VEGF expression in an osteoblast-like cell line is regulated by a hypoxia response mechanism

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Steinbrech, Douglas S., Babak J. Mehrara, Pierre B. Saadeh, Joshua A. Greenwald, Jason A. Spector, George K. Gittes, and Michael T. Longaker. VEGF expression in an osteoblast-like cell line is regulated by a hypoxia response mechanism. *Am J Physiol Cell Physiol* 278: C853–C860, 2000.—Angiogenesis is essential for the increased delivery of oxygen and nutrients required for the reparative processes of bone healing. Vascular endothelial growth factor (VEGF), a potent angiogenic growth factor, has been implicated in this process. We have previously shown that hypoxia specifically and potently regulates the expression of VEGF by osteoblasts. However, the molecular mechanisms governing this interaction remain unknown. In this study, we hypothesized that the hypoxic regulation of VEGF expression by osteoblasts occurs via an oxygen-sensing mechanism similar to the regulation of the erythropoietin gene (EPO). To test this hypothesis, we examined the kinetics of oxygen concentration on osteoblast VEGF expression. In addition, we analyzed the effects of nickel and cobalt on the expression of VEGF in osteoblastic cells because these metallic ions mimic hypoxia by binding to the heme portion of oxygen-sensing molecules. Our results indicated that hypoxia potently stimulates VEGF mRNA expression. In addition, we found that nickel and cobalt both stimulate VEGF gene expression in a similar time- and dose-dependent manner, suggesting the presence of a hemelike oxygen-sensing mechanism similar to that of the EPO gene. Moreover, actinomycin D, cycloheximide, dexamethasone, and mRNA stabilization studies collectively established that this regulation is predominantly transcriptional, does not require de novo protein synthesis, and is not likely mediated by the transcriptional activator AP-1. These studies demonstrate that hypoxia, nickel, and cobalt regulate VEGF expression in osteoblasts via a similar mechanism, implicating the involvement of a heme-containing oxygen-sensing molecule. This may represent an important mechanism of VEGF regulation leading to increased angiogenesis in the hypoxic microenvironment of healing bone.

bone repair; osteogenesis; angiogenesis

VASCULAR DISRUPTION, due to traumatic or surgical injury of bone, together with a cascade of inflammatory events including enzymatic release from necrotic tissues and α-adrenergic-mediated vasoconstriction, leads to the formation of a hypoxic zone of injury (14, 15, 18, 19). This hypoxic zone, in turn, is thought to stimulate angiogenesis to restore blood flow to the fracture site, thus initiating the reparative processes of bone repair (3, 4, 28, 30, 31, 44).

Recent studies have attempted to elucidate the molecular mechanisms regulating angiogenesis in fracture repair (10, 33, 38). These studies have implicated osteoblasts as important mediators of this process. For example, vascular endothelial growth factor (VEGF), a potent direct-acting angiogenic peptide, is highly expressed by osteoblastic cells in vitro (33, 38). In addition, we and others have demonstrated that VEGF expression by these cells is specifically regulated by a number of cytokines and conditions in the wound microenvironment, including inflammatory cytokines (TGF-β1, FGF-2, and PDGF-BB) and hypoxia (33, 35, 37, 38). Furthermore, VEGF mRNA and protein are expressed by osteoblasts during membranous bone repair in vivo (33). The purpose of these experiments was to further analyze the intracellular mechanisms governing VEGF mRNA expression by osteoblasts. Specifically, we sought to identify oxygen-sensing mechanisms used by osteoblastic cells to regulate the expression of VEGF.

Oxygen tension is known to regulate the expression of numerous molecules, of which erythropoietin has been the most extensively investigated (5, 36). Erythropoietin, a dimeric hormone, is the principal regulator of erythrocyte growth and differentiation in response to hypoxia (5). Recent evidence suggests that the hypoxia-induced upregulation of EPO, the gene encoding erythropoietin, is mediated by an oxygen-sensing mechanism whereby a heme-containing molecule changes conformation in response to the redox state of the local microenvironment (12). These findings are supported by evidence that nickel and cobalt stimulate EPO expression in a similar manner. These metallic ions are thought to mimic the hypoxic environment by substituting for Fe^{2+} in the porphyrin ring, thus causing a conformational change resulting in decreased oxygen affinity and effectively locking the heme molecule in a deoxygenated conformation (11). The VEGF gene shares significant homology with the EPO gene in terms of structure, enhancer elements, and behavior in response to hypoxia (21, 24, 40). We, therefore, hypoth-

esized that VEGF gene expression by osteoblasts in response to hypoxic microenvironment may also be regulated via a similar oxygen-sensing mechanism.

To test this hypothesis, we investigated the effects of hypoxia, nickel, and cobalt under a variety of conditions in both primary rat calvarial osteoblast cultures, as well as in MC3T3-E1 cells, a clonal osteoblast-like cell line established from newborn mouse calvaria (39).

MATERIALS AND METHODS

Osteoblast-enriched cell isolation. Fetal rat calvarial (FRC) cell cultures were established using a modification of the techniques described by Owen et al. (27). Briefly, frontal and parietal bones from gestational 21-day-old Sprague-Dawley fetal rats were stripped of their periosteum and dura mater, minced into 1-mm fragments, and washed with sterile PBS. Calvaria were then serially digested with a solution of collagenase/dispase (Life Technologies) in a shaking incubator at 37°C for 10-min cycles (fractions). *Fractions 2–5* were collected, briefly centrifuged, and resuspended in culture media. Cells were plated in 100-mm dishes and allowed to grow to subconfluence. To confirm isolation of osteoblast-enriched cultures, the ability of isolated cells to form mineralized bone nodules and produce alkaline phosphatase was assessed using standard techniques (data not shown).

