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Mechanism of transforming growth factor-β1-induced expression of vascular endothelial growth factor in murine osteoblastic MC3T3-E1 cells

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Abstract

Transforming growth factor-β1 (TGF-β1), an abundant growth factor in bone matrix, has been shown to be involved in bone formation and fracture healing. The mechanism of action of the osteogenic effect of TGF-β1 is not clearly understood. In this study, we found that the addition of TGF-β1 to murine osteoblastic MC3T3-E1 cells induced vascular endothelial growth factor (VEGF) mRNA production. VEGF mRNA levels reached a plateau within 2 h after the addition of TGF-β1. The induction was superinduced by cycloheximide and blocked by actinomycin D. Ro 31-8220, a protein kinase C inhibitor, abrogated the induction. In addition, curcumin, an inhibitor for transcription factor AP-1, also blocked the induction. Electrophoretic mobility shift assay revealed an enhanced binding of transcription factors AP-1 and NF-κB. Transient transfection experiment showed that VEGF promoter activity increased 3.6-fold upon TGF-β1 stimulation. Immunoblot analysis showed that the amount of secreted VEGF was elevated in the medium 4 h after TGF-β1 stimulation. Our results therefore suggest that at least part of the osteogenic activity of TGF-β1 may be attributed to the production of VEGF.

Keywords: Transforming growth factor-β1; Vascular endothelial growth factor; MC3T3-E1 cell; Protein kinase C

1. Introduction

Transforming growth factor-β1 (TGF-β1) is a homodimeric protein with autocrine and paracrine activities found in a variety of cell types [1]. TGF-β1, -β2 and -β3 are differentially expressed in mammalian tissues. In bone, TGF-β1 and -β2 are produced by osteoblasts and incorporated into mineralized bone matrix [2,3]. TGF-β1 stimulates matrix formation including fibronectin, collagen, osteonectin, and integrins [4,5]. Administration of TGF-β has been reported to enhance cancellous bone formation in juvenile and adult rat bone [6] and fracture healing [7,8]. However, the cascade of events that leads to bone formation and repair by TGF-β1 is not clear at present.

Bone, especially trabecular bone, is rich in vascular tissues. Endothelial cells lining the bone are in close proximity with osteoblasts and osteoprogenitor cells [9,10]. These endothelial cells secrete various cytokines, growth factors or soluble mediators that are

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crucial for bone remodeling and formation. Vascular endothelial growth factor (VEGF) plays an important role in bone metabolism and affects both osteoblasts and osteoclasts. In rat osteoblasts, prostaglandin E2 and E1 could induce VEGF at both mRNA and protein level [11]. The expression of VEGF mRNA in primary murine osteoblasts is increased by insulin-like growth factor-1 [12]. Calciotropic hormones upregulate VEGF in osteoblast-like cells [13,14]. VEGF also regulates osteoblast differentiation: when added to fetal bovine osteoblasts, it induces their migration, PTH-dependent cAMP accumulation and alkaline phosphatase increase [15]. VEGF also induces osteoclast recruitment in bones of osteopetrotic mice [16]. Hypoxia regulates the expression of VEGF in osteoblast-like cells in a dose-dependent fashion [17]. Finally, osteogenic protein-1, which is known to stimulate new bone formation in vivo and to induce cell proliferation and differentiation of osteoblasts in vitro, has been shown to increase the steady-state level of VEGF mRNA in a dose- and time-dependent manner [18]. The purpose of the present study was to determine whether TGF-β1 affects bone metabolism through the production of VEGF by the osteoblasts.

VEGF is a heparin-binding, homodimeric protein of 45 kDa [19–21]. Several forms of human VEGF exist as a result of alternative splicing: VEGF 121, 165, 189, and 206 [22,23]. Murine VEGFs are one amino acid shorter than those of humans [24]. Whereas VEGF 121 and VEGF 165 are secreted into the bloodstream, VEGF 189 and VEGF 206 are sequestered in the cell matrix or bound to cell membranes [25]. It is important for normal cell growth, development, differentiation, wound healing and reproduction.

