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Research Article

Hypoxia inhibits the growth, differentiation and bone-forming capacity of rat osteoblasts

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ABSTRACT

We investigated the effect of hypoxia on rat osteoblast function in long-term primary cultures. Reduction of pO_2 from 20% to 5% and 2% decreased formation of mineralized bone nodules 1.7-fold and 11-fold, respectively. When pO_2 was reduced further to 0.2%, bone nodule formation was almost abolished. The inhibitory effect of hypoxia on bone formation was partly due to decreased osteoblast proliferation, as measured by ^{3}H-thymidine incorporation. Hypoxia also sharply reduced osteoblast alkaline phosphatase (ALP) activity and expression of mRNAs for ALP and osteocalcin, suggesting inhibition of differentiation to the osteogenic phenotype. Hypoxia did not increase the apoptosis of osteoblasts but induced a reversible state of quiescence. Transmission electron microscopy revealed that collagen fibrils deposited by osteoblasts cultured in 2% O_2 were less organized and much less abundant than in 20% O_2 cultures. Furthermore, collagen produced by hypoxic osteoblasts contained a lower percentage of hydroxylysine residues and exhibited an increased sensitivity to pepsin degradation. These data demonstrate the absolute oxygen requirement of osteoblasts for successful bone formation and emphasize the importance of the vasculature in maintaining bone health. We recently showed that hypoxia also acts in a reciprocal manner as a powerful stimulator of osteoclast formation. Considered together, our results help to explain the bone loss that occurs at the sites of fracture, tumors, inflammation and infection, and in individuals with vascular disease or anemia.

Introduction

It has long been recognized that angiogenesis is an essential prerequisite for osteogenesis. The processes of endochondral bone formation and fracture repair are dependent upon the invasion of blood vessels [1,2], and exercise-induced bone formation occurs in association with a rich capillary blood supply [3]. However, the effect of oxygenation on the function of osteoblasts, the bone-forming cells, has received little direct attention.

Hypoxia occurs when the blood supply to tissues is reduced or disrupted. Oxygen tension (pO_2) in arterial blood is about 95 mm Hg (~12%), and in venous and capillary blood it is about 40 mm Hg (~5%), approximately a quarter of that in atmospheric air. In normal tissues, median interstitial pO_2 values range from 24 mm Hg to 66 mm Hg (~3–9% O_2) [4,5]. Measurements of bone marrow aspirates from normal human volunteer donors yielded mean pO_2 values of 6.6% [6]. In environments such as the poorly vascularized yellow fatty bone marrow of the elderly, or in inflamed or infected tissue,

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arthritic joints, tumors, wounds, ischemic limbs of diabetics and fracture sites, pO_2 may be considerably lower [4]. In rabbits, measurement of pO_2 within the hematoma 4 days post-fracture indicated mean values of 0.8% O_2, and within the newly formed fibrous bone 2 weeks post-fracture only 3.8% [7].

Lack of oxygen can result in a failure to generate sufficient ATP to maintain essential cellular functions, whereas excess oxygen (hyperoxia) results in the generation of damaging reactive oxygen intermediates. Thus, cellular oxygen concentrations must be tightly regulated within a narrow physiological range. Oxygen-sensitive gene expression is controlled by the hypoxia inducible transcription factor (HIF), which is a heterodimer containing α and β subunits [8]. The oxygen-dependent degradation of HIFα is performed by a family of HIF prolyl hydroxylases, which target conserved proline residues in the HIFα subunit for hydroxylation utilizing molecular oxygen and 2-oxoglutarate, with Fe^{2+} as a cofactor [9]. The hydroxyproline residues are the molecular target for the Von Hippel-Lindau tumor suppressor protein (pVHL), which drives the polyubiquitination of HIFα, targeting it for proteasomal degradation [10]. In low oxygen conditions, HIFα protein is stabilized, dimerizes with HIFβ, and binds hypoxia response elements (HREs) in target gene promoter sequences. These target genes are involved in a variety of cellular processes including angiogenesis (e.g., VEGF), energy metabolism (e.g., glucose transporters 1 and 3), cell proliferation and survival (e.g., IGF-2), pH control (e.g., carbonic anhydrase 9), vasomotor control (e.g., NOS-2), and matrix metabolism (e.g., prolyl 4-hydroxylase) [11]. The oxygen-dependent prolyl hydroxylases that act upon HIFα are members of the same protein family that contains the procollagen prolyl 4-hydroxylase, an enzyme essential for collagen formation [12].

The organic matrix of bone consists of approximately 90% type 1 fibrillar collagen [13]. Collagen is a heterotrimer consisting of two α1 subunits and one α2 subunit [14]; these are synthesized as propeptides that undergo a variety of post-translational modifications to create mature, fibrillar collagen. The initial modification is the hydroxylation of several proline residues, performed by procollagen prolyl 4 hydroxylase (P4OH), the resultant hydroxyproline residues being essential for stable triple helix formation [15]. Like its HIF-modifying counterparts, P4OH also requires molecular oxygen for enzymatic activity [12]. Further hydroxylations are then performed on certain lysine residues by the enzymes procollagen-lysine, 2-oxoglutarate, 5-dioxygenase 1–3 (PLOD1–3), in preparation for secretion into the extracellular space and subsequent cleavage of propeptides [16]. Cleavage of the propeptides renders the collagen triple helix insoluble, and it spontaneously assembles into fibrils, which are then acted upon by lysyl oxidase (LOX) to create covalent cross-links between adjacent lysine and hydroxylysine residues [15]. This binds the fibrils and provides the tensile strength to the collagen fibers. The PLOD and LOX enzymes are also dependent on molecular oxygen for their activity [17].