Cell culture. The primary osteoblast cell cultures were used sparingly because of the technical difficulty in harvesting large numbers of neonatal calvaria. For practical purposes, these primary cell cultures were used only for dose-response protein and mRNA experiments to confirm the hypoxic-induced increase in VEGF in primary cells. For the remainder of the experiments, we used MC3T3-E1 osteoblast-like cells, a standard, well-characterized cell line commonly used in in vitro osteoblast experiments (11) (a gift from Dr. A. Gosain, Medical College of Wisconsin). We have observed no significant differences in behavior between the primary osteoblasts and the MC3T3-E1 cells (33). Both the primary osteoblast cultures and the MC3T3-E1 cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 50 µg/ml streptomycin, and 100 µg/ml amphotericin B (all from Life Technologies, Gaithersburg, MD) (39). Media was changed every 2 days and cells were passaged after trypsinization (0.05% trypsin-EDTA, Life Technologies, Grand Island, NY). All hypoxia experiments were performed using a standard Plexiglas chamber (Bellco Glass, Vineland, NJ). The chamber was deoxygenated by positive infusion of 5% CO_2-95% nitrogen gas mixture. Equal atmospheric pressure was ensured by monitored infusion with a standardized pressure gauge. Cultures were then placed in a standard humidified tissue incubator. During the experiment, continuous O_2% saturation was monitored, and P_{O_2} levels were confirmed by evaluation of culture media using a blood gas analyzer (Ciba-Corning, Norwood, MA). In addition, the blood gas analyzer was used to analyze ambient pH and demonstrated nearly constant neutral pH (7.4–7.5) in all experiments.

Experimental culture conditions. To analyze the temporal expression pattern of VEGF mRNA in response to hypoxia, 5×10^5 FRC cells were plated in 100-mm dishes and allowed to grow in standard conditions for 24 h. Cells were then exposed to hypoxia ($P_{O_2} = 35$–40 mmHg), normoxia ($P_{O_2} = 150$ mmHg), or hyperoxia ($P_{O_2} = 350$ mmHg), and total cellular RNA was isolated after 0, 3, 6, 12, or 24 h. To investigate oxygen-sensing mechanisms, normoxic MC3T3-E1

cells were cultured with varying concentrations of nickel or cobalt and compared with parallel cultures maintained in hypoxia ($P_{O_2} = 35$–40 mmHg). Expression of VEGF in response to nickel or cobalt would implicate heme-containing oxygen-sensing molecules because these elements have been shown to displace iron from the porphyrin ring, thereby locking the sensor in a deoxygenated state (12, 13). Transcription, translation, and mRNA half-life studies were performed with actinomycin D (5 µg/ml) and cycloheximide (10 µg/ml). To investigate the mechanisms of VEGF mRNA upregulation in response to hypoxia, nickel, and cobalt, MC3T3-E1 cells were pretreated with actinomycin D (5 µg/ml), an inhibitor of transcription, for 3 h before exposure to each stimulus. To determine if increases in VEGF mRNA expression in response to hypoxia, nickel, and cobalt required de novo protein synthesis, MC3T3-E1 osteoblastic cells were treated with cycloheximide (10 µg/ml), an inhibitor of translation, immediately before stimulation. Dexamethasone inhibits transcription by inactivating the AP-1 transcription factor in fibroblasts (7). Thus, to evaluate the effect of dexamethasone on VEGF mRNA expression, subconfluent MC3T3-E1 osteoblastic cell cultures were treated with or without dexamethasone (0.5 µM) for 6 h followed by exposure to normoxic ($P_{O_2} = 150$ mmHg) or hypoxic ($P_{O_2} = 35$–40 mmHg) culture conditions. All experiments were performed in duplicate.

Northern blot analysis. Northern blot analyses were performed as previously described by Mehrara et al. (22). Briefly, cells were washed with PBS, lysed with TRIzol reagent (Life Technologies), and total cellular RNA was extracted. RNA was quantified by spectrophotometry (Pharmacia Biotech, Piscataway, NJ), and ethidium bromide staining of 18S and 28S ribosomal RNA bands was performed to confirm RNA integrity. Twenty micrograms of total cellular RNA was fractionated on 1% formaldehyde denaturing gels, transferred to positively charged nylon membranes (Schleicher & Schuell, Keen, NH), and cross-linked by ultraviolet light (Stratagene, La Jolla, CA). Membranes were prehybridized with ExpressHyb solution (Clontech) at 68°C for 1 h, followed by hybridization with [α-^{32}P]dCTP-labeled cDNA probes for 2 h at 68°C. Stringency washes were performed with $2\times$ SSC (sodium saline citrate; $1\times = 15$ mM NaCl, 1.5 mM sodium citrate, pH 7) and 0.1% SDS at room temperature for 15 min followed by $0.1\times$ SSC-0.1% SDS at 50°C for 30 min. Membranes were exposed to phosphorimaging plates overnight and analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Resulting images were quantified using ImageQuant image analysis software (Molecular Dynamics). Equal RNA loading and blot transfer was assessed by stripping and reprobing the same membranes with probe against 18S ribosomal RNA.

