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Vascular Endothelial Growth Factor Principally Acts as the Main Angiogenic Factor in the Early Stage of Human Osteoblastogenesis

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Vascular endothelial growth factor (VEGF)–mediated angiogenesis is essential for bone formation. However, the effect of VEGF on osteoblastic cells during osteoblastogenesis is still controversial. The aim of this study was to clarify the relationship between osteoblastic cells derived from human mesenchymal stem cells (MSCs) and VEGF in the early stage of osteoblastic differentiation. Continuous dexamethasone treatment with a low concentration stimulated osteoblastogenesis of MSCs and the expression of VEGF121 mRNA. The VEGF secretion from osteoblastic cells also increased along with osteoblastogenesis. Neuropilin-1, which mainly binds VEGF165, was detected at all stages during early osteoblastogenesis, but VEGF receptor-1 and -2 were not detected on RT-PCR analyses. In this study, VEGF had no direct effect on the proliferation of osteoblastic cells. However, the secreted VEGF in the conditioned medium of osteoblastic cells exhibited high angiogenic power as to endothelial cell proliferation. Our findings indicated that VEGF121 principally acts as the main angiogenic factor in the early stage of human osteoblastogenesis. The present study also demonstrated the differential expression of VEGF121 during osteoblastogenesis. The increase of VEGF in the early stage might be a useful marker of induction of bone formation due to human MSCs.

Key words: angiogenesis, mesenchymal stem cells, osteoblastic cells, osteoblastogenesis, vascular endothelial growth factor.

Abbreviations: Ab, antibody; ALP, alkaline phosphatase; bFGF, basic fibroblast growth factor; CM, conditioned medium; Dex, dexamethasone; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVECs, human umbilical vein endothelial cells; MSCs, mesenchymal stem cells; MSCGM, mesenchymal stem cell growth medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide; OIM, osteogenesis induction medium; rh-, recombinant human; Runx2/Cbfa1, Runt-related transcription factor–2/core binding factor a1; TGF-β1, transforming growth factor-β1; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Human mesenchymal stem cells (MSCs) derived from bone marrow have the potential to differentiate into the adipocytic, chondrocytic, and osteocytic lineages (*1*). The research on bone formation and tissue engineering techniques involving MSCs is highlighted (*2–4*). Many investigators have reported that bone marrow cell–derived osteogenesis is advantageous for inducing satisfactory bone formation (*5–7*). Sudhakar *et al.* demonstrated that cell differentiation along with the osteoblastic lineage (nonosteoblastic cells, osteoblast precursors, less mature osteoblastic cells, and mature osteoblasts) might be regulated at the level of Runt-related transcription factor–2/core binding factor a1 (Runx2/Cbfa1) expression (*8*). Changes in several marker genes during osteogenesis have also been reported. The expression of alkaline phosphatase (ALP), bone sialoprotein, and osteocalcin in human MSC-derived osteogenesis is determined by the culture duration (*9*). The expression of collagen I, ALP, and bone sialoprotein precedes the expression of osteocalcin during osteogenesis (*10*). However, the phenotypic change of osteoblastic cells derived from human MSCs has not been fully clarified. We therefore investigated the phenotypic differences of human osteoblastic cells in early differentiation stages.

Angiogenesis is also a crucial process in bone formation (*11*). In addition, vascular endothelial growth factor (VEGF)–mediated angiogenesis is an important in fracture repair (*12*) and endochondral ossification (*13*). VEGF has been reported to play a key role in regulation of the interaction between osteogenesis and angiogenesis. Deckers *et al.* have suggested that VEGF regulates bone remodeling by attracting endothelial cells and by stimulating osteoblast differentiation of mouse preosteoblast-like cell line KS483 (*14*). However, the relationship between human MSC-derived osteoblastogenesis and VEGF has not been elucidated yet. In addition, the effect of VEGF on osteoblastic cells is also controversial. Several studies have shown that VEGF stimulates the proliferation, migration, and differentiation of osteoblasts (*14–16*). However, Villars *et al.* demonstrated that VEGF had no proliferative effect on osteoblast progenitors derived from human bone marrow stromal cells (*17*). We there-

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fore examined the differential expression of VEGF and its effect during osteoblastogenesis in an *in vitro* osteogenesis induction model involving human MSCs. To assess the activity of VEGF on osteoblastic cells, we analyzed the expression of receptors for VEGF and performed cell proliferation assays with human MSC-derived osteoblastic cells.

