Bone Cell Responsiveness to Growth and Differentiation Factors Under Hypoxia In Vitro

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Purpose: *Osteogenic cells contribute to the process of osseointegration and graft consolidation. However, whether the cells survive low oxygen tension and maintain their responsiveness to natural and therapeutic growth and differentiation factors remains unknown.* ***Materials and Methods:*** *To determine the effects of low oxygen tension on osteogenic cell viability and responsiveness in vitro, human bone cells were placed into plastic pouches intended to create anaerobic conditions and were either simultaneously or subsequently exposed to supernatants from activated platelets or recombinant bone morphogenetic protein (BMP)-6.* ***Results:*** *Bone cells cultured for up to 72 hours under hypoxia moderately decreased their metabolic activity, which was paralleled by morphologic changes but not by cleavage of the apoptosis markers caspase-3 and poly(ADP)ribose polymerase. Hypoxia suppressed the mitogenic response of bone cells to platelet-released supernatant and the expression of osteogenic differentiation markers alkaline phosphatase and osteocalcin upon incubation with BMP-6. Stimulation of bone cells with platelet-released supernatant and BMP-6 immediately after re-establishment of normoxia caused a moderate cellular response. However, when bone cells were allowed to recover for 7 days under normoxia, their responsiveness was equal to that of cells not previously exposed to low oxygen tension.* ***Conclusions:*** *These findings suggest that osteogenic cells can survive transient hypoxia and retain their potential to respond to growth and differentiation factors once normoxia is re-established. The data also implicate that reoxygenation, and thus blood vessel formation, may be an important determinant for the process of osseointegration and graft consolidation.* Int J Oral Maxillofac Implants 2008;23:417–426

Key words: *bone augmentation, bone regeneration, graft consolidation, hypoxia, osteoblast, oxygen*

The clinical application of growth and differentiation factors has received attention in the field of implant dentistry since the introduction of platelet-rich plasma, a natural source of autologous growth factors,[1] and the clinical approval of recombinant human bone morphogenetic protein-2 (rhBMP-2) for sinus augmentation and filling of extraction sockets by the US Food and Drug Administration (FDA).[2,3] Platelet activation and the expression of BMP-family members during the natural course of bone regeneration underscores the therapeutic potential of these factors.[4–6] The osteogenic response of a recipient site to natural and therapeutic growth and differentiation factors can be modified by physiologic determinants and the type of injury. Aging, clinical conditions such as diabetes, and the defect size have an impact on angiogenesis[7–9] and may thereby affect the process of bone regeneration, for example, during osseointegration of dental implants.[10,11]

It is not until vascular sprouting changes the catabolic hypoxic condition of the blood clot into the anabolic normoxic state of granulation tissue that

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bone formation takes place.[4,12] It can thus be hypothesized that the response of osteogenic cells to growth and differentiation factors depends on oxygen supply. This hypothesis is supported by findings that chronic systemic hypoxia causes a delay in fracture healing[13] and by in vitro studies showing that osteogenic differentiation is reduced under hypoxia and even abolished under anoxic conditions.[14–19] On the other hand, bone regeneration can be improved with hyperbaric oxygen and the local stimulation of blood vessel formation.[14,20–22] It is thus of clinical relevance to investigate whether oxygen tension is among the limiting factors to achieve a full therapeutic response to growth and differentiation factors.

To study the link between oxygen tension and the response of osteogenic cells to growth and differentiation factors, an in vitro approach was utilized in which primary bone cells were cultured in plastic pouches intended to create anaerobic conditions. The effects of hypoxia on proliferation and osteogenic differentiation in response to platelet releasates and BMP-6, respectively, were then examined.

MATERIALS AND METHODS

Bone Cell Culture and Basic Experimental Settings

Primary human bone cells were prepared from femoral heads of 3 adult donors (Department of Traumatology, Danube Hospital/SMZ-Ost, Vienna, Austria) following an established protocol and were stored in liquid nitrogen until further use.[23,24] Bone cells of not more than 8 passages were plated into culture dishes (TPP AG, Trasadingen, Switzerland) at 5 $\times$ 10^4 cells/cm^2 in αMEM (Gibco, Grand Island, NY) and were supplemented with 10% fetal calf serum (FCS) and antibiotics, termed serum-containing medium. The following day, the growth medium was changed to serum-free medium with or without platelet releasates and BMP-6 (30, 100, and 300 ng/mL; R&D Systems, Minneapolis, MN). Culture plates were placed into impermeable pouches (BBL GasPak Pouch System; BD Bioscience, Bedford, MA) intended to produce an atmosphere suitable for the primary isolation and cultivation of anaerobic bacteria or anoxic conditions in cell culture.[25] According to the manufacturer, anaerobic conditions are achieved rapidly with an oxygen concentration of less than 1% and a CO_2 concentration of equal to or greater than 5% within 2 hours of incubation. Anaerobic conditions were confirmed by an indicator strip. The pouch reagent sachet utilizes iron powder and calcium carbonate to produce a CO_2-enriched anaerobic environment in a container designed to hold up to 2 microtiter plates. The developing CO_2 controls the carbonate buffer system in the culture medium. Cells were left in parallel under normoxia in a humidified atmosphere at 37°C and 5% CO_2 for up to 72 hours. In a second set of experiments, bone cells were exposed to hypoxia and normoxia followed by immediate stimulation with platelet-released supernatants and BMP-6 under normoxic conditions for 72 hours. In a third set of experiments, bone cells, which had been exposed to hypoxia for 72 hours, were cultivated with serum-containing medium under normoxia for 7 days before being stimulated with platelet-released supernatants and BMP-6. Biochemical analyses were performed for all 3 settings.