In the skeleton, both chondrocytes and osteocytes occupy matrix-bound sites that are normally avascular; hypoxia is reportedly required for growth arrest and survival of chondrocytes [18] and implicated in mediation of mechanotransduction by osteocytes [19,20]. Diverse responses to hypoxia have been reported for cultured osteoblasts, including increased synthesis of vascular endothelial growth factor (VEGF), insulin-like growth factor II (IGF-II), and transforming growth factor β1 (TGF β1) [21–24], as well as decreased expression of Runx2/Cbfa1 [25,26]. Published data on the effects of hypoxia on bone marrow mesenchymal stem cells include stimulation of fibroblast proliferation [27], increased osteogenic potential of subsequent ectopic implants [28], and stimulation of adipogenesis [29]. In mature osteoblast cultures, 24-h exposure to 1% O_2 was reported not to affect bone nodule formation (or Runx2 expression), although 24-h anoxia was inhibitory [30]. A recent report has indicated that cyclical induction of HIF followed by angiogenic factors such as VEGF may play a critical role in the process of distraction osteogenesis [31].

Disorders such as obstructive pulmonary disease, anemias, tumors, diabetes, and arthritis tend to create chronic tissue hypoxia. We have recently shown that formation of osteoclasts, the bone-resorbing cells, increases strongly with chronic exposure to low ambient oxygen tensions [32]. However, the long-term effects of hypoxia on the function of osteoblasts, the bone forming cells, have received little direct attention. Because active, pathological bone loss often occurs at sites where pO_2 is low, we examined the effect of oxygen tension on the growth and function of osteoblasts, using mineralized bone nodule formation as a key end-point.

Materials and methods

Reagents

Culture medium and buffers were purchased from Gibco (Paisley, UK). Cylinders containing custom mixtures of O_2, CO_2, and N_2 were purchased from BOC Gases (London, UK). All other reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

Bone nodule formation assay

Primary rat osteoblastic cells were obtained by sequential enzyme digestion of excised calvarial bones from 2-day-old neonatal Sprague–Dawley rats using a three-step process (0.25% trypsin for 10 min, 0.2% collagenase type II for 30 min, and 0.2% collagenase type II for 60 min at 37°C), rejecting the first two digests. The cells were washed and plated into a T-75 flask in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum/2 mM L-glutamine/100 U/ml penicillin/100 μg/ml streptomycin/0.25 μg/ml amphotericin (from here on referred to as DMEM). When confluent, cells were removed from the flask using 0.25% trypsin, washed, and counted before being placed into 24-well trays containing 1-cm diameter Melanex discs (Dupont Teijin Films, Dumfries, UK) at a density of 5×10^4 cells/well in DMEM. After 24 h, discs bearing cells were transferred to 25-cm^2 flasks with polyethylene 'plug-seal' caps (Falcon, Becton Dickinson, Oxford, UK), containing 8 ml of DMEM supplemented with 50 μg/ml ascorbate/10^{-8}M dexamethasone/2 mM β-glycerophosphate (six discs per flask). The flasks were then flushed for 2 min with gas mixtures containing 5% CO_2 and 20, 12, 5, 2, 1, or 0.2% O_2 (balance N_2) via a 21-gauge needle inserted through the

loosened polyethylene cap. The cap was then tightened, and the needle hub was closed with a Luer plug. The sealed flasks were incubated at 37°C in a standard incubator containing 5% CO_2/95% atmospheric air and regassed daily. Medium was changed every 3–4 days. Culture medium pH, PCO_2 and pO_2 were monitored at each medium change and at the end of experiments using a clinical blood gas analyzer (ABL 705, Radiometer, Copenhagen, Denmark). Oxygen tensions in the hypoxic range (which is below the measurement capability of blood gas analyzers) were monitored using a fluorescence-based oxygen probe ('FOXY', Ocean Optics, Duiven, Netherlands).

Mineralized bone nodules were visualized by staining with alizarin red S (Sigma, Poole, UK) to detect calcium. Briefly, discs in flasks were washed with PBS and fixed for 3 min in 2.5% glutaraldehyde. Discs were washed three times in 70% ethanol and left to dry inverted for 30 min. 1% w/v alizarin red S in water was then added to flasks for 3–5 min, and discs were washed three times in 50% ethanol and dried before quantification of nodule area. Nodule area was determined by imaging discs at 800 dpi using a high-resolution flatbed scanner (Epson Perfection Photo 3200, Epson, Hemel Hempstead, UK). Images were converted to binary form, and the plan surface area of mineralized bone nodules was determined using image analysis software (Scion Corporation, Frederick, MD, USA; http://www.scioncorp.com/) at constant threshold level and minimum particle size (1 pixel) as described previously [33].

Proliferation assay

Primary osteoblasts were prepared as described above and cultured directly into 24-well trays. Trays were placed into gas-tight fuse boxes which were flushed for 2 min with gas mixtures containing 5% CO_2 and 20 or 2% O_2 (balance N_2) through holes drilled in the lid. Cell proliferation was determined as ^{3}H-thymidine incorporation into deoxyribonucleic acid (DNA), essentially as described previously [34]. Calvarial osteoblasts were labeled with 1 μCi/ml of [6-^{3}H] thymidine (22.0 Ci/mmol; 814 GBq/mmol) for the final 6 h of culture. Cell layers were washed three times with phosphate-buffered saline (PBS) containing 1 mM unlabeled thymidine and then detached using 0.25% trypsin for 5 min at 37°C, and precipitated with 7.5% trichloroacetic acid (TCA) in the presence of 0.2% bovine serum albumin (BSA), followed by centrifugation at 1500×*g*. Pellets were washed with 7.5% TCA/0.2% BSA, recentrifuged, and then digested overnight at 37°C in 0.2 M NaOH and mixed with scintillation cocktail (Wallac Optiphase Hisafe II, Fisher Scientific, Loughborough, UK) for scintillation counting.

