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Bone Morphogenetic Proteins Stimulate Angiogenesis through Osteoblast-Derived Vascular Endothelial Growth Factor A

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During bone formation and fracture healing there is a cross-talk between endothelial cells and osteoblasts. We previously showed that vascular endothelial growth factor A (VEGF-A) might be an important factor in this cross-talk, as osteoblast-like cells produce this angiogenic factor in a differentiation-dependent manner. Moreover, exogenously added VEGF-A enhances osteoblast differentiation. In the present study we investigated, given the coupling between angiogenesis and bone formation, whether bone morphogenetic proteins (BMPs) stimulate osteoblastogenesis and angiogenesis through the production of VEGF-A. For this we used the murine preosteoblast-like cell line KS483, which forms mineralized nodules *in vitro*, and an angiogenesis assay comprising 17-d-old fetal mouse bone explants that have the ability to form tube-like structures *in vitro*.

Treatment of KS483 cells with BMP-2, -4, and -6 enhanced nodule formation, osteocalcin mRNA expression, and subsequent mineralization after 18 d of culture. This was accompanied by a dose-dependent increase in VEGF-A protein levels throughout the culture period. BMP-induced osteoblast differentiation, however, was independent of VEGF-A, as blocking VEGF-A activity by a VEGF-A antibody or a VEGF receptor 2 tyrosine kinase inhibitor did not affect BMP-induced mineralization.

To investigate whether BMPs stimulate angiogenesis through VEGF-A, BMPs were assayed for their angiogenic activity. Treatment of bone explants with BMPs enhanced angiogenesis. This was inhibited by soluble BMP receptor 1A or noggin. In the presence of a VEGF-A antibody, both unstimulated and BMP-stimulated angiogenesis were arrested. Conditioned media of KS483 cells treated with BMPs also induced a strong angiogenic response, which was blocked by antimouse VEGF-A but not by noggin. These effects were specific for BMPs, as TGFβ inhibited osteoblast differentiation and angiogenesis while stimulating VEGF-A production.

These findings indicate that BMPs stimulate angiogenesis through the production of VEGF-A by osteoblasts. In conclusion, VEGF-A produced by osteoblasts in response to BMPs is not involved in osteoblast differentiation, but couples angiogenesis to bone formation. (*Endocrinology* 143: 1545–1553, 2002)

DURING BONE DEVELOPMENT and fracture healing vascularization is observed before bone formation (1, 2). In intramembranous bone formation, osteoblasts arise from mesenchymal precursors and differentiate into mature osteoblasts. At the transition of preosteoblasts to osteoblasts extensive vascularization is observed. In endochondral bone formation a cartilage scaffold is formed, which is subsequently replaced by bone. In the latter process bone formation is also accompanied by invasion of blood vessels (3). Cross-talk between endothelial cells and chondrocytes or osteoblasts has been demonstrated in cocultures (4, 5). In these cultures, endothelial cells stimulated the differentiation of chondrocytes and osteoblasts through production of endothelial-derived growth factors (5, 6), whereas osteoblasts stimulated the proliferation of endothelial cells (5).

One of the factors involved in this communication is vascular endothelial growth factor A (VEGF-A) (5, 7, 8). Although this growth factor was originally identified as an endothelial-specific mitogen and permeability factor (9), actions of VEGF-A on nonendothelial cells have recently been reported (10; reviewed in Refs. 11 and 12). We have previously shown that VEGF-A is expressed during osteoblast differentiation and that exogenously added VEGF-A stimulated the differentiation of osteoblast-like cells (13). It is well documented that VEGF-A production by osteoblast-like cells is further enhanced by stimulators of osteoblast differentiation (IGF and vitamin D) and suppressed by inhibitors of osteoblast differentiation (dexamethasone and PTHrP) (13, 14). Furthermore, osteoblast-like cells (13, 15) and hypertrophic chondrocytes (16) express VEGF receptors.

