differentiation. Current studies are being designed to elucidate the functional mechanisms underlying HIF-1/VEGF-stimulated angiogenesis and osteogenesis.

Acknowledgments

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