Apoptosis assay

Osteoblast apoptosis was assessed using a kit (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics Ltd., Lewes, East Sussex, UK) according to the manufacturer's protocol. This assay measures cytoplasmic histone DNA fragments (mono- and oligonucleosomes) produced after cell death by using mouse monoclonal antibodies against histones and DNA. Briefly, cells were lysed in situ after being cultured in supplemented medium, for 6, 12, and 18 days in 20% or 2% O_2. Mono- and oligonucleosomes released into the cytoplasm were detected by measuring the absorbance at 450 and 490 nm of pooled lysates; duplicate determinations were made. The results were normalized to osteoblast numbers in parallel wells, assessed using a hemocytometer, after sequential treatment with 0.2% collagenase type II for 30 min, and 0.25% trypsin for 10 min to release cells.

Alkaline phosphatase assay

Cells were grown in 24-well trays as described above. Monolayers were washed in PBS and then harvested by scraping into 500 μl of water. The resulting cell suspensions were sonicated at 4°C for 10 min and centrifuged at 500×*g* for 10 min at 4°C; alkaline phosphatase activity and total Lowry protein content of supernatants were determined using kits (Biotron Diagnostics, Hemet, CA, USA; Kit P-5656, Sigma, Poole, UK), according to the manufacturers' instructions.

Transmission electron microscopy

Primary osteoblasts were prepared on Melanex discs as described above and cultured for 35 days in supplemented DMEM in normoxic or hypoxic conditions. Discs were transferred from flasks into 24-well plates, fixed in 2.5% glutaraldehyde, and decalcified by overnight immersion in 1.9% glutaraldehyde 0.15 M EDTA in phosphate-buffered saline (PBS) at 4°C. Discs were rinsed in 0.1 M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide and stained with 1% uranyl acetate before being dehydrated through a graded ethanol series. Ethanol was then replaced with dessicated, degassed embedding resin (agar 100: dodecenyl succinic anhydride: methyl nadic anhydride: *N*-benzyldimethylamine) through a graded series of ethanol:resin mixtures. The resin was cured at 60°C for 48 h, and thin sections (80 to 90 nm) were cut using a diamond knife (Diatome) on a Reichert Ultracut E ultramicrotome. The sections were collected on 300 mesh grids, counterstained with lead citrate, and viewed in a JEOL 1010 transmission electron microscope.

Collagen analysis

Melanex discs with their accumulated collagenous matrix were incubated with 0.5 M acetic acid, pH 2.0, containing pepsin (1 mg/ml; Sigma, Poole, UK) at 4°C for 24 h with gentle agitation. Insoluble material was removed by centrifugation, and the residue was extracted similarly with pepsin for a further 24 h. Collagenous proteins in the combined supernatant solutions were precipitated with NaCl at a final concentration of 1.2 M and collected by centrifugation. Both pepsin-solubilized, precipitated collagen and the insoluble matrix fraction were separately hydrolyzed in 6 M HCl at 107°C for 22 h, after which time the acid was removed by evaporation. Hydroxylation of proline and lysine was assessed by quantifying Hyp, Pro, Hyl, and Lys using an amino acid analyzer (Waters Pico-Tag™ instrument, Manchester, UK). Total collagen was calculated assuming 300 mol Hyp per mole collagen.

Reverse transcriptase polymerase chain reaction (RT-PCR)

RNA for RT-PCR was isolated from primary osteoblasts grown on Melanex discs in gassed 25-cm^2 tissue culture flasks. Monolayers were washed briefly in PBS and 1 ml of TRIzol reagent (Invitrogen, Paisley, UK) was added to each flask and processed according to the manufacturer's instructions. RNA pellets were dissolved in 50 μl of diethyl pyrocarbonate treated water, and 4 μl of total RNA was utilized for cDNA production using the ImPromII Reverse Transcription System (Promega, Southampton, UK), according to the manufacturer's instructions. cDNA levels were normalized to beta-actin expression and used in polymerase chain reactions using primer pairs listed in Table 1.

Statistical analysis

Statistical comparisons were made by one-way analysis of variance (ANOVA) using "Instat" software (version 1.3, Graph Pad, Inc, San Diego, CA); representative data are presented as means ± standard error of the mean (SEM). Bonferroni adjustments were made for multiple comparisons. Significance was assumed at $P < 0.05$. Results are presented for representative experiments that were repeated at least 3 times.

Results

Inhibition of bone nodule formation by hypoxia

Abundant bone nodule formation occurred on Melanex discs bearing osteoblasts cultured in 20% oxygen for 18 to 24 days (Fig. 1). Bone nodule formation decreased as pO_2 was reduced (Figs. 1 and 2). When pO_2 was reduced to 12% or 5%, slight reductions in bone nodule area were seen; as pO_2 was reduced further to 2% O_2 and 1% O_2 (moderate hypoxia), bone formation decreased 10-fold until in extreme hypoxia (0.2% O_2) bone nodule formation was almost completely abolished (Fig. 2). Since hypoxia can cause increased acidification of cell cultures, experiments were performed using large volumes of culture medium to provide excess buffering capacity; medium was monitored regularly to ensure that operating pH (typically close to 7.40 before medium changes) did not differ significantly between cultures exposed to different O_2 tensions.