Preparation of cDNA probes. The mouse VEGF probe was a 420-nucleotide PCR-amplified fragment cloned into a pCR 2.1 plasmid vector (32). The probe for rat 18S RNA was a 334-bp PCR-amplified cDNA fragment. For Northern analysis, 100 ng of each probe was labeled with 50 µCi of [α-^{32}P]dCTP (New England Nuclear Life Sciences, Boston, MA) using the random primer technique (Pharmacia Biochem). Probes were purified of unlabeled probe using Sephadex G-50 DNA grade Nick columns (Pharmacia Biotech). A specific activity of at least 1×10^5 counts·min^{-1}·ml^{-1} of hybridization solution was used for all experiments.

Statistical analysis. All data are expressed as means ± SD of duplicate experiments. Statistical analysis was performed with ANOVA comparing differences between groups with $P \leq 0.05$ considered significant. Post hoc tests were performed using the Tukey-Kramer multiple-comparison test.

RESULTS

VEGF mRNA is increased by hypoxia in a dose-dependent manner in FRC cells. To assess the effects of hypoxia on VEGF mRNA by osteoblast-enriched cultures, subconfluent FRC cells were exposed to hypoxia (P_{O_2} = 35–40 mmHg) for 0, 3, 6, 12, or 24 h. VEGF mRNA levels were compared with cells maintained at normoxia. These results are summarized in Fig. 1. Hypoxic culture conditions resulted in an increase in VEGF mRNA levels beginning at ~6 h after exposure to hypoxia. VEGF mRNA levels in cells exposed to hypoxia became significantly different from normoxia after 12 h of exposure ($P < 0.001$) and peaked after 24 h in hypoxia, with a greater than threefold increase from baseline (i.e., normoxia, $P < 0.001$). Parallel control cultures maintained in normoxia for identical time points did not demonstrate any statistically significant alterations in VEGF mRNA expression as a function of time.

VEGF mRNA expression is not affected by hyperoxia in MC3T3-E1 cells. To assess the effect of hyperoxia on VEGF mRNA expression, MC3T3-E1 cells were exposed to normoxia (P_{O_2} = 150 mmHg) or hyperoxia (P_{O_2} = 350 mmHg) for 24 h. These results, summarized in Fig. 2, demonstrated little change in VEGF mRNA expression in response to hyperoxia. Thus the effects of decreased oxygen tension on VEGF mRNA expression appeared to be gene specific, and not merely related to nonspecific changes in atmospheric oxygen tension. We chose to analyze the effect of hyperoxia at 24 h because this is the time point that showed the greatest change in VEGF expression with hypoxia.

Fig. 2. VEGF mRNA expression is not changed by hyperoxia in MC3T3-E1 cells. To assess effect of hyperoxia on VEGF mRNA expression, MC3T3-E1 osteoblast-like cells were exposed to normoxia (open bar; P_{O_2} = 150 mmHg) and hyperoxia (solid bar; P_{O_2} = 350 mmHg) for 24 h. Increased oxygen tension resulted in no statistically significant change in VEGF mRNA expression.

Nickel upregulates VEGF mRNA expression in a dose- and time-dependent manner. Nickel has been shown to mimic the hypoxic state in several hypoxia-responsive genes, including EPO (12, 13, 21, 24). Nickel is thought to substitute for iron in the porphyrin ring of the protein molecule, binding oxygen with less affinity, thereby locking the heme sensor in a deoxygenated conformation (12, 13). Subconfluent cultures of MC3T3-E1 cells were exposed to standard media or media containing varying concentrations of nickel (1, 10, 50, and 100 ng/ml) for 6 h (13) and VEGF mRNA expression was analyzed. These results are summarized in Fig. 3. Nickel stimulation resulted in a dose-dependent increase in VEGF mRNA, peaking with the second highest dose of nickel used (50 ng/ml; $P < 0.01$). Importantly, peak VEGF mRNA expression in response to nickel was comparable to levels observed in hypoxic cultures.

To assess the time course of VEGF mRNA expression in response to nickel, subconfluent cultures of MC3T3-E1 cells were exposed to standard media or media containing nickel (50 ng/ml) for 3, 6, 12, or 24 h and VEGF mRNA expression was analyzed. These results are summarized in Fig. 4. Stimulation with nickel increased VEGF mRNA expression nearly threefold after 3 h. Expression remained increased at the 6-h time point (2.5-fold increase), and then decreased to below baseline (i.e., normoxia) expression at the 12- and 24-h time points.