Preparation of Platelet Releasates

Platelet concentrates were obtained from volunteer donors at the Department of Transfusion Medicine of the Medical University of Vienna, Austria. Platelets were washed and resuspended in serum-free medium to get a final concentration of 3 $\times$ 10^9 cells/mL. Release of platelet-derived molecules into the supernatant was induced by addition of 1 unit human thrombin/mL (Sigma, St Louis, MO) for 30 minutes at room temperature. After centrifugation, platelet cell releasates (PRS) of multiple donors was pooled, sterile filtered, and stored at –80°C. Platelet-released supernatant was diluted at the 1:5 ratio with serum-free medium before being added to the bone cells.[26]

Trypan Blue Staining

Culture medium was discarded, and 50 µL of a trypan blue solution (0.4%; Sigma) was added to each well of a 48-well plate (Corning Glass, Corning, NY) for 5 minutes at room temperature. Trypan blue was removed, and bone cells in the culture dishes were photographed.

Assays

Metabolic Activity. Thirty µL of MTT (3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide; 5 mg/mL; Sigma) solution were added to each well of a 96-well plate (Corning Glass) for 2 hours at 37°C. Medium was removed, and formazan crystals were solubilized in 100 µL dimethyl sulfoxide. Optical density was measured at 570 nm.

^{3}[H]thymidine Incorporation Assay. Bone cells processed within white 96-well plates (Packard, Meriden, CT) were pulse-labeled with 3[H]thymidine (0.5 µCi/well; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) for at least 6 hours of the cultivation period and subjected to liquid scintillation counting (TopCount; Packard). Data were expressed as percent of counts per minute of unstimulated controls.

Cell Detachment Assay. Bone cells were placed in 96-well plates (Packard) in the presence of 0.5 μCi ^{3}[H]thymidine per well. The following day, unincorporated ^{3}[H]thymidine was removed and cells were further exposed to hypoxia and normoxia for 72 hours under serum-free conditions. Radioactivity, which remains in the culture plates after the incubation period, was measured by scintillation counting.

Western Blot Analysis of Cleaved Caspase-3 and PARP

Bone cells in 6-well plates were exposed to normoxia and hypoxia for 72 hours before being lysed in sodium dodecyl sulfate (SDS) buffer containing protease inhibitors. Cell debris was eliminated by centrifugation. Equal amounts of cell extracts were separated by SDS-PAGE (polyacrylamide gel electrophoresis) and transferred onto nitrocellulose membranes (Amersham). Membranes were blocked in tris-buffered saline (TBS), 0.1% Tween-20 with 5% w/v nonfat dry milk. Anti-cleaved caspase-3 antibody (clone 9661; Cell Signaling Technology, Beverly, MA), and anti-cleaved poly (ADP) ribose polymerase (PARP) antibody (clone 9541; Cell Signaling) were diluted 1:1000 in TBS, 0.1% Tween-20 with 5% bovine serum albumin, and incubated over night at 4°C. The first antibody was detected with the appropriate secondary antibody (Dako, Glostrup, Denmark) using the ECL method (Pierce). Equal loading was confirmed by Ponceau-S staining.

Alkaline Phosphatase Activity

Bone cells were plated at 5×10^4 cells/cm^2 in 96-well plates (Corning Glass) in growth medium. The following day, medium was changed to BMP-6 at 100 ng/mL. The cells were incubated for 3 days. At the end of the culture period, enzymatic activity was determined in cell lysates containing 0.2% Triton X-100 (Sigma). Aliquots of each sample were incubated with alkaline phosphatase substrate buffer (100 mmol/L diethanolamine, 150 mmol/L NaCl and 2 mmol/L $MgCl_2$, 2.5 μg/mL p-nitrophenylphosphate) for 5 to 15 minutes at room temperature. Total cellular protein was determined using the bicinchoninic method (Pierce Chemical, Rockford, IL). Alkaline phosphatase activity is expressed as units per milligram protein, with 1U defined as enzymatic activity that releases 1 nmol p-nitrophenol per minute. For histochemical staining of alkaline phosphatase, bone cells were fixed with 10% neutral buffered formalin and incubated with a substrate solution containing 4 mg of naphthol AS-TR phosphate in 0.15 mL of N,N-dimethylformamide and 12 mg of fast blue BB salt (Sigma) in 15 mL of 100 mmol/L Tris-HCl (pH 9.6). After being rinsed with distilled water, the cells were photographed.