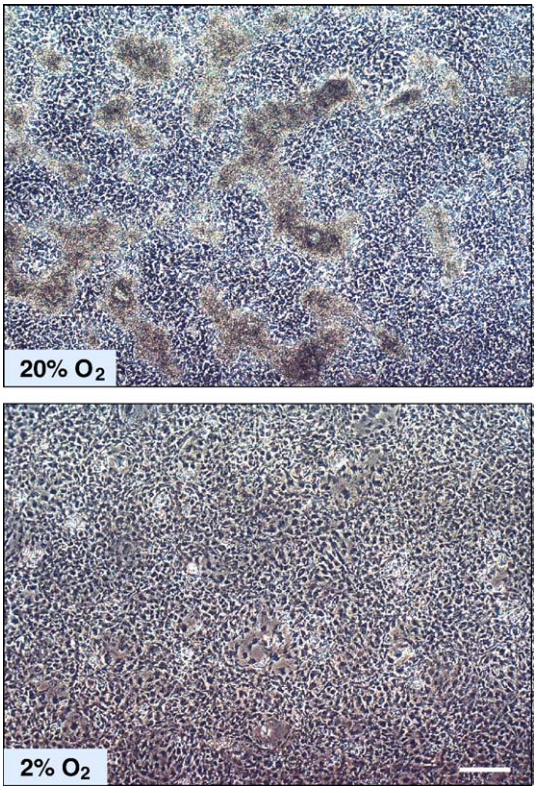

Fig. 1 – Hypoxia inhibits osteoblast growth and bone nodule formation by osteoblasts. Phase contrast micrographs of unstained rat calvarial osteoblasts seeded at an initial density of 5×10^4 cells per well and cultured for 9 days in the presence of 50 μg/ml ascorbate and 2 mM β-glycerophosphate. In 'normoxic' cultures (top), widespread, early bone formation (grey, 'trabecular' features) is evident; however, in hypoxic cultures (bottom), cell numbers are decreased and bone formation is absent. Scale bar = 200 μm.

To determine whether osteoblast function was most sensitive to the inhibitory effect of hypoxia at earlier or later stages of differentiation, osteoblasts were cultured in either 20% or 2% O_2 for 6-day periods for a total of 18

Table 1 – Primer pairs used for RT-PCR analysis of osteoblast mRNA

Gene product	Forward primer 5′–3′	Reverse primer 5′–3′
Beta actin	GTT CGC CAT GGA TGA CGA T	TCT GGG TCA TCT TTT CAC GG
Alkaline phosphatase	CTC ATT TGT GCC AGA GAA	GTT GTA CGT CTT GGA GAC
Osteocalcin	GCA GAC ACC ATG AGG ACC CT	GCA GCT GTG CCG TCC ATA C
$TGF\beta_1$	CGC AAC AAC GCA ATC TAT G	CAT GTC ATG GAT GGT GCC
$TGF\beta_2$	GCA GAG TTC AGG GTC TTT CG	CAC CAC TGG CAT ATG TGG AG
$TGF\beta_3$	TTG CGG AGA GAG TCC AAC TT	CAC ACA GCA GTT CTC CTC CA
PLOD1	CAA GCT GCA GGT GAA CTA C	CCA CAC TGA AGT AGT AGG TGC
PLOD2	CAG TAG ATG TCT ATC CGA	CAG AAC GAG CAT AGT ATC
PLOD3	GAG CCT TAG ATG AAG TGG	TCG TGG TAC ACC TCG TTG
Collagen 1A1	GCG AAG AAG ACA TCC CTG	CTG TCC AGG GAT GCC ATC
P4OH	GAT GCG ACT TCA GGA CAC CT	CCT CTA ACA GCC GCT CAA AG

TGFβ, transforming growth factor beta; PLOD, procollagen, lysine, 2-oxoglutarate, 5-dioxygenase.

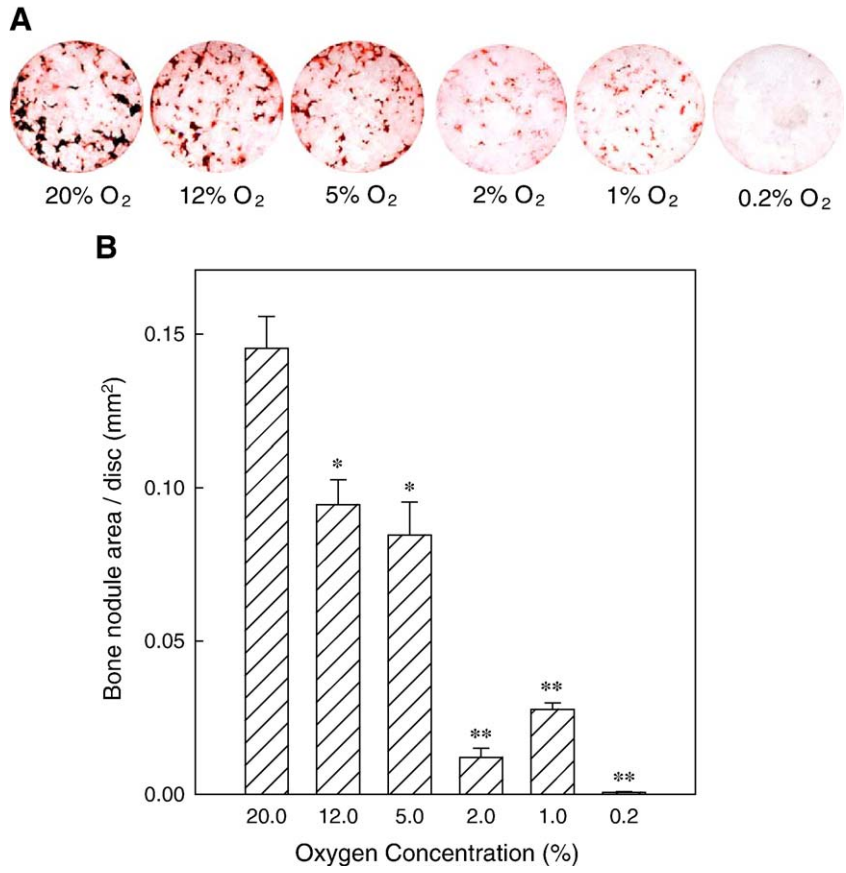

Fig. 2 – Progressive inhibition of mineralized bone nodule formation by osteoblasts with decreasing O_2. (A) Representative low-power images of alizarin red-stained bone nodules formed by rat calvarial osteoblasts cultured for 18 days on 1-cm diameter Melanex discs in sealed tissue culture flasks gassed with 20 – 0.2% O_2/5% CO_2/balance N_2. (B) Bone nodule formation, quantified using image analysis software, was slightly inhibited in 12% or 5% O_2, strongly inhibited in 2% or 1% O_2 (hypoxia) and effectively abolished in 0.2% O_2 (severe hypoxia–ischemia). $^{*}P < 0.5$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. 20% O_2. Values are means ± SEM; $n = 6$.