Cobalt, similar to nickel, increases VEGF mRNA. Cobalt has also been shown to mimic the deoxygenated state in hypoxia-responsive genes such as EPO by substituting for iron in the porphyrin ring, thereby decreasing its oxygen-binding capacity (13). To confirm our findings with nickel, subconfluent MC3T3-E1 cultures were exposed to standard media or media containing varying concentrations of cobalt (1, 10, 50, 100, and 300 ng/ml) for 6 h and VEGF mRNA expression was
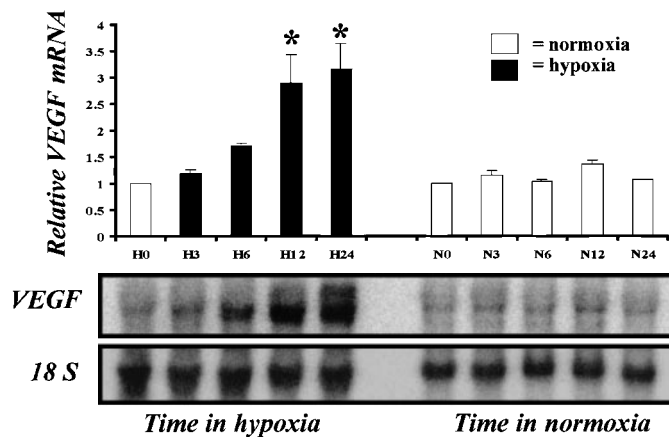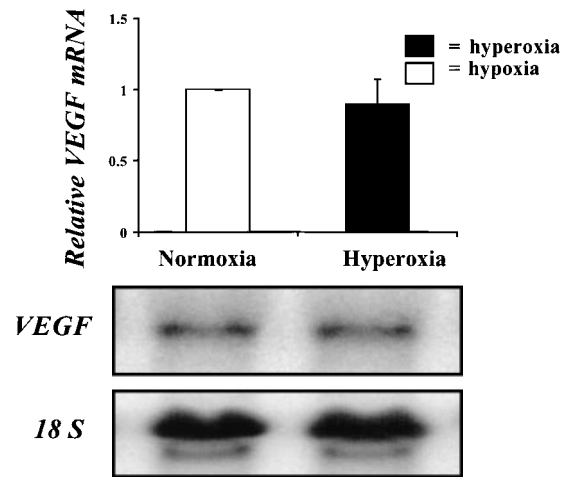

Fig. 1. Vascular endothelial growth factor (VEGF) mRNA expression is increased by hypoxia in a dose-dependent manner in fetal rat calvarial osteoblast-enriched cultures. Hypoxic culture conditions (P_{O_2} = 35–40 mmHg; solid bars) resulted in an increase in VEGF mRNA expression beginning at ~6 h after exposure to hypoxia. VEGF mRNA expression became statistically significantly different from normoxia (open bars) after 12 h (* $P < 0.001$) and peaked after 24 h in hypoxia, with a >3-fold increase from baseline (i.e., normoxia, * $P < 0.001$). Parallel control cultures (normoxia) did not demonstrate any statistically significant alterations in VEGF mRNA expression as a function of time. In Figs. 1–7 and 9, graph represents quantified expressions of VEGF as a mean of duplicate experiments normalized to their corresponding 18S control value ±SD.

Fig. 3. Nickel stimulates a dose-dependent increase in VEGF mRNA accumulation. Nickel stimulation (gray bars) resulted in a significant, dose-dependent increase in VEGF mRNA expression significantly different from normoxic control (open bar; normoxia) at 50 ng/ml (* $P <$ 0.01). Importantly, the level of expression with nickel at 50 ng/ml was within range of those observed in hypoxic conditions (solid bar; * $P <$ 0.001).
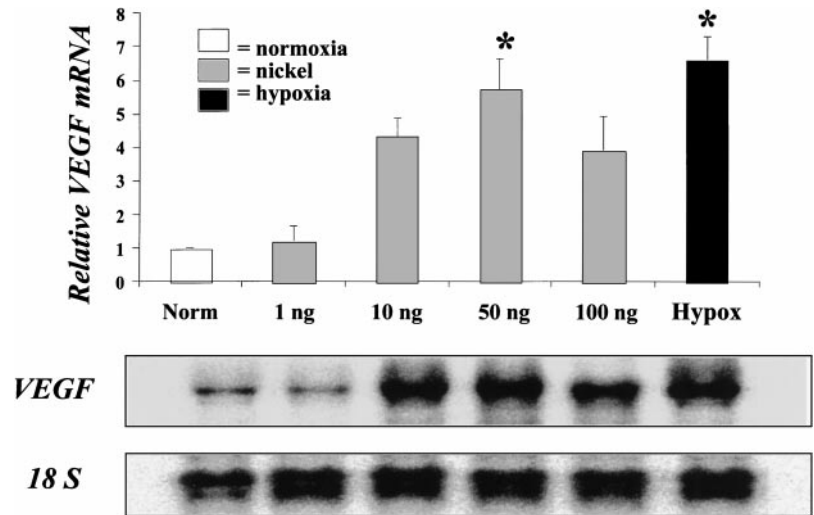
analyzed. These results are summarized in Fig. 5. Maximal VEGF mRNA upregulation by cobalt stimulation was noted when a dose of 10 ng/ml was used (>4-fold increase; $P < 0.01$). Stimulation with higher doses of cobalt (50 or 100 ng/ml) also caused VEGF mRNA upregulation (~50% increase); however, these differences were not as striking. Importantly, VEGF mRNA upregulation secondary to cobalt stimulation, like that of nickel, approached levels similar to the hypoxic cell cultures.

To assess the time course of VEGF expression in response to cobalt, subconfluent MC3T3-E1 osteoblastic cultures were exposed to standard media or media containing cobalt (10 ng/ml) for 3, 6, 12, or 24 h and VEGF mRNA expression was analyzed (Fig. 6). Cobalt stimulation resulted in a marked increase of VEGF mRNA beginning at 3 h after stimulation (2.5-fold increase; $P < 0.05$). Increased VEGF mRNA expression was also noted at the 6-h time point, but decreased to baseline after 12 h.