In this study, we report that TGF-β1 could upregulate VEGF transcripts in murine osteoblastic MC3T3-E1 cells. The induction was due to transcriptional activation. The secreted VEGF level was also elevated after TGF-β1 treatment. Our results suggest that VEGF may contribute to the osteogenic effect of TGF-β1.

2. Materials and methods

2.1. Cell culture and materials

Murine osteoblast MC3T3-E1 cells [26] were cultured in α-modified MEM (Sigma) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine and 25 μg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO_2. Cells were serially subcultured by treatment with trypsin (0.05% trypsin in 0.5 mM Versene) and were used for experimentation between passages 4 and 10. Human recombinant TGF-β1 was purchased from R&D Systems (Minneapolis, MN). Curcumin, polydI–polydC, and salmon testes DNA were obtained from Sigma. Ro 31-8220 was obtained from Calbiochem-Novabiochem (San Diego, CA). [α-^{32}P]dCTP and [γ-^{32}P]ATP were obtained from ICN (Costa Mesa, CA). Fetal bovine serum was obtained from Biofluids (Bethesda, MD). Anti-human VEGF antibody was purchased from Peprotech (Rocky Hill, NJ). T4 polynucleotide kinase, luciferase assay kit, DNA size marker, AP-1 and NF-κB consensus oligomers were purchased from Promega (Madison, WI). RT-PCR kit was obtained from Perkin-Elmer Cetus (Foster City, CA). FuGene 6 transfection reagent was obtained from Boehringer Mannheim (Indianapolis, IN). ECL reagent was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). All other reagents used were of the highest grade commercially available.

2.2. Northern blot analysis

Cells were seeded into 100-mm dishes containing α-MEM and 10% FBS. Near confluent cells were changed to α-MEM containing 0.5% FBS for 16 h before the addition of test agents. After designated time, total RNA was extracted using the acid guanidinium thiocyanate–phenol–$CHCl_3$ extraction method [27]. Fifteen micrograms of RNA was denatured and separated by electrophoresis on 1% agarose gel containing 2.2 M formaldehyde. Following transfer, the RNA was covalently bound to GeneScreen by UV-crosslinking. Hybridization and washing conditions were carried out according to the method of Church and Gilbert [28]. A human VEGF cDNA probe was provided by Dr. Donald Torry (University of Tennessee, Knoxville). The cDNA was labeled

with [^{32}P]dCTP using random primer labeling. The blots were exposed to XAR films with intensifying screens. The intensity of the bands was quantitated by Digital Imaging System (Alpha Innotech). Experiments were repeated at least three times, and representative data are shown.

2.3. RT-PCR analysis of VEGF transcripts

Total RNA isolated from MC3T3-E1 cells was treated with DNase I. The cDNA was synthesized by reverse transcription reaction of 5 μg of RNA. Five μl of cDNA was amplified by Taq DNA polymerase in a 25-μl reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 10 pmol of the two primers: (1) 5′-TGCGATGCGGGGGCTGC-3′ (sense); and (2) 5′-TTTCCTGGTGAGAGTCT-3′ (antisense).

The PCR reaction was allowed to proceed for 40 cycles, each cycle consisting of 45 s at 94°C, 35 s at 54°C, 45 s of polymerization at 72°C and finally a 10-min extension at 72°C. Aliquots of 10 μl of the PCR product were separated on a 2.5% agarose gel. A 100-bp DNA ladder was included as a size marker.

2.4. VEGF promoter activity

MC3T3-E1 cells in 60-mm dishes were transfected with 2 μg promoterless luciferase gene (pxp2) or a luciferase gene-containing VEGF promoter (1.6 kb *Eco*RV–*Pst*I sequence, gift of Drs. Andrew P. Levy and Mark A. Goldberg, Harvard Medical School) [29] in the presence of 5 μl FuGene 6 according to the manufacturer's protocol. After 5 h, the cells were changed to α-MEM containing 10% FBS overnight. The cells were then incubated in serum-free medium containing 10 ng/ml TGF-β1 for 36 h. Cultures were incubated with lysis buffer and luciferase activity in the lysate was measured with a kit from Promega.