days (Fig. 3). Exposure to hypoxia (2% O_2) for the first 6 days of culture followed by 20% O_2 for the final 12 days resulted in a 3-fold decrease in bone nodule formation. Exposure to 20% O_2 for the initial 6 days of culture and 2% O_2 for the final 12 days of culture led to a 2-fold decrease in bone nodule formation. The amount of bone nodule formation in cultures subjected to early hypoxia added to that measured in cultures subjected to late hypoxia approached that of cultures held at 20% O_2 continuously, indicating that osteoblasts are able to recover from hypoxic insult.

Inhibition of osteoblast proliferation and alkaline phosphatase activity by hypoxia

The proliferation assay was performed at days 6, 12, and 18; at all time points culture in 2% O_2 caused ~3-fold decreases in OB proliferation, compared with 20% O_2 (Fig. 4A). Levels of osteoblast apoptosis, detected as nucleosome enrichment in cell monolayers, were generally low and increased with time in culture (Fig. 4B). Continuous culture in 2% O_2 for 6, 12, and 18 days did not increase apoptosis, indicating that the lower cell numbers in hypoxic cultures were attributable to decreased proliferation, not increased cell death.

Alkaline phosphatase activity was greatly decreased in hypoxic osteoblasts during the mineralizing phase of culture (Fig. 5). Osteoblasts grown in 20% O_2 showed a robust 3-fold increase in alkaline phosphatase activity from day 6 to day 12 in culture, but in hypoxic cultures, this increase was much reduced.

Effect of hypoxia on collagen ultrastructure and biochemistry

Because the enzymes involved in collagen formation utilize molecular oxygen for their activity, we examined the effect of hypoxia on the ultrastructure of collagen deposited in bone nodules by osteoblasts. Transmission electron microscopy revealed the presence of abundant, dense, regular bundles of collagen fibrils in normoxic (20% O_2) cultures after 35 days. However, in cultures maintained for 35 days in 2% O_2, collagen fibrils were deposited at much lower density and in a less-organized manner (Fig. 6).

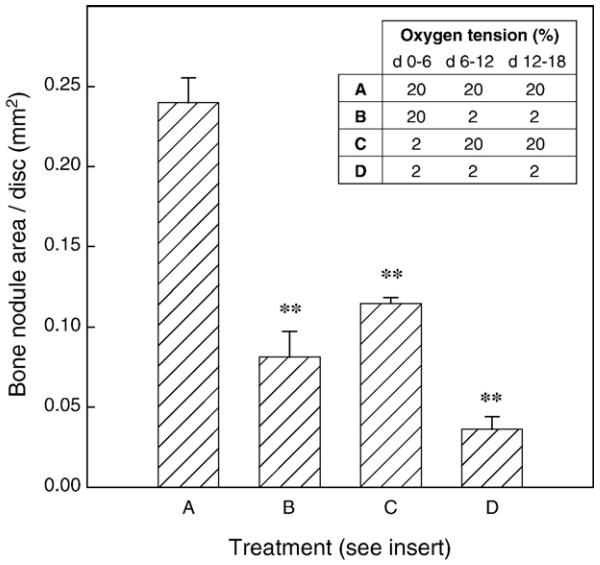

Fig. 3 – Reversibility of the inhibitory action of hypoxia on bone formation by osteoblasts. Cultures were maintained in either 20% or 2% O_2 during the time periods indicated in the key. Exposure to 2% O_2 for the maturation and matrix production stages (days 6 to 18; treatment group B) led to 3-fold reductions in bone nodule area. Exposure to 2% O_2 for the initial proliferation stage (days 0 to 6; treatment group C) resulted in 2-fold reductions in nodule area. The cumulative level of bone nodule formation in treatment groups B and C together approached that observed when osteoblasts were cultured continuously at 20% O_2 (treatment group A). $^{*}P < 0.5$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. treatment group A (20% O_2). Values are means ± SEM; $n = 6$.

We also found that the small amount of collagen deposited in hypoxic cultures was much more susceptible to pepsin degradation than collagen formed in 20% O_2: approximately 95% of collagen formed in 20% O_2 was insensitive to pepsin, compared to only 55% of 2% O_2 collagen. Moreover, hydroxylation of lysine residues was decreased by about 30% in hypoxic collagen, although the proportion of hydroxyproline residues was unaltered (Table 2).

Hypoxia delays expression of osteoblast markers and genes involved in collagen formation

We observed delays in osteoblast differentiation in response to hypoxia (Fig. 7A). Expression of mRNA for osteocalcin, a marker of mature osteoblasts, was strong by day 12 of culture in osteoblasts maintained in 20% O_2, increasing further as cultures matured; however, in hypoxic cultures, osteocalcin expression remained low throughout the culture. A similar effect of hypoxia was observed on the expression pattern of mRNA for alkaline phosphatase.

The 3 procollagen lysine, 2-oxoglutarate, 5-dioxygenases (PLOD1–3) responsible for the formation of hydroxylysine residues were differentially expressed during osteoblast maturation. Hypoxia (2% O_2) caused decreases in expression of all of the PLOD genes (Fig. 7A), in line with the delay observed with ALP and OCN expression. In contrast, expression of mRNAs for TGFβ isoforms was only slightly reduced in osteoblast cultures exposed to acute (3–48 h) or chronic (6–18 days) hypoxia (Figs. 7A and B). Similarly, *COL1A1* and P4OH mRNA levels showed only small decreases in hypoxic osteoblasts (Fig. 7A).