Actinomycin D inhibits VEGF mRNA upregulation by hypoxia, nickel, and cobalt. To investigate the mechanisms of VEGF mRNA upregulation in response to hypoxia, nickel, and cobalt, MC3T3-E1 osteoblastic cells were pretreated with actinomycin D (5 µg/ml), an inhibitor of transcription, for 3 h before exposure to hypoxia. These results are summarized in Fig. 7. As expected, exposure of cells to hypoxia, nickel (10 ng/ml), or cobalt (10 ng/ml) resulted in markedly increased VEGF mRNA expression compared with unstimulated cells (4-fold, 2-fold, and 2.5-fold increases, respectively). Treatment with actinomycin D abolished this response, suggesting that active RNA transcription was required for the increased levels of VEGF mRNA secondary to hypoxia, nickel, or cobalt. This conclusion was further supported by the finding that hypoxic culture conditions did not cause significant alterations in VEGF mRNA stability, as shown in Fig. 8. Thus addition of actinomycin D 3 h after exposure to hypoxia inhibited new RNA transcription and did not demon-
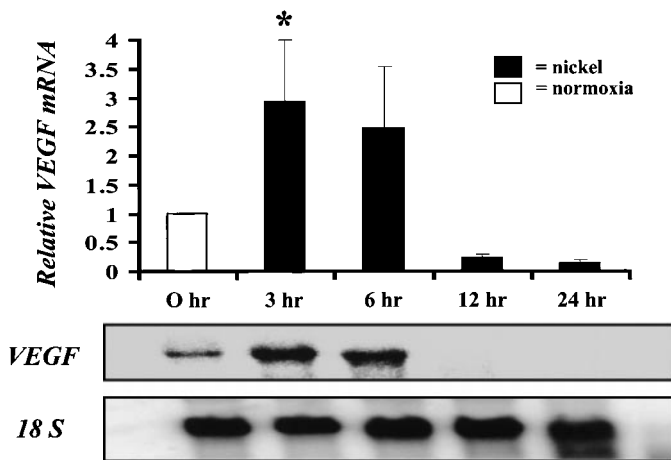

Fig. 4. Nickel stimulates a time-dependent increase in VEGF mRNA accumulation. Stimulation with nickel (solid bars) resulted in an increase in VEGF mRNA expression most dramatically at 3-h time point (* $P < 0.05$). Expression remained elevated for 6 h, then decreased to below baseline (i.e., normoxia; open bar) expression at 12- and 24-h time points.
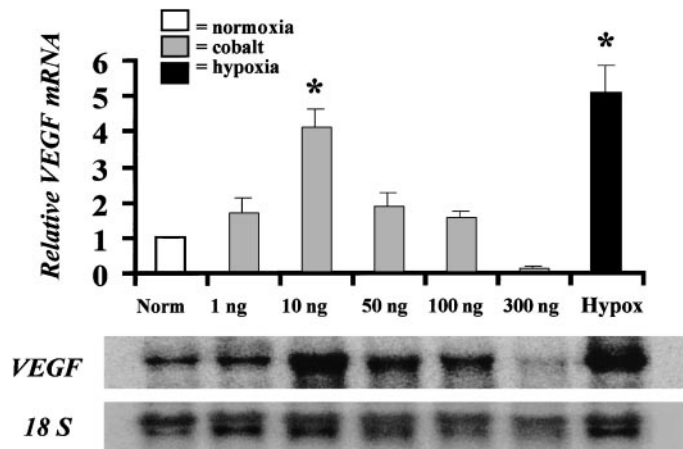

Fig. 5. Cobalt stimulates a dose-dependent increase in VEGF mRNA accumulation. Cobalt (gray bars) stimulation resulted in a dose-dependent increase in VEGF mRNA expression beginning at only 1 ng/ml and peaking in expression to >4-fold at 10 ng/ml (* $P < 0.01$), then returning to baseline (open bar; normoxia) expression at 300 ng/ml.
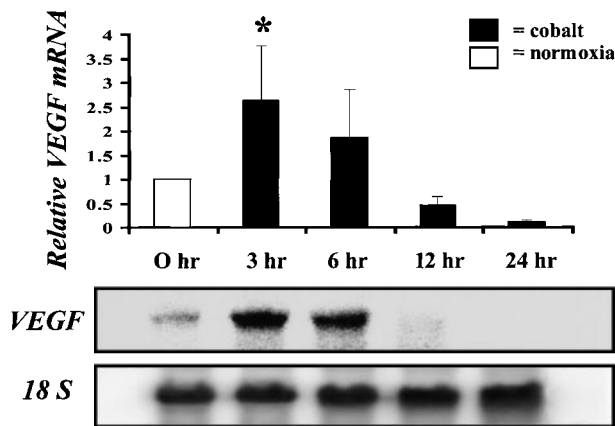
Fig. 6. Cobalt stimulates a time-dependent increase in VEGF mRNA accumulation. Cultures stimulated with cobalt (solid bars) resulted in an increase in VEGF mRNA expression most dramatically at 3-h time point (* $P < 0.05$). Expression remained relatively elevated to 6th h, and then decreased to below baseline normoxic expression (open bar) at 24-h time point.

strate significant differences in the stability (i.e., rate of degradation) of existing VEGF mRNA over time.