Another family of growth factors highly expressed in bone and involved in chondrocyte and osteoblast differentiation is the TGFβ superfamily, which consists of TGFβ, bone morphogenetic proteins (BMPs), activins, inhibins, and others. Within the TGFβ superfamily, BMPs comprise a unique subfamily of low mol wt polypeptides (17) that induce ectopic calcification when implanted in muscle (18, 19). BMPs are divided into distinct groups. BMP-2 and BMP-4 are highly homologous. Another group is composed of BMP-5, BMP-6, BMP-7 (or OP-1), and BMP-8 (or OP-2) (17). Although the nomenclature of the BMPs suggests that they are involved in bone development, BMPs are pleiotropic growth factors that

Abbreviations: ActD, Actinomycin D; amVA, goat antimouse VEGF-A; BMP, bone morphogenetic protein; CHX, cycloheximide; PECAM-1, platelet-derived endothelial cell adhesion molecule 1; P/S, penicillin/streptomycin; rh, recombinant human; strBMPR-1A, soluble truncated bone morphogenetic protein receptor 1A; VEGF, vascular endothelial growth factor.

play a role in the growth and differentiation of various organs (Ref. 20 and references herein).

Application of BMPs *in vivo* not only increases bone formation, but also enhances angiogenesis (21, 22). The mechanism involved in BMP-induced angiogenesis, however, has been little studied. Given the coupling between bone formation and angiogenesis, this study was undertaken 1) to examine the effects of different classes of BMPs on osteoblastogenesis and angiogenesis, and 2) to determine whether these effects are mediated by VEGF-A. To address these issues, the mouse osteoblast-like cell line KS483 and an angiogenesis assay comprised of fetal mouse metatarsals were used. We propose a model in which BMPs induce osteoblast differentiation and simultaneously induce VEGF-A expression, thereby establishing the coupling between osteoblastogenesis and angiogenesis.

Materials and Methods

Cell culture

To study the effects of BMPs on osteoblast differentiation the preosteoblastic mouse KS483 cell line was used, a nontransformed stable subclone of the parental preosteoblast-like cell line KS4 (23). An additional feature of this cell line is that it secretes VEGF-A in a differentiation-dependent manner (13). Cells were seeded at a density of 15,000 cells/cm^2 in phenol red-free αMEM (Life Technologies, Inc., Breda, The Netherlands) supplemented with 10% FBS (Life Technologies, Inc.) and penicillin/streptomycin (P/S). After the cells reached confluence, cells were cultured in medium containing ascorbic acid (50 μg/ml; Merck & Co., Amsterdam, The Netherlands), growth factors [recombinant human (rh) BMP-2, rhBMP-4, rhBMP-6, and rhTGFβ2, R&D Systems, Inc., ITK (Uithoorn, The Netherlands)], or vehicle at 37 C at 5% CO_2/95% O_2. Medium was replaced every 3–4 d until nodules appeared (d 14). To further stimulate osteogenic differentiation medium was replaced from then on with medium containing also 5 mM β-glycerophosphate (Sigma, Zwijndrecht, The Netherlands) until the end of the culture period (d 18) (13). Goat antimouse VEGF-A (AF-493) and the BMP antagonists noggin and soluble truncated BMP receptor 1A (strBMPR-1A) were all obtained from R&D Systems, Inc. Goat antimouse VEGF-A (amVA) showed less than 10% cross-reactivity to rhVEGF165 and rhVEGF121 and no cross-reactivity to other cytokines. Moreover, we recently demonstrated species specificity of the neutralizing antibody (24). A selective VEGF receptor 2 tyrosine kinase inhibitor (CT6697) was provided by Celltech Chiroscience (Cambridge, UK). Cultures were performed in triplicate, and each experiment was repeated at least twice. At the end of the experiment (d 18) alkaline phosphatase activity and VEGF-A production were measured in conditioned media, and calcium deposition was determined in the cell layer. Other cultures were used for histochemistry, RNA extraction, or protein extraction.

VEGF-A ELISA

VEGF-A levels were assessed in the culture media by a quantitative sandwich ELISA (R&D Systems, Inc.) as previously described (13). Values were assayed in triplicate and calibrated against a VEGF standard (7.8–500 pg/ml). The sensitivity of the assay is 3 pg/ml, and it detects both $VEGF_{120}$ and $VEGF_{164}$.