Discussion

The results presented here demonstrate that osteoblast function and bone formation are strongly oxygen-dependent.

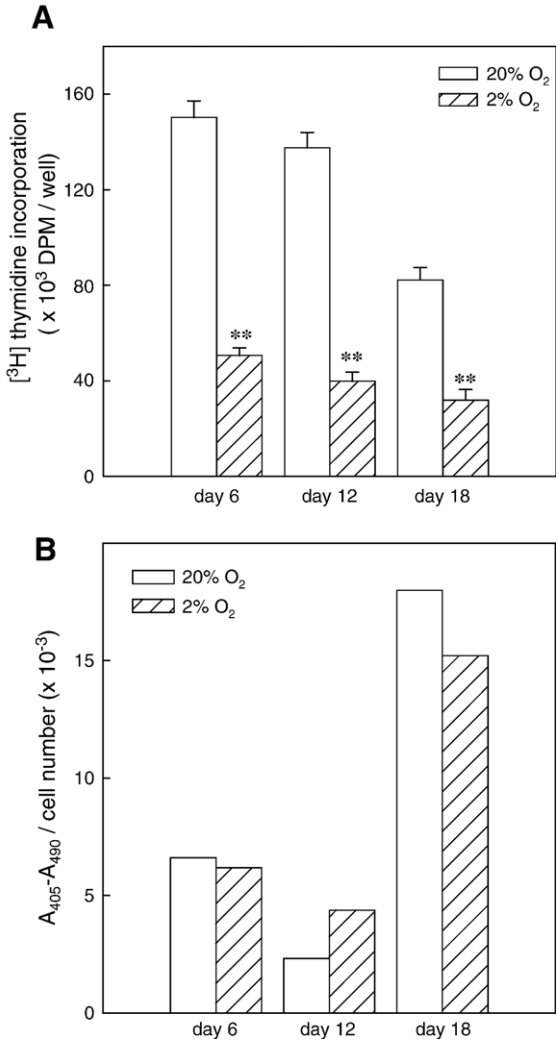

Fig. 4 – Hypoxia inhibits osteoblast proliferation but does not increase apoptosis. (A) Hypoxia inhibits the proliferation of primary rat calvarial OBs at days 6, 12, and 18 as measured by tritiated thymidine incorporation. $^{*}P < 0.5$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. 20% O_2. Values are means ± SEM; $n = 6$. (B) Apoptosis increases with time in culture but is not induced by continuous exposure to low pO_2 (2%). Osteoblasts were grown in 20% or 2% O_2 for 6, 12, or 18 days before apoptosis was determined colorimetrically in pooled cell lysates using enzyme-linked immunosorbent assay (ELISA) that detects cytoplasmic histone DNA fragments; results are normalized to cell number. Values are means of duplicate determinations.

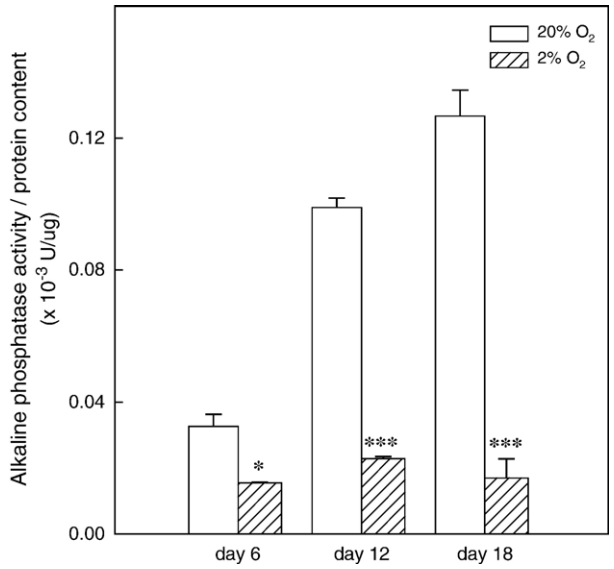

Fig. 5 – Hypoxia inhibits alkaline phosphatase activity in rat calvarial osteoblasts. Alkaline phosphatase activity, normalized to cell protein content, failed to increase from day 6 levels in osteoblast grown continuously in 2% O_2. A robust increase was observed over the same time period for osteoblasts cultured in 20% O_2. $^{*}P < 0.5$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. 20% O_2. Values are means ± SEM; $n = 6$.

Mineralized bone nodule formation by cultured osteoblasts was strongly inhibited when pO_2 was <5% and almost completely prevented when pO_2 was <1%. Bone formation in vivo normally occurs in environments where pO_2 is between 12% and 5% (corresponding to arterial and venous blood, respectively). Thus, atmospheric oxygen levels (i.e., 20% O_2) correspond to hyperoxia; our findings indicate additionally that bone formation by osteoblasts in 20% O_2 (which may be considered as hyperoxia) is stimulated by about 50% relative to the physiological 5–12% O_2 range.

Formation of mature, mineralized bone matrix involves a series of discrete steps. Hypoxia inhibits the proliferation of immature osteoblast precursors, leading to failure to achieve the 'critical mass' of differentiated cells needed for bone formation in vitro. It also prevents the production of mineralized matrix by disrupting collagen formation and alkaline phosphatase activity. Delayed osteoblastic differentiation associated with hypoxia has been reported elsewhere; this effect has been ascribed to decreased expression and activity of the transcription factor, Runx2 [25,26,30]. We also show evidence of delayed osteoblast differentiation in hypoxia through the inhibition of alkaline phosphatase gene expression and protein activity and of osteocalcin gene expression. Alkaline phosphatase (ALP) activity was observed to increase slightly in hypoxic cultures from days 6 to 12; however, expression of ALP mRNA was not detectable at the later time point. The expression of ALP mRNA at day 6 could account for the increase in enzyme activity observed at day 12. Overall, however, ALP mRNA expression and enzyme activity were inhibited strongly in hypoxic osteoblast cultures relative to cells maintained in 20% O_2. A previous report described upregulation of collagen and TGFβ gene expression [24] in short-term cultures of rat calvarial osteoblast cultures exposed to ~5% O_2; in the present study, we observed no obvious changes in gene expression of either collagen or TGFβ in short-term osteoblast cultures exposed to 2% O_2 and modest decreases in longer-term cultures.