2.5. Nuclear extract and electrophoretic mobility shift assay

Nuclear extracts from control and TGF-β1-treated cells were prepared according to Andrews and Faller [30]. AP-1 and NF-κB consensus sequences were end-labeled with [γ-^{32}P]ATP and T4 kinase. Binding reaction (20 μl) containing 10 μg of nuclear extract, 1 μg testes DNA, and 1 μg polydI–polydC was preincubated at room temperature with or without 100-fold excess of unlabeled oligomers as competitors for 15 min. ^{32}P-Labeled AP-1 or NF-κB (20 000 cpm) was then added to the reaction and incubated at room temperature for another 20 min. The samples were resolved on native 4% polyacrylamide gel at 4°C with 0.5×TBE. The gel was dried and analyzed by autoradiography.

2.6. Immunoblot analysis of VEGF

Near confluent MC3T3-E1 cultures in 100-mm dishes were incubated with α-MEM supplemented with 0.5% FBS. Cells were treated with or without TGF-β1 for designated times. Conditioned media were collected in the presence of protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin and 0.5 mM phenylmethylsulfonyl fluoride). Secreted proteins in the conditioned medium were precipitated with quinine sulfate–SDS as described previously [31]. The precipitates were resuspended in Laemmli SDS gel sample buffer and electrophoresed on 10% SDS–PAGE. Immunoblots were carried out with rabbit anti-human VEGF antibodies (1:500) at room temperature for 1.5 h and then with goat anti-rabbit IgG conjugated to HRP (1:10 000, Sigma) for 1 h. Chemiluminescence was detected using ECL reagent.

3. Results

Steady-state levels of VEGF mRNA were determined in MC3T3-E1 cultures by Northern blot analysis. The basal level of VEGF mRNA (3.7 kb) was low. The mRNA level increased rapidly upon the addition of TGF-β1. Levels of VEGF mRNA increased 3.4-fold at 2 h (Fig. 1, lane 4) and then declined. As shown in Fig. 2, VEGF mRNA responded to TGF-β1 in a dose-dependent manner.

The next series of experiments was performed to examine the mechanism of VEGF mRNA induction. Inhibitors of protein and RNA synthesis were included singly or in combination with TGF-β1. Cycloheximide alone minimally induced VEGF mRNA (Fig. 3, lane 3). In the presence of TGF-β1 and cycloheximide (lane 4), the induction was higher than TGF-β1 alone (lane 2), implying that the induction

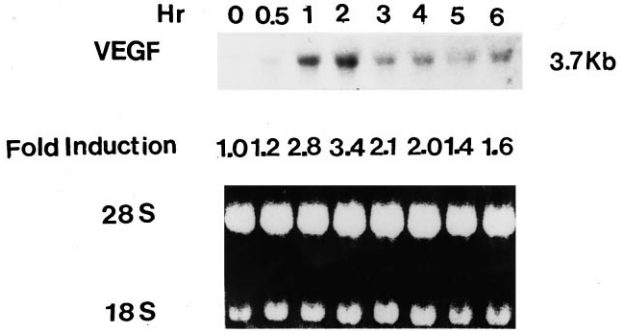

Fig. 1. Time course of VEGF mRNA induction in MC3T3-E1 cells by TGF-β1. Near confluent cultures were exposed to 5 ng/ml TGF-β1 for the times indicated. Fifteen μg of total RNA was hybridized with a cDNA probe for human VEGF. Hybridization patterns of cells treated with TGF-β1 for 0 min (lane 1); 30 min (lane 2); 1 h (lane 3); 2 h (lane 4); 3 h (lane 5); 4 h (lane 6); 5 h (lane 7); 6 h (lane 8); are shown. Ethidium bromide staining pattern of 28S and 18S ribosomal RNA bands are shown in the lower panel. The intensity of VEGF band was quantitated by image analysis. Fold induction was calculated by comparing with the VEGF level in untreated cells.

does not require de novo protein synthesis. In the presence of actinomycin D, the induction was abolished (lane 6), indicating that de novo RNA synthesis is required.