One-step Quantitative RT-PCR Analysis of Gene Expression

Cellular RNA was extracted from spin columns (RNeasy; Qiagen, Hilden, Germany). The reverse transcription-polymerase chain reaction (RT-PCR) was performed in a 1-step method (SuperScript III Platinum SYBR Green One-Step quantitative RT-PCR (qRT-PCR) Kit; Invitrogen, Carlsbad, CA) in a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). The protocol was performed according to the 1-step qRT-PCR kit. The following primers were used: alkaline phosphatase 5′-cgtggctaagaatgtcatcatgtt-3′ (sense primer); 5′-tggtggagctgacccttga-3′ (antisense primer); osteocalcin 5′-gcaaaggtgcagcctttgtg-3′ (sense primer); 5′-ggctcccagccattgatacag-3′ (antisense primer); β2 microglobulin 5′-actttgtcacagcccaagatagttaa-3′ (sense primer); and 5′-aaatgcggcatcttcaacct-3′ (antisense primer). The following RT-PCR conditions were used: 1 cycle of 50°C for 3 min; followed by 1 cycle of 95°C for 5 minutes; 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds; followed by a final cycle of 40°C for 1 minute. Melting curve analysis showed a single peak for the primer sets of all genes, indicating no primer-dimer formation. Amplification plots were obtained for each target gene as well as for β2 microglobulin. Ct value (threshold cycle) was used to calculate the fold-upregulation by subtracting the Ct value for β2 microglobulin from the Ct value for the target gene and comparing the experimental (E) result with the control (C) using the equation fold-upregulation = 2^(–ΔΔCt), where ΔΔCt = ΔE – ΔC, ΔE = CtE target – CtE β2 microglobulin and ΔC = CtC target – CtC β2 microglobulin. PCR amplification was performed in triplicate and repeated with cells from 3 independent donors.

Statistical Analysis

Experiments were performed with cells from at least 3 donors. Differences of the means were compared by Student *t* test. Significant difference was assigned at the 5% level.

RESULTS

Characterization of Bone Cells Cultured Under Hypoxia

The influence of hypoxia on parameters of bone cell survival was examined. Hypoxia caused a shrinking and rounding of a small fraction of bone cells (Fig 1a). Trypan blue staining indicated positive cell fragments, which were more prominent in the hypoxia group (Fig 1b). Bone cells cultured for at least 48 hours under anoxic conditions showed a weak