Calcium deposition

Calcium deposits were determined at the end of the culture period using an *o*-cresolphthalein complexone, which reacts with calcium by forming a red complex. Calcium was extracted from the cell layer by incubating with 3 N HCl for 8–16 h while shaking. Aliquots (10 μl) were diluted five times in distilled water, and 10 μl of this mixture were transferred to a 96-well flat-bottom microtiter plate. Subsequently, 90 μl reagent solution consisting of 0.012% *o*-cresophtalein complexone and 250 mM 2-amino-2-methyl-1,3-propanediol (Sigma) were added per well. Samples were calibrated against a calcium/phosphorus standard (5–80 mg/ml), and absorption was measured at 550 nm using a spectrophotometer. Values were assessed in triplicate and depicted as milligrams of calcium per well.

Transcriptional control

To determine whether BMP-induced VEGF-A mRNA expression requires *de novo* protein synthesis or RNA synthesis, cells were plated in six-well plates. After 3 d, culture medium was replaced by fresh medium containing ascorbic acid (50 μg/ml). After 24 h, 10 μg/ml cycloheximide (CHX; Sigma), actinomycin D (ActD; 5 μg/ml in methanol; Sigma), or vehicle (0.2% methanol) were added. After 30 min, BMP-2 (100 ng/ml) or TGFβ (25 ng/ml) was added for 120 min. Cells were lysed, and RNA was extracted and stored until Northern analysis.

RNA isolation

RNA was isolated using phenol-chloroform extraction according to the method of Chomczynski and Sacchi as previously described (13, 25).

Competitive RT-PCR

Total RNA was reversed transcribed into cDNA as previously described (26). To correct for differences in cDNA levels, 4-fold serial dilutions of an internal standard plasmid pMUS that encode for the housekeeping gene β_2-microglobulin were coamplified with 5 ng cDNA by competitive PCR over 33 cycles. Samples were regarded to contain equal amounts of cDNA by a cDNA/construct ratio of 1:1 as described in detail previously (26).

Semiquantative PCR

To study VEGF-A, -B, and -D and osteocalcin mRNA expression in KS483 cells, primer pairs were designed, and protocols were carried out as previously described (13). The PCRs were performed in a Gene Amp PCR system 9700 (Perkin-Elmer Corp., Nieuwerkerk aan de IJssel, The Netherlands). Every PCR was repeated at least twice, and representative pictures of each experiment are shown.

Northern blotting

RNA samples were denatured, and 15 μg total RNA were separated on a 1.2% agarose gel containing 7.5% formaldehyde and stained with ethidium bromide. The gel was transferred to a nylon membrane (Hybond N, Amersham Pharmacia Biotech, Den Bosch, The Netherlands) overnight. The full-length human VEGF-A probe was provided by Dr. Monique Duyndam (Free University Medical Center, Amsterdam, The Netherlands). Blots were hybridized overnight with a ^{32}P-labeled full-length VEGF-A probe in hybridization mixture containing 10% dextran sulfate, 5× Denhardt's solution, 100 μg/ml single-stranded DNA, 0.5% SDS, 3× SSC, and 10 mM EDTA at 65 C, followed by two washes, with 2× SSC containing 0.1% SDS for 30 min at 65 C and with 1× SSC containing 0.1% SDS for 30 min at 65 C. The blots were exposed to Kodak X-OMAT films (Eastman Kodak, Rochester, NY) with intensifying screens at −80 C. The size of the VEGF-A product is 3.7 kb.

Angiogenesis assay

Angiogenesis assay was performed using 17-d-old fetal mouse metatarsals as described previously (24). The isolated metatarsals (n = 6) were cultured in 24-well plates in αMEM (Life Technologies, Inc.) supplemented with 10% heat-inactivated FBS (Life Technologies, Inc.) and P/S. After 72 h, medium was replaced by 250 μl fresh medium in the presence or absence of BMPs, BMP antagonists, amVA, or conditioned medium of KS483 cells (10%, vol/vol) as indicated. Medium was replaced every 7 d. After 14 d of culture, the explants were fixed and stained for PECAM-1 (platelet-derived endothelial cell adhesion molecule 1), as described in detail previously (24), and the area of PECAM-1-positive tubular structures was determined by image analysis using Image Pro Plus 3.0 for Windows 95/NT (Media Cybernetics, Carlsbad, CA) (24). Images were obtained using a digital camera with a fixed window of 768 × 576 pixels. Data are depicted as number of pixels per area.