We next investigated whether TGF-β1 exerted its effect through a protein kinase C (PKC)-dependent pathway. Ro 31-8220, a PKC inhibitor [32], was included in our experiment. Cells were pretreated with 15 μM Ro 31-8220 for 30 min prior to the addition of TGF-β1. Ro 31-8220 was able to block the induction of VEGF mRNA (Fig. 4, lane 4). Our results

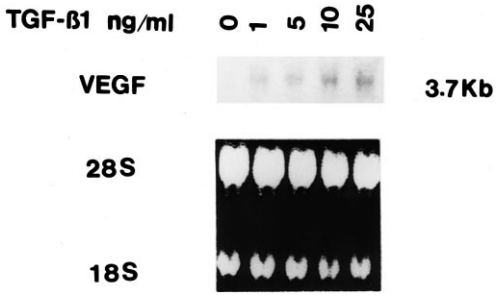

Fig. 2. Dose-dependent induction of VEGF mRNA by TGF-β1. Near confluent cultures of MC3T3-E1 cells were treated with different concentrations of TGF-β1 for 2 h. Fifteen μg of total RNA was hybridized with a cDNA probe for human VEGF. Hybridization patterns of control cells (lane 1); cells treated with 1 ng/ml TGF-β1 (lane 2); 5 ng/ml TGF-β1 (lane 3); 10 ng/ml TGF-β1 (lane 4); 25 ng/ml TGF-β1 (lane 5); are shown.

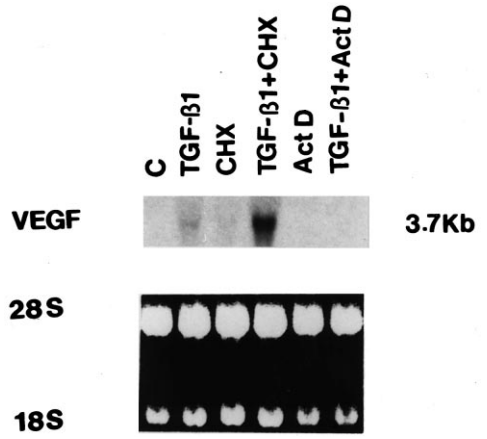

Fig. 3. Effect of cycloheximide (CHX) and actinomycin D (Act D) on the induction of VEGF mRNA by TGF-β1. Near confluent cultures of MC3T3-E1 cells were treated with 5 ng/ml TGF-β1 for 2 h in the absence or presence of CHX (20 μg/ml) or Act D (1 μg/ml). Fifteen μg of total RNA was hybridized with a human VEGF cDNA probe. Hybridization patterns of control cells (lane 1); cells treated with TGF-β1 (lane 2); CHX (lane 3); TGF-β1 and CHX (lane 4); Act D (lane 5); TGF-β1 and Act D (lane 6); are shown.

suggest that the effect of TGF-β1 is mediated by a PKC-dependent pathway. The promoter of the mouse VEGF gene has been shown to contain AP-

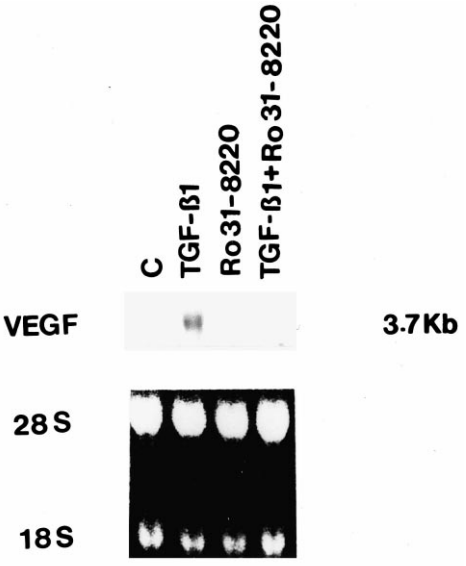

Fig. 4. Effect of a protein kinase C inhibitor, Ro 31-8220, on the induction of VEGF mRNA by TGF-β1. Near confluent MC3T3-E1 cells were pretreated with 15 μM Ro 31-8220 for 30 min before the addition of 5 ng/ml TGF-β1 for 2 h. Hybridization patterns of control cells (lane 1); cells treated with TGF-β1 (lane 2); Ro 31-8220 (lane 3); TGF-β1 and Ro 31-8220 (lane 4); are shown.