VEGF expression in osteoblasts or osteoblastic cells is stimulated by the following factors: prostaglandin E1 and E2 (*18*), insulin-like growth factor–I (*14*, *19*, *20*), 1,25-dihydroxyvitamin D3 (*20*), parathyroid hormone (*20*), fibroblast growth factor-2 (*21*), transforming growth factor–β1, –2, and –3 (*21*), bone morphogenetic proteins–2, –4, and –6 (*22*), and hypoxic conditions (*23*, *24*). Dexamethasone (Dex) treatment induces the differentiation of human bone marrow osteogenic stromal cells into osteoblast-like cells (*25*). However, whether or not VEGF expression is affected by Dex treatment in osteoblastic cells has not been clarified yet. Harada *et al.* reported that Dex treatment inhibited the expression of VEGF mRNA upregulated by prostaglandin E2 in immortalized rat calvaria-derived osteoblast-enriched cells (*18*). We consider that VEGF expression in human osteoblastic cells is regulated by the differentiation stage during Dex-induced osteoblastogenesis.

In the present study, we demonstrate that VEGF is not an osteoblastic cell growth factor but the main angiogenic factor in early osteoblastogenesis. Moreover, the augmentation of VEGF mRNA and protein are dependent on the differentiation stage of osteoblastic cells.

MATERIALS AND METHODS

Cells and Cell Culture—Human MSCs were obtained from BioWhittaker (Walkersville, MD). According to the osteogenesis protocol, 3×10^5 cells were plated in 75 cm^2 tissue culture flasks (Greiner, Frickenhausen, Germany) and then cultured in mesenchymal stem cell growth medium (MSCGM, BioWhittaker) for 2 days. MSCs were trypsinized and then sub-cultured in the osteogenesis induction medium (OIM) based on α-minimum essential medium (Sigma, St. Louis, MO) comprising 10% fetal bovine serum (Sigma), 10 mM Na-β-glycerophosphate (Sigma), 82 μg/ml of ascorbic acid phosphate (Wako, Osaka), and 1×10^{-8} M dexamethasone (Dex, Sigma). After 1-week culture with OIM, the flasks and cells (Dex-1wk cells) were randomly divided into two groups. One group was maintained in the Dex-containing OIM for an additional 2 weeks (Dex-3wk cells), and the other one was cultured in the Dex-starved OIM for 2 weeks (Dex-1-2wk cells). Cells between passages 2 and 4 were used for experiments. Non-induced control human MSCs were maintained in MSCGM with the same schedule (MSCGM-1wk cells). Human umbilical vein endothelial cells (HUVECs) were obtained from Kurabo (Osaka), and maintained and sub-cultured as described previously (*26*). HUVECs between passages 2 and 6 were used for experiments. Cell morphology was observed by phase contrast microscopy.

RNA Isolation, RT-PCR, Southern and Northern Blot Analyses—Total cellular RNA from the cells at each osteoblastic stage was extracted with TORIZOL reagent (Invitrogen, Carlsbad, CA). RNA samples (5 μg) were reverse-transcribed to cDNA as described previously (*26*). The cDNAs were subjected to PCR amplification in the presence of 10 pmol of the following specific primer sets: 5′-TCATCTGGAACCGCACGGAA-3′ and 5′-TGTTGTGAGCATAGTCCACC-3′ for ALP, 5′-CAGAGGCATAAAGGGTCACC-3′ and 5′-GAGTGGCACATCTTGAGGTC-3′ for collagen I, 5′-CGCGGATCCACACCGTGGCTTCACTGGTC-3′ and 5′-CGGAATTCCTCGGCGTCATGCTGTCTCA-3′ for collagen II, 5′-TCTTCCTGGTCTGGCTGGTA-3′ and 5′-TCCTCGATGTCCTTTGATGC-3′ for collagen III, 5′-ACGATGAATGTGGCGATACT-3′ and 5′-AGTGCATTCAAGGCTGTTGG-3′ for neuropilin-1, and 5′-ATGAGAGCCCTCACACTCCT-3′ and 5′-CTAGACCGGGCCGTAGAAGC-3′ for osteocalcin. The primer sets for VEGF (exons 1–5 and exons 1–8), VEGF receptor (VEGFR)-1, and VEGFR-2 were presented in our previous report (*26*). The 452-bp fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using a GAPDH 0.45-kb control amplimer set (Clontech Laboratories, Palo Alto, CA). The PCR reactions were allowed to proceed for 30 cycles, each cycle consisting of 30 s at 94°C, 30 s at 58°C (64°C for osteocalcin), and 40 s at 72°C. Their signals were then normalized as to the levels of the GAPDH signals. Southern blot analyses of VEGF isoforms amplified with exons 1–8 primers were performed according to our previous report (*26*). Northern blot analyses were performed using 10 μg of RNA samples and a cDNA probe for VEGF exons 1–5 as described previously (*26*).