Statistics

Results are depicted as the mean ± SEM. Differences among groups were determined by one-way ANOVA for multiple comparison followed by a Fisher's least significant difference test and were considered to be significantly different if $P < 0.05$.

Results

BMPs stimulate VEGF-A production by KS483 cells

To investigate whether besides their stimulatory effect on osteoblast differentiation BMPs also have an effect on VEGF-A production by osteoblastic cells, KS483 cells were continuously treated with various concentrations of BMP-4. Treatment of KS483 cells with 25 and 100 ng/ml BMP-4 increased calcium deposition from 3.9 ± 0.4 mg calcium/well in unstimulated cells to 18.7 ± 3.6 and 17.1 ± 1.0 mg calcium/well ($P < 0.05$), respectively. Treatment of KS483 cells with a lower dose of BMP-4 (10 ng/ml) did not increase calcium deposition (3.06 ± 0.35 mg calcium/well). In contrast, all three doses of BMP-4 significantly stimulated VEGF-A levels after 96 h of treatment (d 7), which remained elevated thereafter (Fig. 1). The stimulation of VEGF-A production was most pronounced between d 7 and 10. VEGF-A levels in BMP-treated cells further increased between d 14 and 18 after plating, as was the case in control cells (Fig. 1).

To study the time course of VEGF-A protein levels after BMP-4 stimulation, KS483 cells were treated with BMP-4 (10, 25, and 100 ng/ml) for 6, 24, and 72 h. No significant increase in VEGF-A production was found after 6 h of stimulation. Treatment with 25 and 100 ng/ml BMP-4 for 24 h stimulated VEGF-A production from 211 ± 7.5 pg/ml in control cultures to 281 ± 4.0 and 380 ± 1.0 pg/ml, respectively. After 72 h, all concentrations of BMP-4 used stimulated VEGF-A production.

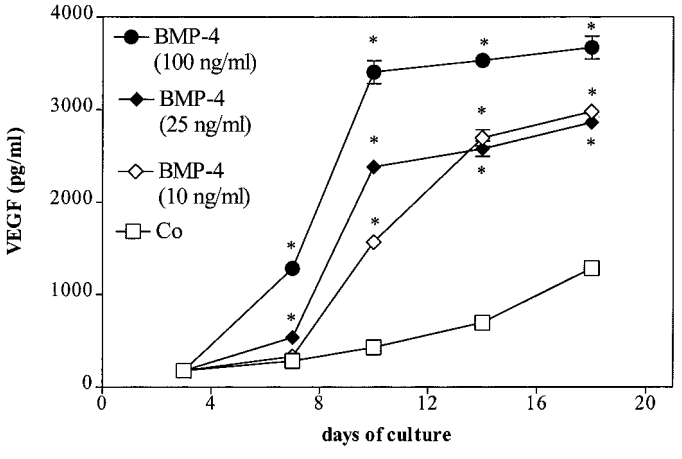

FIG. 1. BMP-4 stimulates VEGF-A production during differentiation of KS483 cells. KS483 cells were plated at a density of 15,000 cells/cm^2 in 12-well plates and cultured in phenol red-free αMEM containing 10% FBS and P/S. After 3 d, medium was replaced by differentiation medium containing 50 μg/ml ascorbic acid in the presence or absence of BMP-4. Medium was replaced every 3–4 d, and after 14 d β-glycerophosphate was also added to the differentiation medium. Cultures were performed in triplicate. VEGF-A levels were measured in the supernatants by ELISA at the time points indicated. Values were significantly different from control as indicated (*, $P < 0.05$). Co, Control.

Given the observation that BMPs exert their stimulatory effect on VEGF-A production within 96 h, we studied whether a short exposure of KS483 cells to BMPs was sufficient to stimulate VEGF-A production and calcium deposition after 18 d of culture. Treatment of KS483 cells with BMP-2, -4, or -6 for 72 h (d 4–7) dose-dependently increased VEGF-A production within 72 h after treatment, and production remained elevated thereafter (Table 1). To study whether this response was specific for the BMP family of growth factors, KS483 cells were treated with 0.5, 2.5, 5, or 25 ng/ml TGFβ for 72 h. Treatment with 0.5 ng/ml TGFβ significantly increased VEGF-A production from 282 ± 9.2 to 1508 ± 23 pg/ml after 72 h of treatment. Maximum levels were obtained with 5 ng/ml TGFβ (3580 ± 83 pg/ml) and remained elevated up to doses of 25 ng/ml TGFβ.