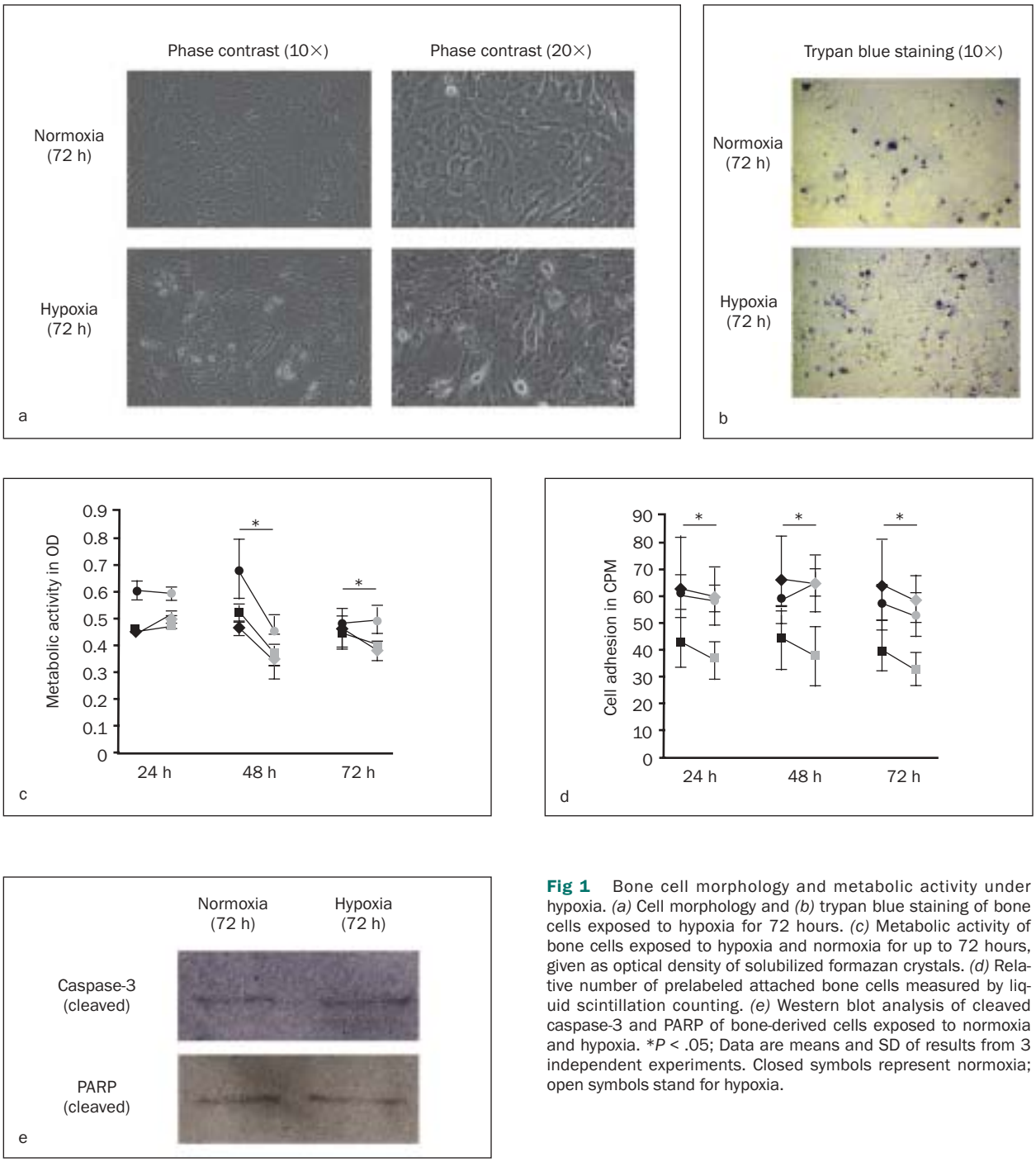

Fig 1 Bone cell morphology and metabolic activity under hypoxia. *(a)* Cell morphology and *(b)* trypan blue staining of bone cells exposed to hypoxia for 72 hours. *(c)* Metabolic activity of bone cells exposed to hypoxia and normoxia for up to 72 hours, given as optical density of solubilized formazan crystals. *(d)* Relative number of prelabeled attached bone cells measured by liquid scintillation counting. *(e)* Western blot analysis of cleaved caspase-3 and PARP of bone-derived cells exposed to normoxia and hypoxia. *$P < .05$; Data are means and SD of results from 3 independent experiments. Closed symbols represent normoxia; open symbols stand for hypoxia.