Enzyme-Linked Immunosorbent Assay (ELISA)—The conditioned medium (CM) of each cell group was replaced with the respective fresh medium. The cells were allowed to grow for 48 h, and then the CM was collected and centrifuged to remove all cellular fragments. The adhered cells were sub-cultured for further *in vitro* assays or detached for RNA extraction. VEGF proteins were measured with a Quantikine human VEGF ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Three samples under the same conditions were collected. Each of the CM was added to two wells and the mean value for the two wells was regarded as a single value. After collecting the culture supernatants, cells were trypsinized and counted with a hemocytometer. The levels of VEGF protein were calculated as nanograms of VEGF/10^6 cells/48 h as described (*27*).

Cell Proliferation Assays—Cells were trypsinized and seeded at 1×10^4 cells/well on 96-well plastic tissue culture plates (Coster, Cambridge, MA). Then the plates were incubated with fresh OIM for 24 h. In cell proliferation assays, the medium was replaced with fresh OIM, VEGF-containing OIM, or anti-VEGF antibody-containing OIM. HUVECs were cultured for 24 h on 96-well plates as described (*28*). The medium of HUVECs was replaced with fresh OIM, the CM of Dex-1wk cells, or the CM of Dex-3wk cells. To assess the efficacy of VEGF as to cell proliferation, we used recombinant human (rh-) VEGF proteins and neutralizing antibodies against human VEGF. The rh-VEGF121 and -VEGF165 proteins (both obtained from R&D Systems) were added to the medium to a final concentration of 5 ng/ml. A goat anti-human VEGF polyclonal antibody (VEGF-Ab. R&D Systems), which has the ability to neutralize the biological activity of VEGF121 and VEGF165, was added to the media at a final concentration of 10 μg/ml. To evaluate

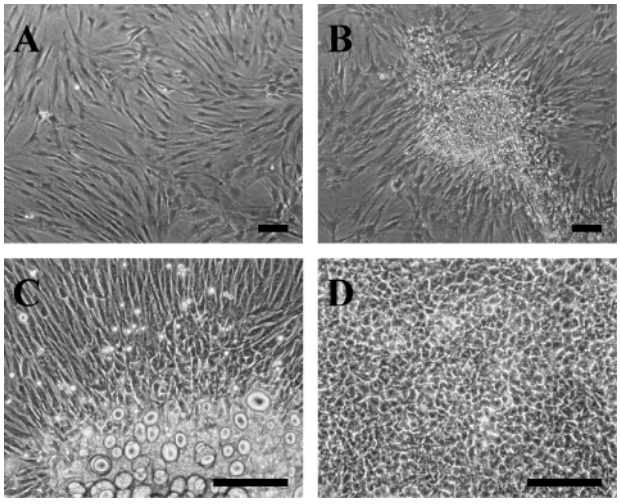

Fig. 1. **Dex treatment stimulated cell morphology conversion.** (A) Spindle-shaped cells maintained in MSCGM for 1 week (MSCGM-1wk cells). (B) Cuboidal-shaped cells induced by Dex treatment for 1 week (Dex-1wk cells). (C) Dex-1wk cells-derived cell clusters cultured in Dex-depleted medium for 2 weeks (Dex-1-2wk cells). (D) Continuous Dex treatment for 3 weeks increased the formation of cell clusters (Dex-3wk cells). Scale bars = 200 μm.

the proliferative activity of osteoblastic cells, we added 10 ng/ml of rh-basic fibroblast growth factor (bFGF. Pepro Tech, London, UK) in the fresh OIM. We also performed inhibition assays using a rabbit anti-human bFGF polyclonal antibody (bFGF-Ab. Pepro Tech) at a concentration of 10 μg/ml as described previously (*26*). The plates were incubated for 24 h to allow proliferation and then the cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT. Chemicon, Temecula, CA) according to the manufacturer's protocol. Each of the test samples was added to four wells (n = 4). The optical density was measured at a wavelength of 595 nm as described (*28*). The osteoblastic cell proliferation data were evaluated as relative cell proliferation, which is the percentage compared with the mean value for OIM of Dex-1wk cells. For HUVECs, the data were compared