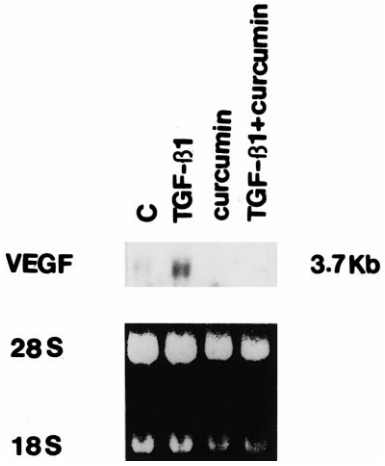

Fig. 5. Effect of curcumin on the induction of VEGF mRNA by TGF-β1. Near confluent MC3T3-E1 cells were pretreated with 20 μM curcumin for 30 min before the addition of 5 ng/ml TGF-β1 for 2 h. Hybridization patterns of control cells (lane 1); cells treated with TGF-β1 (lane 2); 20 μM curcumin (lane 3); TGF-β1 and curcumin (lane 4); are shown.

1 sites [33]. Curcumin [34], an inhibitor of AP-1, was also tested. Fig. 5 shows that preincubation of cells with curcumin blocks the induction of VEGF mRNA (lane 4).

To characterize the transcription factors that were activated by TGF-β1 treatment, EMSA was performed on nuclear extracts prepared from MC3T3-E1 cultures treated with or without TGF-β1. Fig. 6 shows that both AP-1 and NF-κB were activated by TGF-β1 in a time-dependent manner. The optimal activity was observed at 15 min after stimulation. The specificity of binding was demonstrated by the

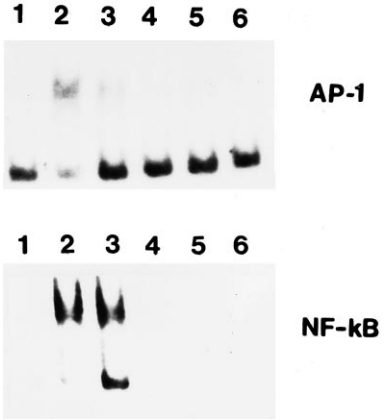

Fig. 6. TGF-β1 induced activation of AP-1 and NF-κB binding. Nuclear extracts from control and TGF-β1 (5 ng/ml) treated MC3T3-E1 cells were incubated with labeled oligomer containing an AP-1 binding site (top panel) or an NF-κB binding site (bottom panel). Gel shift patterns of nuclear extract from control cells (lane 1); cells treated with TGF-β1 for 15 min (lane 2); and cells treated with TGF-β1 for 30 min (lane 3); are shown. Lanes 4–6 represent the binding pattern of the same samples in the presence of 100-fold excess cold oligomer.

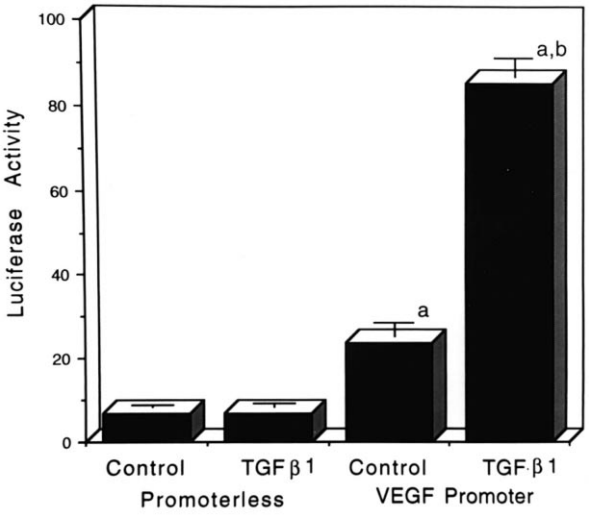

Fig. 7. Stimulation of VEGF promoter activity by TGF-β1. MC3T3-E1 cultures in 60-mm plates were transfected with 2 μg of promoterless luciferase gene or luciferase gene containing VEGF promoter and 5 μl of FuGene 6 transfection reagent as described in Section 2. Luciferase activity in the cell lysates was expressed as light units $\times 10^3$/μg protein. Results were expressed as mean ± S.E.M. of three plates for each variable. Statistical analysis was made by Student's *t*-test. [a] $P < 0.05$ vs. control (promoter less), [b] $P < 0.05$ vs. control (VEGF promoter).