All three BMPs at a dose of 100 ng/ml increased calcium deposition after 18 d of culture from 4.4 ± 0.4 mg calcium/well in unstimulated cells to 18.2 ± 0.1, 19.9 ± 2.0, and 18.9 ± 0.8 mg calcium/well in BMP-2-, BMP-4-, and BMP-6-stimulated KS483 cells, respectively. Moreover, this was accompanied by enhanced expression of the differentiation marker osteocalcin after 18 d of culture (Fig. 2B). This stimulation of differentiation was specific for BMPs, as treatment with TGFβ resulted in inhibition of osteocalcin mRNA expression (Fig. 2B). Collectively, these data demonstrated that a single treatment of KS483 cells with BMPs at an early stage of osteoblast differentiation is sufficient to increase osteoblast differentiation during the whole culture period. In the following experiments only short-term exposures of 72 h or less were used.

BMPs stimulate VEGF-A mRNA expression

To study the effect of BMPs on expression of VEGF-A isoforms at the mRNA level, KS483 cells were treated with BMP-2 (100 ng/ml) for 30 min, 60 min, 3 h, or 6 h, followed by RNA isolation. Housekeeping gene β_2-microglobulin was analyzed by competitive PCR (Table 2). Samples were regarded to contain equal amounts of cDNA by a cDNA/construct ratio of 1:1 as described in detail previously (26). The number of copies of the internal standard at which this ratio was 1 are depicted in Table 2 for all samples. As can be

TABLE 1. VEGF-A production by KS483 cells after 72 h of BMP treatment

BMP treatment (ng/ml)	VEGF-A (pg/ml)	
	7	18
Control	507 (12.3)	2597 (185)
BMP-2		
10	532 (76)	3415 (386)[a]
30	1067 (27)[a]	4447 (50)[a]
100	1435 (60)[a]	4302 (83)[a]
BMP-4		
100	1012 (30)[a]	4410 (325)[a]
BMP-6		
10	656 (84)	3415 (386)
30	1112 (40.7)[a]	3733 (50)[a]
100	1670 (35)[a]	4070 (311)[a]

VEGF-A production in KS483 cells after 72 h of BMP treatment was measured after 7 or 18 d of culture. Data are depicted as the mean of triplicate cultures ± SEM.

[a] $P < 0.05$ *vs.* control cultures.