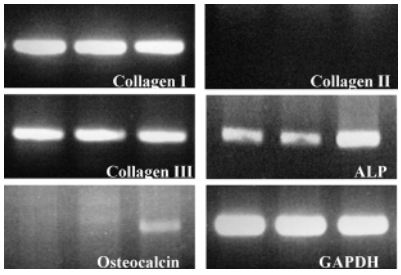

Fig. 2. **Expression of osteoblastic marker genes during osteoblastogenesis.** The RT-PCR fragments in the left, middle, and right lanes of each panel reflect the gene expression in Dex-1wk, Dex-1–2wk, and Dex-3wk cells, respectively. The signals of collagen I (489 bp) and collagen III (334 bp) were observed, however, the collagen II gene was not expressed in any of the groups. ALP fragments (516 bp) were detected in all cell groups. Note that the expression of ALP was slightly upregulated in Dex-3wk cells. The expression of osteocalcin (303 bp) was only observed in Dex-3wk cells. The 452-bp fragment of GAPDH was equally amplified in all the groups.

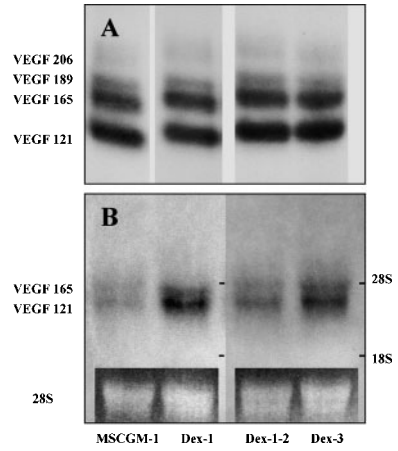

Fig. 3. **VEGF121 expression was stimulated by Dex treatment along with early osteoblastogenesis.** (A) The expression of VEGF121, 165, 189, and 206 was observed in all groups on RT-PCR-Southern blot analyses. Note that the VEGF121 and 165 signals were stronger than the others. (B) The amounts of VEGF mRNA were determined by Northern blot analyses. MSCGM-1wk cells expressed a low level of VEGF mRNA. Note that the expression of VEGF121 (the lower band, 3.7 kb) was increased in Dex-1wk and Dex-3wk cells, however, VEGF165 signals (the upper band, 4.5 kb) remained at a low level. The expression of VEGF121 decreased under Dex-depleted condition in Dex-1-2wk cells. VEGF expression in Dex-3wk cells was maintained by the additional Dex treatment. No densitmetric difference between lanes was observed in the signal of 28S rRNA. The lanes were loaded with MSCGM-1wk, Dex-1wk, Dex-1-2wk, and Dex-3wk cells, from the left. The loading positions of VEGF isoforms are shown on the left of these figures. Bars in (B) denote the loading position of human rRNA.

with the OIM. The assays were performed in triplicate and similar results were obtained.

Statistical Analyses—Data were expressed as means ± SD. Differences among groups were compared using the Mann-Whitney U-test. Statistical significance was established at $p < 0.05$.

RESULTS

Dex-Containing Conditioning Stimulate Further Osteoblastogenesis—We investigated the effect of Dex on osteoblastic differentiation. Dex affected the cell morphology conversion from a spindle shape to a cuboidal shape, and produced many cell clusters (Fig. 1, A and B). The morphology of Dex-1-2wk cells cultured in Dex-depleted OIM was not different from that of Dex-1wk cells (Fig. 1C). Continuous Dex treatment increased the formation of cell clusters by Dex-3wk cells (Fig. 1D). To examine the osteoblastic differentiation in this culture model and to confirm the different phenotypes of these three groups, we performed RT-PCR analyses using the marker genes expressed in osteoblastic cells and other cell types. A representative chondrocytic marker, collagen II, was not observed (Fig. 2). Although collagen I (489 bp), collagen III (334 bp), and ALP (516 bp) expression was detected in all groups, the PCR fragment of osteocalcin (303 bp) was only detected in Dex-3wk cells (Fig. 2). We considered that this culture model induced not chondrogenic or adipogenic differentiation but osteogenic differentiation. These findings also demonstrate that the initial Dex