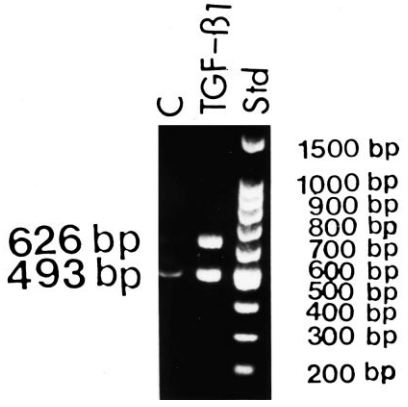

Fig. 8. RT-PCR analysis of the expression of VEGF transcripts. RT-PCR was performed as described in Section 2. Control and TGF-β1-treated RNA are presented in lanes 1 and 2, respectively. Lane 3 shows the 100-bp DNA ladder. Two VEGF transcripts of 493 and 626 bp are detected.

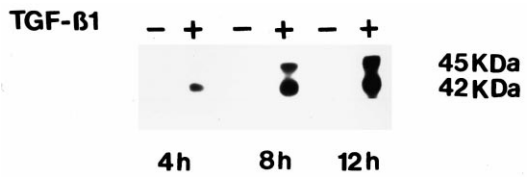

Fig. 9. Appearance of VEGF in the conditioned medium of MC3T3-E1 cells. Cells were treated with or without 5 ng/ml TGF-β1 for different times and the conditioned medium was precipitated with quinine sulfate–SDS. Proteins were separated by 10% SDS–PAGE. Immunoblot was carried out with rabbit anti-human VEGF antibodies. Immunostaining patterns of cells treated with TGF-β1 for 4, 8 and 12 h (lanes 2, 4 and 6) and their corresponding controls (lanes 1, 3 and 5), are shown.

disappearance of bands after the addition of excess unlabeled oligomers (lanes 4–6).

In order to examine whether the induction of VEGF mRNA is a result of transcriptional activation, transfection experiments were carried out with a construct containing VEGF promoter and luciferase in the absence or presence of TGF-β1. Fig. 7 shows that TGF-β1 increased luciferase activity of VEGF gene 3.6-fold, whereas it had no effect on the luciferase gene itself.

Although only a 3.7-kb VEGF mRNA was detected by the Northern blot analysis, RT-PCR experiment revealed two subtypes of mRNA 493 and 626 bp in length in MC3T3-E1 cells (Fig. 8, lane 1). These two products were enhanced in the presence of TGF-β1 (lane 2). The sizes of the mRNA match murine VEGF 120 and VEGF 164 [35].

In order to examine whether the increase of VEGF mRNA correlated well with the appearance of VEGF in the conditioned medium, immunoblot analysis was carried out. The conditioned media of control and TGF-β1-treated cells were precipitated by quinine sulfate–SDS. In the absence of TGF-β1, MC3T3-E1 cells secreted only trace amounts of VEGF (Fig. 9, lane 1). A protein band of 42 kDa appeared in the medium 4 h after the addition of TGF-β1 (lane 2). At 8 h, another 45-kDa band also appeared (lane 4). The level of these two bands was increased at 12 h (lane 6), whereas the basal levels remained low (lanes 3 and 5).

4. Discussion

The present study demonstrates that TGF-β1 acts on mouse osteoblast MC3T3-E1 cells to induce VEGF mRNA in a dose- and time-dependent manner. The induction involves a PKC-dependent pathway. In addition, the increase of VEGF mRNA correlates well with the release of VEGF into the conditioned medium. The effect of TGF-β1 is not unique to murine osteoblasts, similar induction was observed in human osteosarcoma MG-63 cells (C.C. Chua, R.C. Hamdy and B.H.L. Chua, unpublished data).