Cycloheximide does not diminish VEGF mRNA upregulation by hypoxia, nickel, or cobalt. To determine if increases in VEGF mRNA expression in response to hypoxia, nickel, and cobalt requires de novo protein synthesis, MC3T3-E1 osteoblastic cells were pretreated with cycloheximide (10 µg/ml), an inhibitor of protein translation, immediately before stimulation. These results are summarized in Fig. 7. In contrast to actinomycin D, treatment with cycloheximide did not demonstrate significant differences in VEGF mRNA expression in response to hypoxia, nickel, or cobalt compared with the noncycloheximide controls. These data suggest that de novo protein synthesis is not required for increased expression of VEGF mRNA in MC3T3-E1 cells in response to hypoxia, nickel, and cobalt.

Dexamethasone does not alter the expression of VEGF mRNA in response to hypoxia. Dexamethasone, a potent corticosteroid, has been shown to block transcrip-

tional activation of specific genes by inhibiting the transcriptional activator AP-1 in fibroblasts (7). We sought to evaluate the effect of dexamethasone on VEGF mRNA expression in response to hypoxia because AP-1 is involved in the expression of VEGF secondary to growth factor stimulation (7). Subconfluent MC3T3-E1 cell cultures were treated with or without dexamethasone (0.5 µM) for 6 h followed by exposure to normoxic ($P_{O_2} = 150$ mmHg) or hypoxic ($P_{O_2} = 35$–40 mmHg) culture conditions. The results are shown in Fig. 9. Again, as expected, hypoxia induced a statistically significant increase in VEGF mRNA expression compared with normoxia (4.5-fold increase; $P < 0.01$). This expression pattern was essentially unchanged by the addition of dexamethasone, suggesting that the increase in VEGF mRNA expression by MC3T3-E1 cells in response to hypoxia is independent of AP-1.

DISCUSSION

A number of studies have explored the relationship between angiogenesis and osteogenesis both in vivo and in vitro (1, 2, 33, 38). For example, Aronson et al. demonstrated a ninefold increase in blood flow in osteotomized tibial bone segments compared with non-osteotomized controls (1, 2). Ganey et al. studied the expression of laminin and type IV collagen in blood vessel formation in a mandibular model, histologically demonstrating the role of angiogenesis in distraction osteogenesis (8). Also, Trueta et al. demonstrated a relationship between epiphyseal angiogenesis and the corresponding level of ossification in endochondral bone and concluded that osteogenesis was dependent on oxygen supply (41–43).

More recently, in a rat model of mandibular fracture repair, Saadeh et al. demonstrated increased VEGF mRNA expression during fracture repair (33). This increase in VEGF mRNA corresponded temporally to a period associated with increased angiogenesis (1–2 wk postfracture), and the VEGF protein was immunolocalized to proliferating osteoblasts near the osteotomy
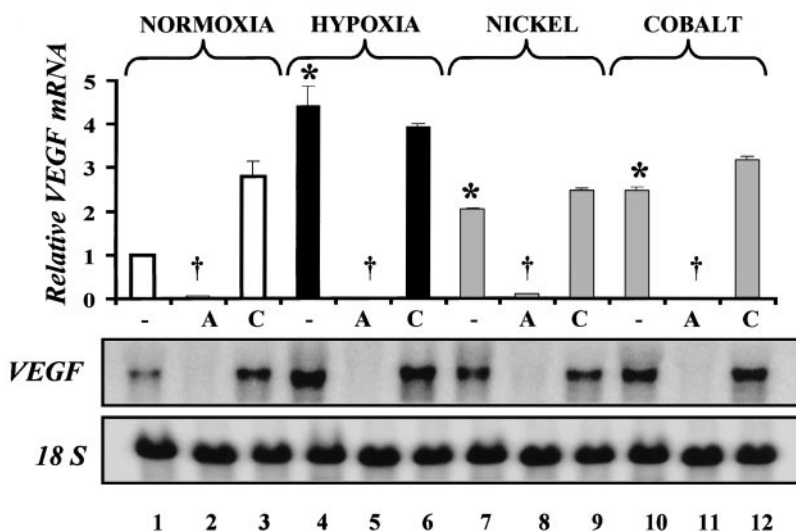