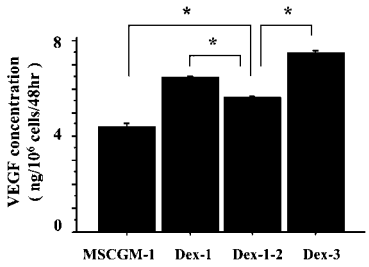

Fig. 4. **VEGF secretion also increased along with osteoblastic differentiation.** The concentration of VEGF in each CM was measured by ELISA. The mean VEGF concentrations in the CM of MSCGM-1wk, Dex-1wk, Dex-1-2wk, and Dex-3wk cells were 4.42 ± 0.15, 6.44 ± 0.09, 5.62 ± 0.08, and 7.48 ± 0.11 ng/10^6 cells/48 h, respectively. A statistical significance (*, $p < 0.05$) was observed for each group with the Mann-Whitney *U*-test. Error bars, SD.

treatment for 1 week is not sufficient for the induction of matured osteoblasts in this culture model. In addition, Dex-starved conditions for osteoblast precursors might possibly cause them to differentiate into nonosteoblastic cells.

VEGF Expression Increased Along with Osteoblastic Differentiation—To assess the angiogenic power in an early stage of osteoblastic cells, we investigated the relationship between VEGF expression and each cell group. On RT-PCR-Southern blot analyses, we detected four VEGF isoforms, which arise through alternative splicing, and which encode human VEGF having 121, 165, 189, and 206 amino acids (Fig. 3A). On Northern blot analyses, however, two isoforms of VEGF (VEGF121 and VEGF165) were observed in all groups (Fig. 3B). Moreover, we detected the augmentation of VEGF121 expression (3.7 kb) in Dex-1wk and Dex-3wk cells (Fig. 3B). We surprisingly found that the VEGF expression on Dex-1-2wk cells was lower than that on Dex-1wk cells (Fig. 3B). In addition, we investigated the level of VEGF protein in each CM by ELISA. The mean VEGF concentrations for MSCGM-1wk, Dex-1wk, Dex-1-2wk, and Dex-3wk CM were 4.42 ± 0.15, 6.44 ± 0.09, 5.62 ± 0.08, and 7.48 ± 0.11 ng/10^6 cells/48 h, respectively (Fig. 4). Statistical significance ($p < 0.05$) was observed for each concentration. These results show that Dex treatment stimulates VEGF121 expression and osteoblastogenesis.

VEGF Has No Effect on the Proliferation of Osteoblastic Cells—The effect of VEGF on osteoblastic cells is still controversial. Therefore, we investigated the expression of receptors for VEGF on osteoblastic cells. The PCR fragments of VEGFR-1 and -2 were not detected in any of the groups (Fig. 5). However, neuropilin-1 was found to be expressed in all groups on RT-PCR analyses (Fig. 5). These results suggest that VEGF121 secreted from osteoblastic cells might not have an autocrine effect. To investigate the response of osteoblastic cells to VEGF stimulation, we performed cell proliferation assays using Dex-1wk and Dex-3wk cells. Neither of the cell proliferation rates was affected by the addition of rh-VEGF or VEGF-Ab to the medium (Fig. 6A). However, we observed a difference in relative cell proliferation between Dex-1wk and Dex-3wk cells (Fig. 6A). The cell proliferation rate of Dex-3wk cells was approximately 0.8 times lower than that of Dex-1wk cells ($p < 0.05$). To evaluate the proliferative activities of osteoblastic cells, we also analyzed their responses to another growth factor, bFGF. The addition of bFGF stimulated their proliferation contrary to that of VEGF (Fig. 6A). However, Dex-3wk cell proliferation was lower than that of Dex-1wk cells (Fig. 6A). These findings also suggest that osteoblastic cells at an early differentiation stage might have a stronger proliferative potential than ones at a late stage.

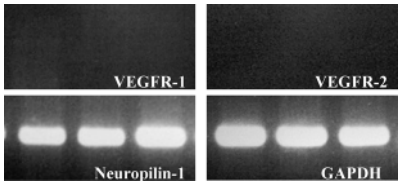

Fig. 5. **Expression of VEGF receptors in osteoblastic cells.** The RT-PCR fragments in the left, middle, and right lanes of each panel reflect the gene expression in Dex-1wk, Dex-1-2wk, and Dex-3wk cells, respectively. Although VEGFR-1 and -2 were not detected, the expression of neuropilin-1 (452 bp) was observed in all the groups. The expression of VEGFR-1, -2, and neuropilin-1 was not dependent on the differentiation stage of osteoblastogenesis. The 452-bp fragment of GAPDH was equally amplified in all the groups.