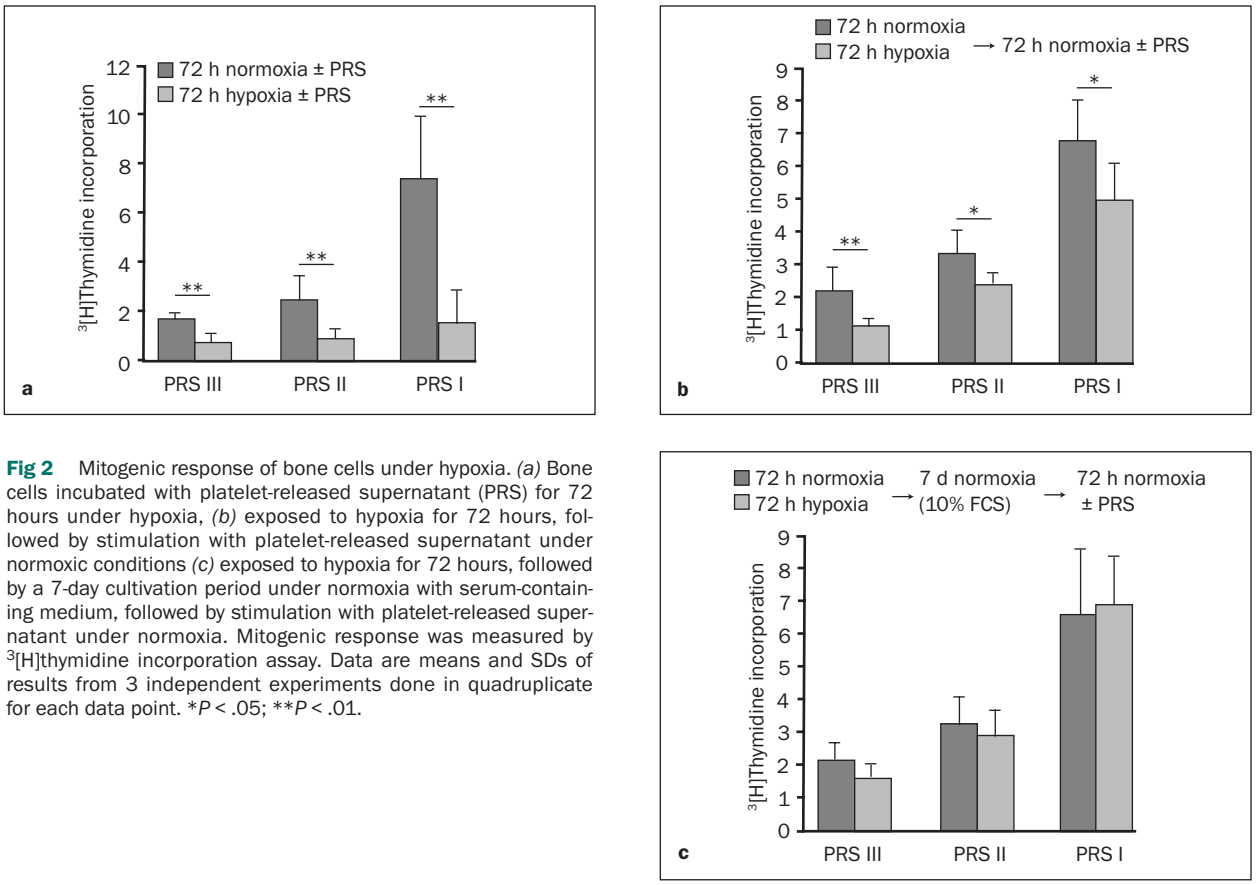

Fig 2 Mitogenic response of bone cells under hypoxia. *(a)* Bone cells incubated with platelet-released supernatant (PRS) for 72 hours under hypoxia, *(b)* exposed to hypoxia for 72 hours, followed by stimulation with platelet-released supernatant under normoxic conditions *(c)* exposed to hypoxia for 72 hours, followed by a 7-day cultivation period under normoxia with serum-containing medium, followed by stimulation with platelet-released supernatant under normoxia. Mitogenic response was measured by ^{3}[H]thymidine incorporation assay. Data are means and SDs of results from 3 independent experiments done in quadruplicate for each data point. *$P < .05$; **$P < .01$.

decrease in the metabolic activity based on MTT conversion into formazan crystals when compared to cells cultured under normoxia ($P < .05$, Fig 1c). Bone cells prelabeled with ^{3}[H]thymidine increasingly detached from the culture plate when exposed to hypoxia for at least 24 hours ($P < .05$, Fig 1d). Incubation of bone cells for 72 hours under anoxic conditions did not increase cleavage of the apoptosis markers capase-3 and PARP (Fig 1e).

Responsiveness of Bone Cells to PRS and BMP-6

Under Hypoxia. The proliferation and differentiation status of bone cells under hypoxia was assessed in vitro by their incubation with platelet-released supernatant and BMP-6 for 72 hours in plastic pouches intended for creating anaerobic conditions. Incubation of bone cells with platelet-released supernatant under hypoxia did not result in an increased bone cell proliferation, and the absolute values reflecting the number of counts per minute was about 8 times lower in the hypoxia group when compared to bone cells cultured under normoxia (Fig 2). Bone cells that were exposed to normoxia but not to hypoxia responded to BMP-6 with increased alkaline phosphatase activity and increased expression of osteocalcin (Fig 3).

Immediately Following Hypoxia. The second approach was performed to investigate whether bone cells that have previously been exposed to hypoxia are capable of responding to growth and differentiation factors under normoxic conditions. Bone cells that were transiently exposed to hypoxia for 72 hours showed a marked responsiveness to platelet-released supernatant under normoxia, based on the results of the proliferation assay. Again, absolute values for proliferation were lower in the hypoxia group when compared to bone cells cultured under normoxia (Fig 2b). Bone cells that were transiently exposed to hypoxia did not regain their full potential to express alkaline phosphatase activity and osteocalcin under normoxia when cultured in the presence of BMP-6 (Fig 4).