To investigate whether, conversely, hypoxia might promote the formation of cartilage, an avascular tissue, we measured the effect of oxygen tension on cartilage formation in micromass cultures of mesenchymal cells from embryonic chick limb buds. However, we observed that in 5% and 2% O_2, cartilage nodule formation was reduced by 3-fold and 5-fold, respectively [35]; these inhibitions were thus of similar magnitude to those observed for bone nodule formation in hypoxia. Analogous inhibitory responses to hypoxia have also recently been reported for adipocytes (reduced proliferation, differentiation, and adipogenesis), a related connective tissue cell type [36,37].

Hypoxia may exert more important inhibitory effects on osteoblast collagen production at the post-translational level. Hydroxyproline residues within collagen provide thermal

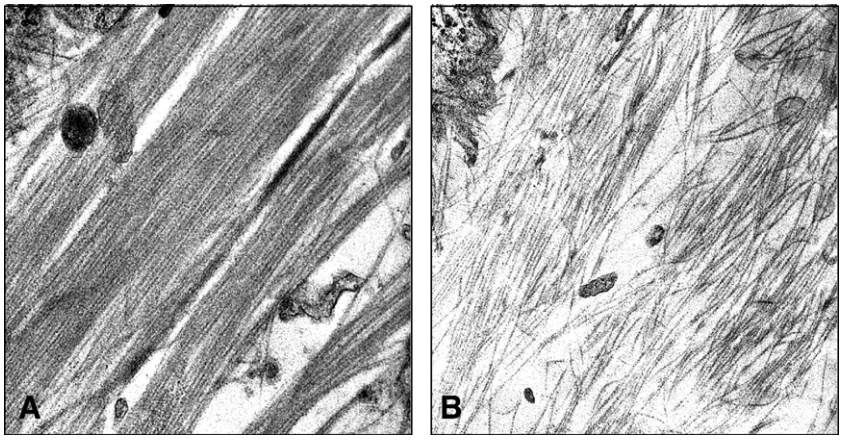

Fig. 6 – Hypoxia induced changes in the ultrastructure of collagen fibrils. Transmission electron microscopy was performed on demineralized bone nodules formed by rat calvarial OBs grown in 20% O_2 (A) and 2% O_2 (B). Collagen formed in 20% O_2 is deposited as dense, regular fibrils, whereas the collagen formed in low O_2 is characterized by a lower fibril density and a disorganized appearance (original magnification = 3000×).

Table 2 – Hypoxia inhibits collagen formation and lysine hydroxylation

Oxygen (%)	Collagen (nmol/ml)	Pepsin-insoluble collagen (%)	Lysine hydroxylation (%)	Proline hydroxylation (%)
20	4.96	94.9	31.6	43.9
2	0.22	54.6	22.3	42.2

Collagen was extracted from bone nodules formed on Melanex discs by rat calvarial OBs and analyzed to determine the percentage lysine hydroxylation (expressed as 100*Hyl/[Hyl + Lys]), proline hydroxylation (expressed as 100*Hyp/[Hyp + Pro]), and the total amount of collagen. Collagen formed in 2% O_2 was more susceptible to pepsin digestion than that formed in 20% O_2. Hydroxylysine residues were decreased in collagen extracted from hypoxic cultures, whereas hydroxyproline residues were unchanged relative to 20% O_2.

stability, enabling correct intracellular triple helix formation, whereas hydroxylysine residues provide covalent linkage sites between adjacent collagen molecules within deposited fibrils. The enzymes responsible for proline and lysine hydroxylation are oxygen-dependent [17,38]. The collagen-specific prolyl-4-hydroxylase (P4OH) requires 50 mm Hg O_2 (~5%) to display 50% of its maximal activity and 150 mm Hg O_2 (~15%) for 90% of maximal activity [38]. Considering the central role of prolyl hydroxylases in oxygen sensing [39], it might be expected that the proportion of hydroxylated proline residues would be decreased in collagen formed by hypoxic osteoblasts. However, collagen molecules with insufficient proline hydroxylation are unstable at body temperature and would not proceed to the triple helical conformation and deposition [14]. Underhydroxylated procollagen is retained intracellularly with some degradation, and upon resumption of permissive conditions for hydroxylation, this procollagen is then processed normally [40]. These findings suggest that if proline hydroxylation is compromised in hypoxia, it would be difficult to detect such changes in any collagen deposited; rather, the phenomenon would present as a generalized decrease in total collagen. Such a mechanism would also be consistent with the reversibility of the hypoxic inhibition of collagen deposition.

The decreased percentage of hydroxylysine residues in hypoxia may reflect the requirement of the PLOD enzymes for molecular oxygen [41], together with the observed reduction in PLOD expression in the hypoxic cultures. These findings contrast with reported increases in PLOD-2 expression during hypoxic culture of dermal fibroblasts [42] and increased PLOD-1 and -2 gene expression in muscle cells in response to hypoxia in vitro [43]. Fibroblasts have been reported to increase their production of collagen when exposed to hypoxia in vitro [44], in vivo, however, wound-healing evidence suggests that collagen deposition is related directly to wound oxygen tension [38]. The changes in pepsin sensitivity of the collagen formed by hypoxic osteoblasts could be accounted for by decreased activity of lysyl oxidase (LOX) and thus decreased covalent cross-linking [17]. This notion is supported by the report that collagen isolated from rats treated with the specific LOX inhibitor, β-aminoproprionitrile (BAPN) had increased sensitivity to pepsin digestion [45]. Decreased activity of LOX, leading to impaired intermolecular cross-linking, could also help to account for the disorganized collagen ultrastructure observed by transmission electron microscopy. Decreased collagen cross-linking has clear implications for bone strength and has been associated with osteoporosis [46,47].