VEGF121 Is the Main Angiogenic Factor during Early Osteoblastogenesis—To assess the biological activity of VEGF present in the CM of osteoblastic cells, we performed endothelial cell proliferation assays using HUVECs. The VEGF concentrations in the CM obtained from Dex-1wk and Dex-3wk cells were 1.27 and 1.48 ng/ml, respectively. The CM of Dex-1wk and Dex-3wk cells increased HUVEC proliferation up to approximately 150% of the OIM level (Fig. 6B). Moreover, VEGF-Ab treatment inhibited the proliferative effect of the CM on HUVECs to the control level (Fig. 6B). These results demonstrate that the angiogenic activity of osteoblastic cells mainly depended on the secretion of VEGF at an early differentiation stage.

DISCUSSION

Our results illustrate the differential expression of VEGF on osteoblastic cells derived from human MSCs. The increase in VEGF expression during osteoblastogenesis was dependent on the maturity of the osteoblastic cells. However, VEGF had no effect on the proliferation of osteoblastic cells in our culture model. We propose here that VEGF121 is the key angiogenic factor in the early stage of human osteoblastogenesis.

In this study, we demonstrated that Dex treatment stimulated VEGF expression during human osteoblastogenesis. Deckers *et al.* detected the expression of VEGF120 and VEGF164, which correspond to human VEGF121 and 165, respectively, in murine preosteoblast-like cell line KS483 on RT-PCR analyses (*14*, *22*). However, on Northern blot analyses, only the single band of VEGF120 was detected (*22*). Although the RT-PCR detection of VEGF121 and VEGF165 in human osteoblast progenitor cells has been reported (*17*), Wang *et al.* have shown that only the VEGF121 signal was observed on Northern blot analyses using human osteoblast-like cells

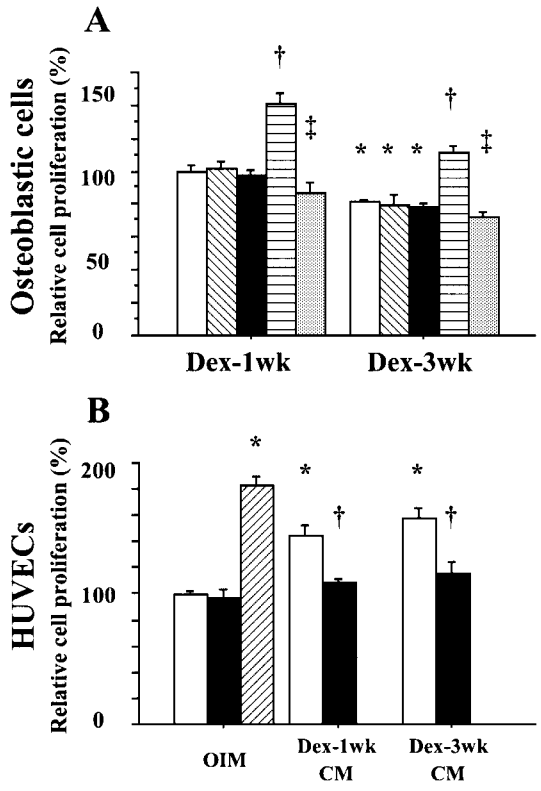

Fig. 6. **VEGF had no direct effect on osteoblastic cell proliferation in early stages of osteoblastogenesis.** (A) Cell proliferation assays involving Dex-1wk (left columns) and Dex-3wk (right columns) cells. Rh-VEGF treatment (5 ng/ml) did not affect the proliferation of either type of cells. In addition, VEGF-Ab (10 μg/ml) had no inhibitory effect on their proliferation. However, rh-bFGF stimulated their proliferation up to approximately 140% of the OIM level in both cell types. The increase in their proliferation with rh-bFGF was suppressed to the basal level by bFGF-Ab (10 μg/ml). Dex-1wk cells had a greater proliferative potential than Dex-3wk cells. * indicates $p < 0.05$ compared with the index for Dex-1wk cells cultured under the same conditions. † and ‡ denote $p < 0.05$ compared with each open and horizontal-hatched bar, respectively. Open, diagonal-hatched, filled, horizontal-hatched, and gray bars indicate the percentages of relative cell proliferation with the OIM, rh-VEGF-containing OIM, VEGF-Ab-containing OIM, rh-bFGF-containing OIM, and rh-bFGF/bFGF-Ab-mixed OIM, respectively. Error bars, SD. (B) HUVEC proliferation was assessed using the OIM (left columns), CM of Dex-1wk (middle columns), and Dex-3wk (right columns) cells. Rh-VEGF-containing OIM (5 ng /ml) stimulated HUVEC proliferation up to approximately 180% of the OIM level. The VEGF concentrations for Dex-1wk and Dex-3wk CM were 1.27 and 2.48 ng/ml, respectively. The CM of Dex-1wk and Dex-3wk cells increased HUVEC proliferation up to approximately 150% of the OIM level. Note that VEGF-Ab treatment (10 μg/ml) inhibited the proliferative effect of the CM on HUVECs to the control level. * and † indicate $p < 0.05$ compared with the OIM control and each original CM, respectively. Open, filled, and diagonal-hatched bars indicate the percentages of relative cell proliferation with the OIM (CM), VEGF-Ab-containing OIM (CM), and rh-VEGF-containing OIM, respectively. Error bars, SD.