Fig. 7. Effect of actinomycin D and cycloheximide pretreatment on VEGF mRNA induction by hypoxia, nickel, and cobalt. To determine if increased VEGF mRNA expression in response to hypoxia, nickel, and cobalt was transcriptionally mediated, MC3T3-E1 osteoblast cells were stimulated with actinomycin D (A), an inhibitor of transcription. Hypoxia (*lane 4*), nickel (*lane 7*), or cobalt (*lane 10*) all increased VEGF mRNA expression (* $P < 0.05$), compared with normoxic control (*lane 1*). In contrast, with the addition of actinomycin D, hypoxia (*lane 5*), nickel (*lane 8*), and cobalt (*lane 11*), in each case, demonstrated a dramatic decrease in expression of VEGF mRNA († $P < 0.001$, 0.01, 0.001, respectively). To determine if increase in VEGF mRNA in response to hypoxia, nickel, and cobalt required de novo protein synthesis of an intermediate molecule, MC3T3-E1 osteoblast-like cells were stimulated with cycloheximide, an inhibitor of translation. In contrast to the actinomycin D groups, with addition of cycloheximide, hypoxia (*lane 6*), nickel (*lane 9*), and cobalt (*lane 12*), in each case, demonstrated no statistically significant change in expression of VEGF mRNA compared with their respective controls.
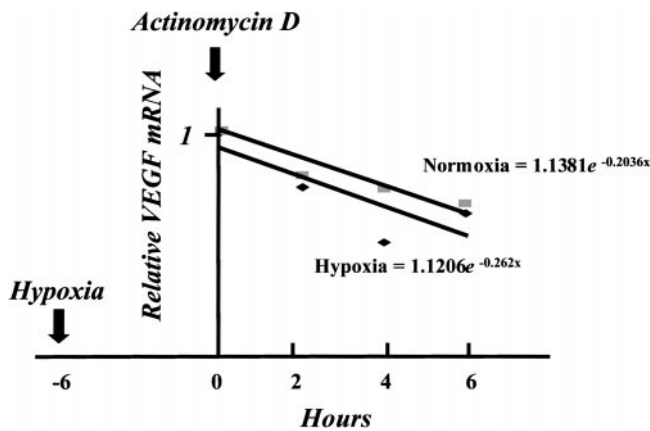
Fig. 8. VEGF mRNA stability is unaffected by hypoxic conditions in MC3T3-E1 cells. To determine influence of hypoxia on VEGF mRNA stability, subconfluent MC3T3-E1 cultures were exposed to normoxia or hypoxia followed by introduction of actinomycin D after 6 h, discontinuing further mRNA synthesis to quantify mRNA degradation over time. In these experiments, no significant differences were noted between mRNA half-life of normoxic and hypoxic cultures.

front within the fracture callus (33). Also, Li et al. have recently investigated vascular formation during distraction osteogenesis in a rabbit leg lengthening model, and have implicated the role of VEGF in the angiogenesis associated with new long bone formation (21).

In vitro, we have demonstrated that hypoxia has a variety of phenotypic effects on MC3T3-E1 cells, including alterations in cellular proliferation, maturation, and production of VEGF mRNA and protein synthesis (38). Taken together, these findings support the hypothesis that osteoblasts may be responsible, at least in part, for the increased VEGF expression and subsequent angiogenesis during osseous repair.

Several genes have been found to be regulated by local oxygen tension (6, 11, 36). One hypoxia response mechanism that has been extensively studied is the EPO gene, which encodes the erythropoietin protein. Erythropoietin is a dimeric hormone that acts as the principal regulator of erythrocyte production. Expressed predominantly in the kidney and fetal liver, erythropoietin binds to erythroid progenitor cell receptors to induce cell growth and differentiation in response to hypoxia (6). Hypoxia-induced upregulation of EPO expression has been hypothesized to be specifically mediated by an oxygen-sensing mechanism, whereby a heme molecule changes conformation in response to the redox state of the local microenvironment (11, 12). Evidence for this mechanism includes the fact that carbon monoxide inhibits the hypoxic induction of erythropoietin by binding and locking the heme protein in an oxygenated state (11).

Recent reports have demonstrated structural similarities between VEGF and the EPO gene (6, 20, 36). For example, Ladoux and Frelin found a 3′ sequence in the human VEGF gene that is highly homologous to a 12-bp fragment of the hypoxia-responsive element downstream to the coding region of the EPO gene (20). Minchenko et al., studying the 5′ end of the VEGF gene in fibroblasts, found a 160-bp enhancer fragment 60 bp

downstream from the polyadenylation sequence (24). Thus there is molecular evidence suggesting similarities in the transcriptional regulation of these two genes.

EPO expression is induced by cobalt and nickel, metallic ions that mimic the mechanism of hypoxia by substituting for Fe^{2+} in the porphyrin ring. Altered conformation of the heme group mimics the deoxygenated state due to a lowered affinity for oxygen (12, 13). In our studies, we observed both a dose- and time-dependent response of VEGF mRNA expression to nickel and cobalt in osteoblast-like cells. The decrease seen in VEGF mRNA after 12 or 24 h may demonstrate an autoregulatory pathway with time, or alternatively may reflect inherent cellular toxicity of these metals with time that is not as apparent with the ample 18s RNA control samples. Interestingly, both cobalt and nickel induced maximal expression of VEGF mRNA in a similar range as cells cultured in hypoxic conditions. These data agree with previous studies of hypoxia-induced VEGF expression in other cell types including cardiac myocytes, glioblastoma cells, and endothelial cells (21, 24, 26, 29). Taken together, our experiments support the hypothesis that the induction of VEGF mRNA expression by osteoblasts in response to hypoxic culture conditions is modulated by an oxygen-sensing hemelike protein similar to the regulation of the EPO gene.

In previous work, the hypoxia-induced increase in EPO gene expression has been shown to be mediated by enhanced transcription (13). In our study, virtually all VEGF mRNA was eliminated after 6 h of transcriptional inhibition by actinomycin D in the hypoxic, nickel, or cobalt cultures. This dramatic decrease in VEGF mRNA by osteoblasts treated with actinomycin D supports the hypothesis that these stimuli exert their effects in a similar manner by stimulating increased
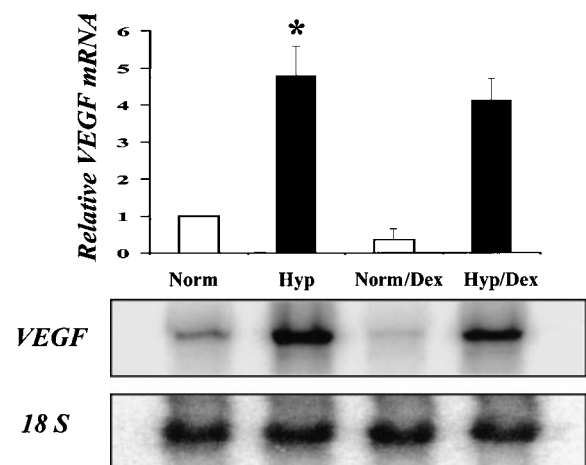