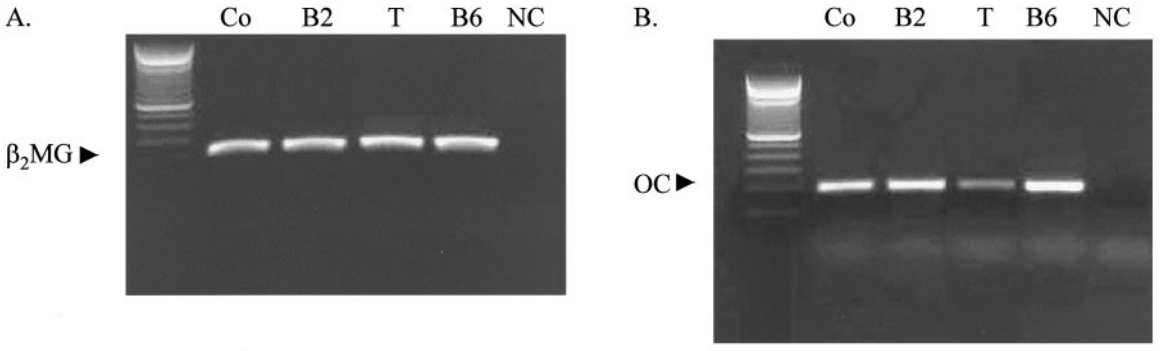

FIG. 2. Treatment of KS483 cells with BMP-2 and -6 stimulates osteoblast differentiation. KS483 cells were plated at a density of 15,000 cells/cm^2 in 6-well plates and cultured in phenol red-free αMEM containing 10% FBS and P/S until confluence. On d 3, medium was replaced by differentiation medium containing ascorbic acid (50 μg/ml). Cells were treated with BMP-2 or -6 (100 ng/ml) or TGFβ2 (25 ng/ml) for 72 h. After 18 d of culture, RNA was isolated and reversed transcribed into cDNA. A, Expression of the housekeeping gene β_2-microglobulin after 25 cycles of PCR in control cultures (lane 2) and in BMP-2-treated (lane 3), TGFβ2-treated (lane 4), and BMP-6-treated (lane 5) cultures. B, Expression of osteocalcin mRNA after 30 cycles of PCR in control cultures (lane 2) and in BMP-2-treated (lane 3), TGFβ2-treated (lane 4), and BMP-6-treated (lane 5) cultures.

TABLE 2. Analysis of housekeeping gene expression

	0 (min)	30 (min)	60 (min)	180 (min)	360 (min)
Undiff, −BMP	40,000	50,000	45,000	60,000	50,000
Undiff, +BMP		70,000	60,000	70,000	50,000
Diff	45,000	60,000	50,000	60,000	55,000
Diff, +BMP		80,000	70,000	75,000	70,000

KS483 cells were plated at a density of 15,000 cells/cm^2 in six-well plates and cultured in phenol red-free αMEM containing 10% FBS and P/S until confluence. On d 3 medium was replaced by differentiation medium containing ascorbic acid (50 μg/ml). On d 4 (undifferentiated cells) or d 10 (differentiated cells), BMP-2 (100 ng/ml) was added. RNA was isolated before BMP treatment (0 min) and after 30, 60, 180, and 360 min of stimulation and reversed transcribed into cDNA. β_2-Microglobulin expression was analyzed after 33 cycles of quantitative PCR. Relative differences in mRNA levels were determined by comparing the number of copies of the internal standard with which cDNA would compete. The number of copies of internal standard pMUS at which the ratio of cDNA: construct is 1, in control cells and/or after treatment of the cells with BMPs at various time points (0, 30, 60, 180, or 360 min) is depicted. In all groups the difference in copy number of the internal standard did not exceed a 2-fold difference. Undiff, Undifferentiated cells; Diff, differentiated KS483 cells; BMP, 100 ng/ml BMP-2.

appreciated from this table, none of the samples exceeded a 2-fold difference in copy number; thus, all were regarded as equal. This was followed by amplification of VEGF-A mRNA levels by semiquantitative RT-PCR. Primers were used that amplify three splice variants of the mouse VEGF-A gene, namely VEGF-A$_{120}$, VEGF-A$_{164}$, and VEGF-A$_{188}$ (13). Confluent KS483 cells (d 4) expressed low levels of VEGF-A$_{120}$ and VEGF-A$_{164}$ mRNA, whereas, consistent with our previous reported observations (13), VEGF-A$_{188}$ was undetectable (Fig. 3). Treatment with BMP-2 increased VEGF-A mRNA levels after 30 min. BMP-induced VEGF-A mRNA levels reached their maximal induction after 60 min of treatment and remained elevated until at least 6 h after treatment. VEGF-B and -D mRNA levels were not affected by BMP treatment (data not shown).

To test whether differentiated KS483 cells are equally responsive to BMP treatment, KS483 cells were treated with BMPs after 10 d of culture. Stimulation of VEGF-A mRNA expression by BMP-2 in differentiated cells was less pronounced compared with stimulation in undifferentiated cells, again with maximal stimulation after 60 min of treatment (Fig. 3). This demonstrated that KS483 cells are more sensitive to BMP-induced VEGF-A mRNA expression in the undifferentiated stage (d 4) than in the differentiated stage (d 10).