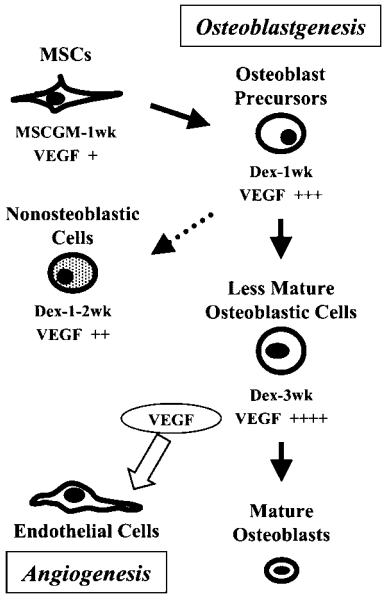

Fig. 7. **Schematic characterization of the relationship between VEGF expression and osteoblastogenesis.** Arrows indicate the direction of the differentiation into the osteoblastic lineage. The dotted arrow indicates the loss of their characters. The paracrine effect of VEGF on endothelial cells is shown as an open arrow. MSCGM-1wk cells, which express a low amount of VEGF, represent human MSCs. Dex treatment stimulates VEGF expression along with osteoblastic differentiation. Osteoblast precursors correspond to Dex-1wk cells. Dex-depleted conditions at this stage may induce irreversible dedifferentiation into nonosteoblastic cells. VEGF expression in Dex-1-2wk cells is slightly suppressed. Continuous Dex treatment with a low concentration induces further differentiation into less mature osteoblastic cells corresponding to Dex-3wk cells. Osteocalcin expression is observed at this stage. The expression of VEGFs, especially VEGF121, is also increased in Dex-3wk cells. However, receptors for VEGF on osteoblastic cells are still limited to neuropilin-1. Proliferation of osteoblastic cells in these early stages is not affected by VEGF stimulation. VEGF secreted in these stages mainly acts as an angiogenic factor for bone formation. Mature osteoblasts might respond to VEGF for their own survival.

and several human osteoblastic osteosarcoma cell lines (*20*). We detected the four isoforms of VEGF (VEGF121, 165, 189, and 206) in each differentiation stage of osteoblastic cells on RT-PCR analyses (Fig. 3A). On Northern blot analyses, we observed the two isoforms of VEGF mRNA (VEGF121 and 165) and an increase in VEGF121 expression along with osteoblastic differentiation (Fig. 3B). We consider that the discrepancy in the different VEGF isoforms between the previous reports and the present study lies in: (i) the cell species, (ii) the VEGF primer set, and (iii) the experimental method. Our findings indicate that the expression of VEGF121 is upregulated along with the progress of Dex-induced osteoblastogenesis.

VEGF receptors have been reported to be endothelial cell-specific receptors against VEGF (*29*). In the present study, the expression of VEGFR-1 and -2 was not observed in human osteoblastic cells on RT-PCR analyses (Fig. 5). However, neuropilin-1 expression was detected (Fig. 5). Harper *et al.* have indicated that immortalized murine osteoblast cell line MC3T3-E1 does not express VEGFR-1 and -2, but neuropilin-1, an isoform-specific receptor for VEGF165 containing exon 7 (*30*, *31*), was detected. They also showed that VEGF binding to osteoblasts was strictly dependent on neuropilin-1 and suggested that neuropilin-1 might directly modulate osteoblast function (*32*). In contrast, Mayr-Wohlfart *et al.* found that the effect of VEGF165 on primary human osteob-

lasts derived from cancellous bone fragments was mediated *via* VEGFR-1 (*16*). Deckers *et al.* reported that the expression of VEGFR-1 and -2 increased along with the mineralization of KS483 cells, and proposed that VEGFR-2 was involved in the regulation of osteoblastic differentiation (*14*). In our study, we demonstrated that VEGF had no proliferative effect on human osteoblastic cells in an early differentiation stage (Fig. 6A). However, bFGF stimulated osteoblastic cell proliferation, unlike VEGF (Fig. 6A). We also detected bFGF and its major receptor (FGF receptor 1) mRNA on RT-PCR analyses (data not shown). These findings suggest that immature osteoblastic cells have no potential to use the autocrine effect of VEGF121 for their own growth. We consider that VEGF receptors and their effects on osteoblastic cells might depend on the differentiation stage during osteoblastogenesis. Further studies involving the differential expression of receptors for VEGF are required.