Fig. 9. Dexamethasone does not significantly affect expression of VEGF mRNA. To determine involvement of AP-1 in hypoxic-induced expression of VEGF in osteoblast-like cells, subconfluent cultures were pretreated with or without dexamethasone (0.5 µM) for 6 h with or without hypoxia. Hypoxia induced a significant increase in VEGF (* $P < 0.01$). Addition of dexamethasone did not have any statistically significant effect on VEGF mRNA synthesis. Norm, normoxia; hyp, hypoxia; dex, dexamethasone.

VEGF mRNA transcription. Additionally, these data agree with work by Namiki et al., who observed a decrease in VEGF mRNA accumulation in hypoxia-induced endothelial cultures incubated with transcriptional inhibitors such as actinomycin D (26). Similar decreases in VEGF mRNA have been observed in cultures of cardiac myocytes and hepatoma tumor cells pretreated with actinomycin D before stimulation with nickel, cobalt, or manganese (11, 21).

Inhibiting translation with cycloheximide allows the determination of whether de novo protein synthesis is required for increased VEGF mRNA synthesis (8, 26). In our cycloheximide-pretreated MC3T3-E1 cells, in each case (exposure to hypoxia, nickel, and cobalt), there was no statistically significant decrease in the expression of the VEGF mRNA compared with their respective controls. These results demonstrated that de novo protein synthesis was not required for the observed increase in VEGF mRNA in MC3T3-E1 cells in response to any of these stimuli. These data suggest that the hypoxic regulation of VEGF expression is either via direct activation of VEGF mRNA or mediated by preexisting protein modifiers, but not by hypoxia-induced de novo synthesis of intermediate protein regulators.

The above cycloheximide results could also be explained by two alternative posttranscriptional hypotheses: *1*) hypoxia stimulates the production of mRNA-stabilizing factors, that, when not produced, allow more rapid degradation of the message, or *2*) cycloheximide decreases production of degradative enzymes allowing a continued elevation of the message. To further clarify whether the increased steady state of VEGF mRNA was due specifically to transcriptional upregulation or to changes in the posttranscriptional mRNA stability, we performed mRNA half-life studies with actinomycin D. In these experiments, no significant differences were noted between the mRNA half-life of the normoxic and hypoxic cultures. These data, in combination with the above cycloheximide results, strongly suggest that increased VEGF mRNA in osteoblasts in response to hypoxia is predominantly transcriptionally mediated. This is in contrast to previous work in glioma cells, where Ikeda et al. found increased stabilization of mRNA in the presence of hypoxia (16). Possible explanations for this difference may include tissue-specific differences in mRNA stability and phenotypic differences in gene expression between a malignant cell type (glioblastoma) and the more osteoblast-like (MC3T3-E1) cells used in our study.

The VEGF gene has been reported to have 5′ AP-1 binding sites similar to that of the EPO gene (24). AP-1, a dimeric transcriptional factor composed of the Fos and Jun proteins, has been shown to be potently inhibited by dexamethasone (17, 34). Similar to findings by Finkenzeller et al. in NIH/3T3 fibroblasts (7), we found no appreciable reduction in VEGF mRNA levels with the addition of dexamethasone, suggesting that AP-1 is not significantly involved in VEGF mRNA induction in osteoblasts.

Interestingly, in contrast to the hypoxia-induced increase in VEGF mRNA expression in osteoblasts, hyperoxic conditions did not inversely decrease VEGF mRNA production. This finding may suggest that the osteoblast oxygen-sensing mechanism is a "one-way" regulatory phenomenon acting only to upregulate VEGF and angiogenesis in states of oxygen deficiency.

In summary, the results of this study demonstrated that hypoxia stimulates a time-dependent increase in VEGF mRNA synthesis in primary rat calvarial osteoblasts. In addition, similar increases were observed when MC3T3-E1 osteoblast-like cultures were stimulated by nickel and cobalt, suggesting the presence of a hemelike oxygen-sensing mechanism. Moreover, actinomycin D, cycloheximide, and mRNA stabilization studies collectively demonstrated that this regulation acts predominantly at the level of transcription and does not require de novo protein synthesis. Finally, dexamethasone studies suggested that the AP-1 transcriptional activator does not appear to mediate the hypoxia-induced increase in VEGF mRNA synthesis.

Furthermore, these findings add support to the central hypothesis that, in vivo, a hypoxia-response mechanism may be responsible for the increased VEGF production by osteoblasts seen in the hypoxic zone of the fracture. This increase in VEGF may subsequently promote angiogenesis required for successful osteogenesis after fracture. The complex sequence of events in the hypoxic induction of VEGF affords multiple opportunities for therapeutic intervention by genetic manipulation. Indeed, the addition of recombinant protein or viral constructs may allow a tissue-directed delivery of gene products to augment or accelerate osteogenesis by enhancing angiogenesis in scenarios of poorly healing bone (5, 6, 23, 25).

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