Effects of CHX and ActD

To test whether the effect of BMPs on VEGF-A mRNA levels requires *de novo* protein synthesis, KS483 cells were treated with CHX for 30 min before treatment with BMP or TGFβ. After 90 min of treatment with BMP-2 or TGFβ, samples were analyzed by Northern blotting. The image of the ethidium bromide-stained gel is depicted in Fig. 4A to show equal loading. In the absence of CHX or ActD, BMP and TGFβ stimulated VEGF-A mRNA expression compared with the control (Fig. 4B). Cotreatment of KS483 cells with CHX and BMP-2 or TGFβ resulted in a superinduction of VEGF-A mRNA levels (Fig. 4B). To study the possibility that induction of VEGF-A mRNA levels by BMPs was due to increased mRNA stability, cells were treated with the transcription inhibitor ActD. Cotreatment of KS483 cells with BMP-2 and ActD completely abrogated VEGF-A mRNA expression (Fig. 4B), indicating transcriptional control. Similar results were obtained using TGFβ (Fig. 4B).

Endogenously produced BMPs stimulate VEGF-A production by KS483 cells

The observations that VEGF-A production is stimulated by BMPs and that KS483 cells produce VEGF-A at confluence suggest that endogenous BMPs regulate VEGF-A expression in undifferentiated osteoblasts. KS483 cells were treated with various doses strBMPR-1A (62.5–1000 ng/ml) or the BMP-2 and -4 antagonist noggin (12.5–500 ng/ml) to block the activity of endogenous BMPs. As expected, nodule formation was strongly inhibited in the presence of BMP blockers (data not shown). Moreover, VEGF-A production was significantly inhibited at concentrations of 500 and 1000 ng/ml strBMPR-1A (Fig. 5A) or

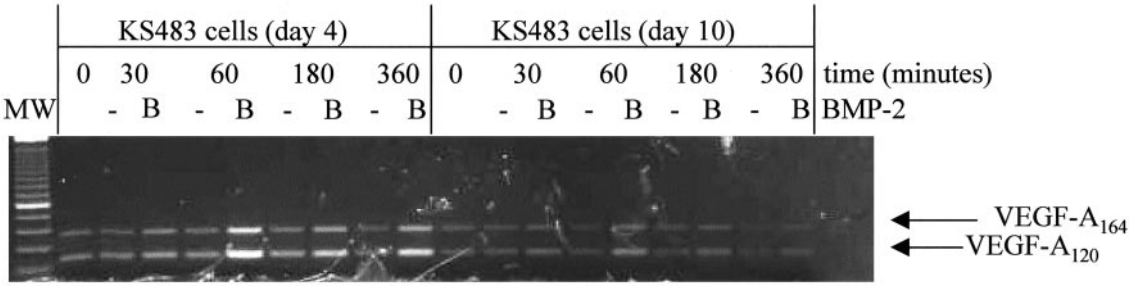

FIG. 3. BMP-2 stimulates the expression of VEGF-A. KS483 cells were plated at a density of 15,000 cells/cm^2 in 6-well plates and cultured in phenol red-free αMEM containing 10% FBS and P/S until confluence. On d 3, medium was replaced by differentiation medium containing ascorbic acid (50 μg/ml). On d 4 (undifferentiated cells) or d 10 (differentiated cells), BMP-2 (100 ng/ml) was added. RNA was isolated before BMP treatment (time zero) and after 30, 60, 180, and 360 min of stimulation and reversed transcribed into cDNA. In lane 1 a mol wt marker is shown (MW). Undifferentiated KS483 cells (lanes 2–10) in the absence of BMP-2 (lanes 2, 3, 5, 7, and 9) or in the presence of BMP-2 (lanes 4, 6, 8, and 10) *vs.* differentiated KS483 cells (lanes 11–19) in the absence of BMP-2 (lanes 11, 12, 14, 16, and 18) or in the presence of BMP-2 (lanes 13, 15, 17, and 19) are shown. The size of the products is indicated by *arrows*. VEGF-A expression was determined after 35 cycles of PCR, yielding products of 360 bp (VEGF-A$_{164}$) and 230 bp (VEGF-A$_{120}$), respectively. B, BMP-2 (100 ng/ml).