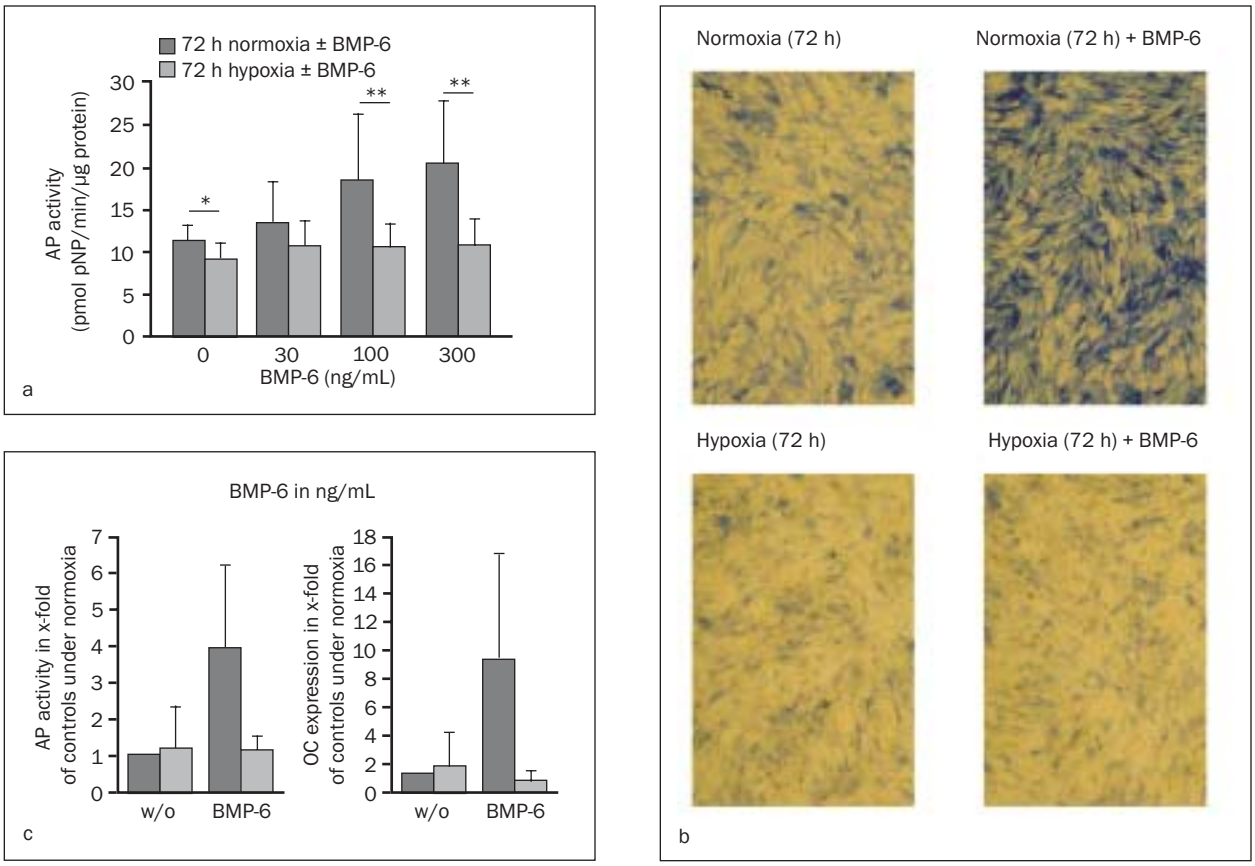

Fig 3 Differentiation of bone cells under hypoxia. *(a)* Alkaline phosphatase (AP) activity, *(b)* alkaline phosphatase histochemistry, and *(c)* gene expression of bone cells incubated with BMP-6 for 72 hours under normoxia and hypoxia. Data are means and SD of results from 3 independent experiments. OC = osteocalcin; w/o = without. *$P < .05$, **$P < .01$.

After Hypoxia and a 1-week Recovery Period. The third approach was performed to examine the effect of PRS and BMP-6 on bone cells that were allowed to recover for 7 days after exposure to hypoxia. These cells regained their full potential to proliferate in response to platelet-released supernatant (Fig 2c). These cells were also exposed to BMP-6, resulting in similar levels of alkaline phosphatase activity and expression compared to hypoxia. The expression of osteocalcin, however, was not increased (Fig 5).

DISCUSSION

The study is based on the assumption that the response of osteogenic cells to growth and differentiation factors is modified by the local oxygen tension. Growth and differentiation factors are naturally expressed at defect sites, and the introduction of PRP as well as the approval of BMP-2 by the FDA to boost the process of bone regeneration has gained increasing attention in implant dentistry.[1–3] Local oxygen tension is considered to be a limiting factor for the process of bone regeneration in vivo[27,28] and in vitro,[14–19] and possibly affects the cellular response to a therapy with growth and differentiation factors. In order to optimize the therapeutic outcome and to improve the design of scaffolds for timing of growth factor delivery, an increased understanding of the influence of oxygen tension in the biology of bone regeneration is demanding.

The in vitro model used simulates an anoxic situation based on observations that early after injury, oxygen tension drops to levels below 1% at fracture sites.[27,28] In arterial and venous blood, oxygen tension reaches about 12% and 5%, respectively. Tissue levels are reported to range between 3% and 9% oxygen tension.[29,30] In an in vitro plastic pouch