We demonstrated that the VEGF secreted by osteoblastic cells into the CM caused endothelial cell proliferation of HUVECs (Fig. 6B). Although HUVECs are not truly representative of capillaries, we consider that HUVECs are the best *in vitro* model for estimating the responses of endothelial cells to angiogenic factors such as VEGF and bFGF. Human osteoblastic cells also express the mRNAs of bFGF, transforming growth factor-β1 (TGF-β1), and their receptors (data not shown), so we had to consider the effects of a negative regulator (TGF-β1) on endothelial functions. Several reports have been described the interactions between the activities of endothelial cells and TGF-β1. HUVEC proliferation was not affected, but bovine capillary endothelial cell proliferation was decreased by TGF-β1 (*33*, *34*). The proliferation of bovine aortic endothelial cells and rat epididymal fat pad microvascular endothelium was inhibited by TGF-β1 (*35*). In contrast to the stable capillary tube networks with HUVECs, the network formation of bovine aortic endothelial cells was inhibited by TGF-β1 because of their apoptosis (*36*). Rhim *et al.* reported that immortalized HUVECs developed a capillary-like tube structure and thus were a useful model for studying angiogenesis (*37*). Moreover, HUVECs within three-dimensional gels implanted subcutaneously into immunodeficient mice formed arteriole-, venule-, and capillary-like structures *in vivo* (*38*). Therefore, we selected HUVECs as an *in vitro* model to avoid the inhibitory effect of TGF-β1 as much as possible, and considered that HUVECs partially represented microvessels. In cell proliferation assays involving HUVECs, bFGF-Ab exhibited a lower inhibitory effect on HUVEC proliferation than VEGF-Ab (data not shown). These findings suggest that VEGF acts as the main angiogenic factor during early osteoblastogenesis.

Dex-depleted conditions did not induce morphological changes of osteoblastic cells (Fig. 1C). However, VEGF expression was found to be decreased on Northern blot analyses and ELISA. In addition, the mineralization of Dex-1-2wk cells was not observed even when we stimulated them again with Dex-containing OIM (data not shown). These findings suggest that continuous Dex stimulation, with a low concentration, is important for the early stage of osteoblastogenesis. The ability of dedifferentiation into the osteoblastic lineage might also disappear under Dex-depleted conditions (Fig. 7). Dex stimulation with a low concentration of 1×10^{-8} M has been reported to upregulate the expression of Runx2/Cbfa1 protein, and to induce the growth and differentiation of immortalized human osteoblast cell lines (*39*). In contrast, Chang *et al.* reported that Dex treatment with the high level of 1×10^{-4} M might induce glucocorticoid-dependent bone loss by suppressing Runx2/Cbfa1 functions in osteoblast-enriched cultures derived from fetal rat parietal bones (*40*). Liu *et al.* demonstrated that Runx2/Cbfa1 stimulates the function of less mature osteoblasts, but inhibits osteoblast differentiation at a late stage (*41*). These previous reports also support our suggestions.

We obtained three cell groups; Dex-1wk, Dex-1-2wk, and Dex-3wk cells, by controlling the duration of Dex treatment. We additionally showed that the proliferative activity of osteoblastic cells decreased along with their differentiation (Fig. 6A). We propose that Dex-1wk cells, which express ALP but not osteocalcin, represent osteoblast precursors, and that Dex-3wk cells, which express both genes, are representative of less mature osteoblasts, which have less proliferative potential than precursors (Fig. 7). In contrast, VEGF expression increased along with osteoblastogenesis (Figs. 3B and 4). These findings also suggest that osteoblastic cell proliferation is not dependent on an increase in VEGF secretion.

In conclusion, the present study demonstrated that the expression of VEGF121 increases along with the differentiation of osteoblastic cells derived from human MSCs, and suggests that secreted VEGF might play no role in the osteoblastic cell proliferation in the early stage.

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