It is interesting that VEGF mRNA is 'superinduced' in the presence of cycloheximide and TGF-β1, indicating that de novo synthesis of protein factors is not necessary for the induction. This could be due to cycloheximide inhibiting the synthesis of either labile ribonucleases involved in degrading VEGF mRNA, or repressor protein(s) involved in the regulation of VEGF gene expression. Our data demonstrated that the induction of VEGF mRNA level by TGF-β1 could be mainly attributed to transcriptional activation. Electrophoretic mobility shift assay showed that TGF-β1 enhanced AP-1 and NF-κB binding. The promoter of mouse VEGF gene has been shown to contain NF-κB, AP-1, AP-2 and SP-1 binding sites [33]. The involvement of transcription factors, such as SP-1 or AP-2, in VEGF induction is under investigation.

Bone is rich in vascular tissues. Bone endothelial cells are in close proximity with osteoblasts and osteoprogenitor cells [9]. These endothelial cells secrete various cytokines, growth factors or soluble mediators that are crucial for bone remodeling and formation. Accumulating evidence indicates an important role of VEGF in bone metabolism. Midy and Plouet [15] demonstrated that VEGF is chemotactic and could induce migration and differentiation of osteoblasts. Several growth factors, cytokines and calciotropic hormones have been demonstrated to induce the gene expression of VEGF. Hirada et al. [11] reported that prostaglandin E_2 and E_1 could upregulate VEGF at both mRNA and protein level in rat calvaria-derived osteoblasts. The induction could be blocked by dexamethasone. Goad et al. [12] demonstrated that the addition of physiological level of insulin-like growth factor-1 (10 nM) resulted in a fourfold stimulation of mRNA level in both human SaOS-2 osteoblast-like cells and primary murine osteoblasts. The induction of VEGF mRNA was ac-

companied by an increase in immunoreactive VEGF protein. In addition, Wang et al. [13] showed that 1,25-dihydroxyvitamin D3 (10^{-11}–10^{-10}M) could up-regulate VEGF mRNA level by 1.7- and 2.3-fold in human osteoblast-like cells (HobLC) and human osteosarcoma SaOS-2 cells, respectively. There is a two-fold increase in the immunoreactive VEGF in the conditioned medium. Schlaeppi et al. [14] also found that 1,25-dihydroxyvitamin D3 (1–10 nM) could stimulate VEGF mRNA expression by approximately three-fold in human osteoblast-like cells. The up-regulation of VEGF protein was detected after 6 h of treatment. Combined treatment of parathyroid hormone (PTH-1–34) and 1,25-dihydroxyvitamin D3 resulted in approximately five-fold stimulation of VEGF mRNA.

The pathophysiological role of VEGF in bone diseases has recently been investigated. Gerber and colleagues found high levels of VEGF mRNA expression in terminal hypertrophic and relatively ischemic chondrocytes [36]. These investigators have also shown that VEGF stimulates endochondral bone formation by inducing capillary invasion of the cartilagenous growth plate and by recruiting chondroclasts to resorb the apoptotic hypertrophied chondrocytes. Steibrech and colleagues reported that hypoxia during fracture healing was a stimulus for the osteoblasts to increase the expression of VEGF and thus enhance the healing process [17]. Our results demonstrate that TGF-β1 could regulate the expression of VEGF by the osteoblasts in the absence of hypoxia.

TGF-β1 has been classified as an indirect angiogenic factor, because it stimulates angiogenesis by facilitating capillary formation in vivo [37], but fails to stimulate the mitogenic activity and migration of endothelial cells in vitro [38]. The present study clearly shows that a direct angiogenic factor VEGF could be regulated by TGF-β1. A similar phenomenon has been observed in smooth muscle cells where TGF-β1 stimulates the gene expression of two direct angiogenic factors, VEGF and basic FGF [39].

In summary, we found that TGF-β1 upregulated the gene expression of VEGF in murine osteoblasts. The elevated production of VEGF could act on neighboring endothelial cells and lead to proliferation and increased blood supply, contributing to bone formation, remodeling and repair.

Acknowledgements

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