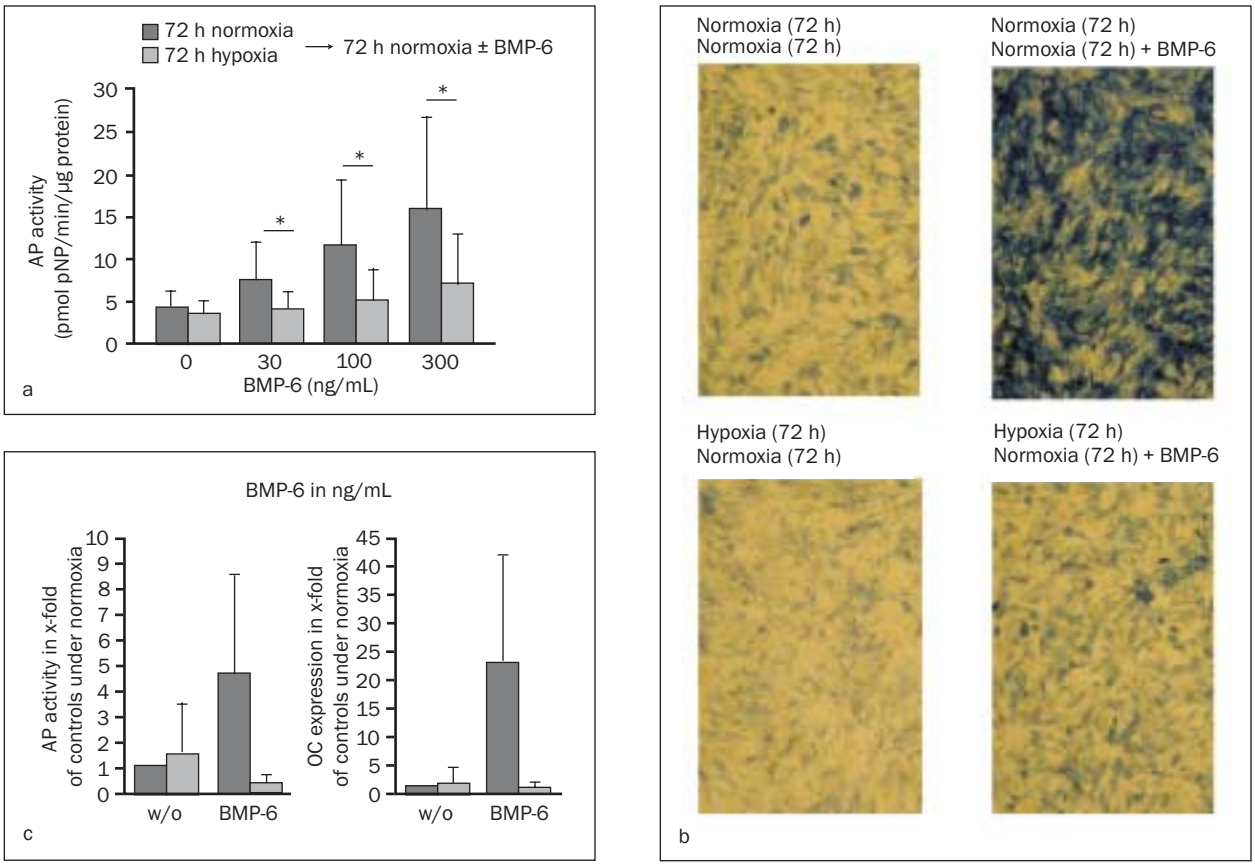

Fig 4 Differentiation of bone cells after 72 hours of hypoxia. *(a)* Alkaline phosphatase activity, *(b)* alkaline phosphatase histochemistry, and *(c)* gene expression of bone cells exposed to hypoxia for 72 hours followed by stimulation with BMP-6 under normoxic conditions. Data are means and SDs of results from 3 independent experiments. *$P < .01$.

model, dissolved oxygen concentrations in culture medium continuously decrease to 2% to 3% within 24 hours and further decrease until hypoxia is reached.[31] In the present study, it was found that the viability of osteogenic cells was not severely affected following 3 days of exposure to this anoxic condition. Only a weak increase of membrane disruption revealed the occurrence of necrosis as indicated by trypan blue staining. Programmed cell death (ie, apoptosis) was not activated under hypoxia, which is similar to findings observed with bone marrow stromal cells.[16] Under low-oxygen tension, osteogenic cells may enter a quiescent state of anaerobic metabolism where energy supply is restricted to glycolysis.[16,17] The in vitro findings support preclinical studies showing that the contribution of transplanted osteogenic cells to bone formation is apparent in small, highly vascularized areas[32] but hampered in large defects.[33] However, this does not necessarily mean that osteogenic cells in large defects have lost their bone-forming competence. The findings presented support the view that osteogenic cells can survive at least transient low oxygen tension. However, during this time, they fail to respond to local cues that are required to control their capacity to form bone.

To test this assumption in vitro, osteogenic cells were exposed to growth and differentiation factors under hypoxia compared to normoxia. The aim was to simulate the organizing blood clot, where osteogenic cells are exposed to mitogens released from activated platelets and to BMP-6 expressed throughout the healing sequence under low oxygen tension.[4–6] The key finding was that under anoxic conditions proliferation of osteogenic cells in response to platelet releasates was suppressed and BMP-6 failed to induce their differentiation into a more mature phenotype. When transferred to nor-