A well-documented response of osteoblasts to hypoxia is upregulation of the potent angiogenic agent, vascular endothelial growth factor (VEGF) [21,22]. These responses could be

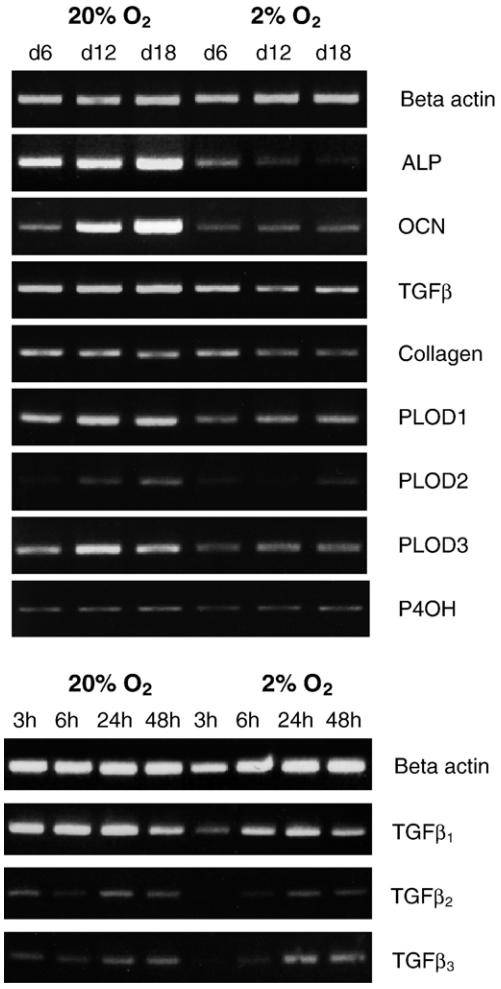

Fig. 7 – Expression of differentiation markers and collagen modifying enzymes is delayed in hypoxic osteoblasts. (A) Long-term hypoxia delays or decreases the expression of mRNAs for osteocalcin and alkaline phosphatase by rat calvarial osteoblasts. The expression of PLOD1–3 mRNAs was also reduced in hypoxic cultures; small decreases in expression of collagen, P4OH, and TGFβ mRNAs were additionally evident. RT-PCR analysis was performed on RNA extracted from rat calvarial osteoblasts cultured for 6, 12, 18, and 24 days in normoxia (20% O_2) or hypoxia (2% O_2). (B) Hypoxia does not alter expression of TGFβ isoforms in short-term cultures. RNA was extracted from rat calvarial osteoblasts maintained for 3, 6, 24, and 48 h in 20% or 2% O_2. Abbreviations: ALP, alkaline phosphatase; OCN, osteocalcin; TGFβ$_{1–3}$, transforming growth factor β$_{1–3}$; PLOD1–3, procollagen lysine, 2-oxyglutarate, 5-dioxygenase 1–3; P4OH, prolyl 4 hydroxylase.

mediated via the stabilization of HIF1α and HIF2α that occurs in hypoxic osteoblasts in vitro [47,48]. VEGF is required for fracture healing [49] and is essential for normal bone development [50]. Osteoblasts express VEGF receptors, and VEGF increases mineralized nodule formation in osteoblast cultures [49,51]. However, despite the increased VEGF expression reported by others in hypoxic osteoblasts, bone nodule formation was inhibited strongly by hypoxia in our cultures, suggesting that in the absence of sufficient oxygen, osteoblasts cannot mount an anabolic response to VEGF. In vivo, however, hypoxia would induce expression of VEGF (and other factors), leading to angiogenesis and, once sufficient oxygen was available, increased osteoblastic bone formation.

Bone nodule formation by osteoblasts was susceptible to inhibition by hypoxia at both early and late stages of culture but recovered well when cells were transferred to atmospheric oxygen levels following early exposure to hypoxia. We found that osteoblast apoptosis was low but increased slightly with time in culture, in line with another recent study [33]; surprisingly, however, long-term hypoxia did not increase apoptosis. These observations are consistent with the notion that hypoxia induces a state of quiescence or 'suspended animation' in osteoblasts. It seems reasonable to suppose that such a response to hypoxia, which could be mediated via the involvement of HIF proteins [11], would serve to ensure osteoblast survival in vivo until (re)vascularization occurred.

The inhibitory response of osteoblasts to hypoxia is reciprocal with the powerful stimulatory action of hypoxia on osteoclast formation (and thus, bone resorption). It is noteworthy that even in severe, chronic hypoxia (0.2% O_2), mouse or human osteoclast formation is increased 2- to 3-fold compared with 20% O_2 [32,33]. Hypoxia in vivo also results in local decreases in tissue pH due to increased anaerobic metabolism and reduced perfusion. We have recently shown that mineralization of organic matrix deposited by osteoblasts is extremely sensitive to inhibition by small pH reductions (although osteoblast proliferation and collagen production were unimpaired) [33]. Therefore, in the present in vitro study, culture medium acidification was carefully controlled in order to eliminate pH as a variable. Importantly, osteoclast activation is now known to be dependent on extracellular acidification [52–54]. Thus, in vivo, hypoxia and its attendant acidosis would be expected to exert a 'quadruple-negative' action on bone cell function by blocking osteoblast growth/matrix deposition and subsequent mineralization while increasing both the formation and resorptive activity of osteoclasts.

The results presented here show that bone formation by osteoblasts in vitro is critically dependent on oxygen and provide further evidence for the vital role of the vasculature in maintaining bone health.

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