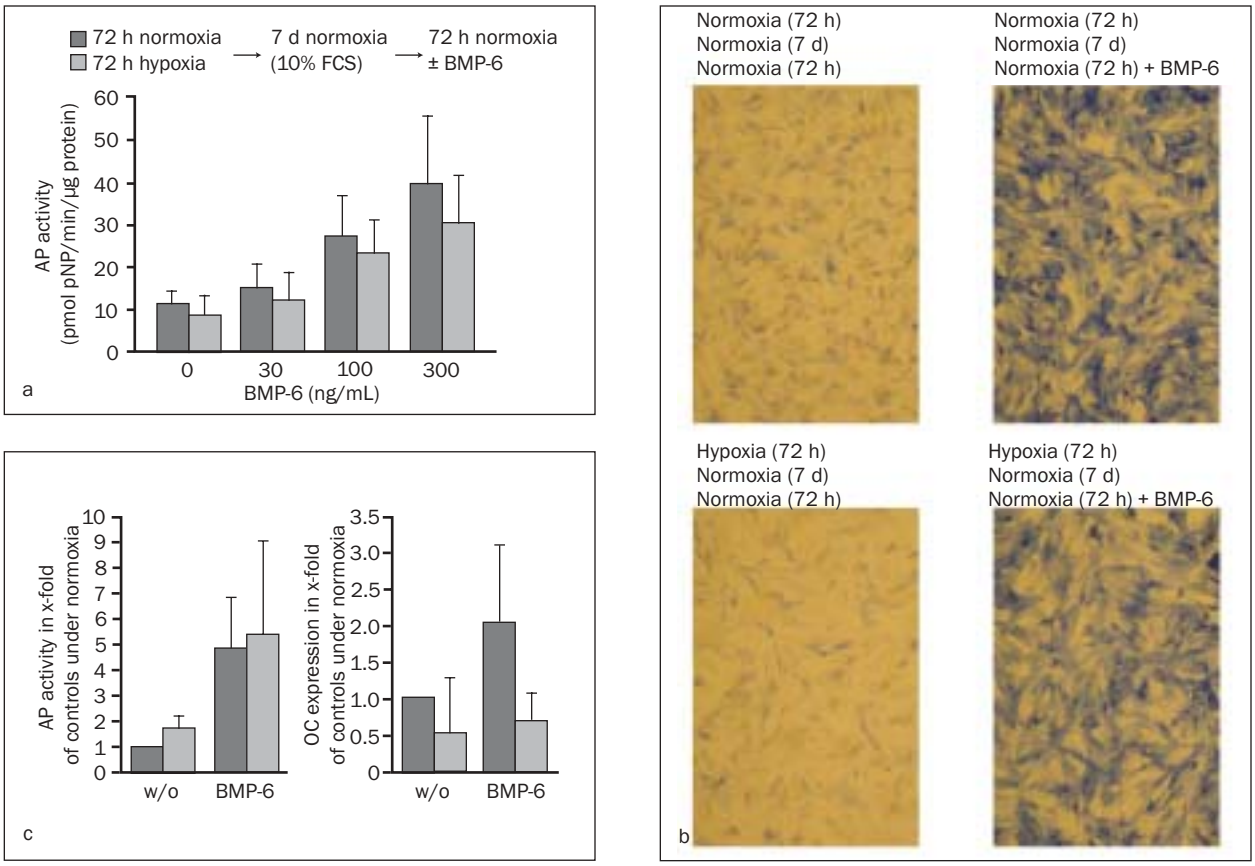

Fig 5 Differentiation of bone cells after 72 hours of hypoxia with 7 days of recovery. *(a)* Alkaline phosphatase activity, *(b)* alkaline phosphatase histochemistry, and *(c)* gene expression of bone cells exposed to hypoxia for 72 hours followed by a 7-day cultivation period under normoxia with serum-containing medium, followed by stimulation with platelet-released supernatant under normoxia. Data are means and SDs of results from 3 independent experiments.

moxia, osteogenic cells increasingly proliferate and differentiate in response to the applied stimuli, although the effect was weak. However, responsiveness was almost fully regained following one week of cultivation under normoxia. These findings suggest that during their reversible quiescent state, osteogenic cells are incapable of responding to mitogenic stimuli and BMPs. The in vitro data provide a feasible explanation for why bone formation is restricted to vascular and thus oxygen supply.[34] Under physiologic conditions, hypoxia is transient and is a key stimulus of blood clot organization.[12,35] However, chronic or at least prolonged hypoxia is considered pathologic rather than beneficial in bone regeneration. For example, blood vessels and mechanically stable conditions are mandatory for osseointegration; otherwise, implants are encapsulated by a fibrous tissue.[36,37] This mechanism may also at least partially explain the compromised bone regeneration capacity in the elderly, under pathologic conditions, and in preclinical models associated with impaired blood vessel formation.[10,11] To clarify the mechanisms that control cell fate via oxygen tension, further experiments are indicated.

If the responsiveness of osteogenic cells to growth and differentiation factors is oxygen-dependent, various implications for the therapy of bone regeneration can be suggested. The present data support the clinical demand of providing stable conditions for osseointegration and graft consolidation that allow blood vessel sprouting into the defects area. It would thus be reasonable to determine if recombinant BMPs are more effective when applied after granulation tissue has formed. Preclinical studies showing that the local application of angiogenic molecules, eg, recombinant vascular endothelial growth factor-A (VEGF), or the respective plasmids cause increased bone regeneration.[21,22] This may be due to the increasing responsiveness of osteogenic cells to locally expressed growth and differentiation factors. Findings that the BMPs more effectively induce bone formation when blood vessel supply is

simultaneously stimulated by VEGF[38] are in agreement with the present hypothesis. Moreover, recombinant basic fibroblast growth factor failed to show an effect in ischemic wounds, further supporting the idea of a modulation of a cellular response by oxygen tension.[39] This may explain and support strategies that target blood vessel formation to promote the process of osseointegration and graft consolidation in implant dentistry and other disciplines dealing with bone regeneration.

CONCLUSIONS

The in vitro data presented demonstrate that even when osteogenic cells survive transient hypoxia, they require sufficient oxygen tension to respond to growth and differentiation factors. These data underline the role of sufficient oxygen tension and consequently blood vessel formation to allow osteogenic cells to respond to local cues, eg, during the process of osseointegration and consolidation of augmented areas. The data further underline the clinical relevance of situations affecting angiogenesis, such as aging, diabetes, and the defect size. Moreover, the data can contribute to development of therapies considering that the response of osteogenic cells depends on the level of oxygen tension.

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