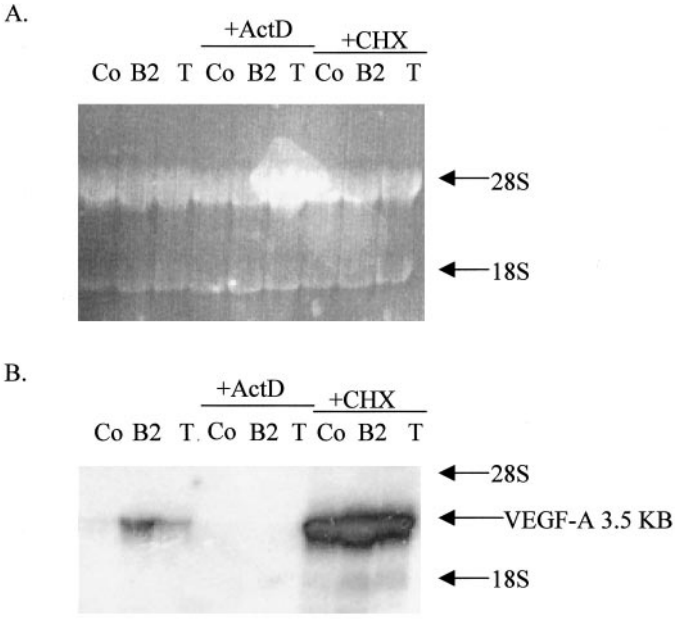

FIG. 4. BMP-induced VEGF-A mRNA levels depend on RNA transcription. KS483 cells were plated at a density of 15,000 cells/cm^2 in 6-well plates and cultured in phenol-red free αMEM containing 10% FBS and P/S until confluence. On d 3, medium was replaced by differentiation medium, and after 24 h CHX (10 μg/ml) or ActD (5 μg/ml) was added. After 30 min of incubation, BMP-2 (100 ng/ml) or TGFβ (25 ng/ml) was added to the cultures as indicated. RNA was isolated after 90 min of treatment. Samples were analyzed by Northern blotting and probed with a ^{32}P-labeled full-length VEGF-A probe. A, Ethidium bromide-stained gel of control cells (lanes 1, 4, and 7) and after BMP-2 (lanes 2, 5, and 8) and TGFβ (lanes 3, 6, and 9) treatment in the presence of ActD (lanes 4–6) or in the presence of CHX (lanes 7–9). B, VEGF-A mRNA expression in control cells (lanes 1, 4, and 7) and after BMP-2 (lanes 2, 5, and 8) and TGFβ (lanes 3, 6, and 9) treatment in the presence of ActD (lanes 4–6) or in the presence of CHX (lanes 7–9). The sizes of 18S and 28S products are indicated by *arrows*. Co, Control; B, BMP-2; T, TGFβ; A, ActD; C, CHX.

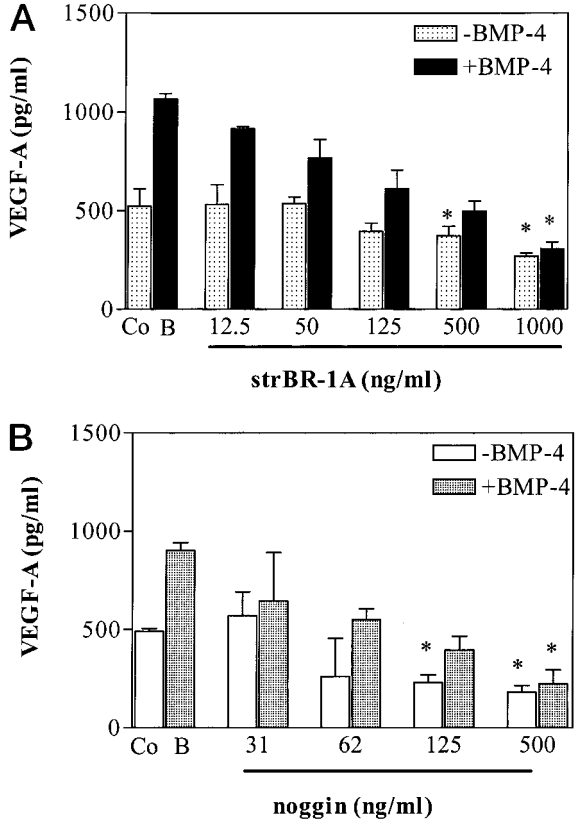

FIG. 5. Endogenous BMPs regulate VEGF-A activity in KS483 cells. KS483 cells were plated at a density of 15,000 cells/cm^2 in 12-well plates and cultured in phenol red-free αMEM containing 10% FBS and P/S until confluence. On d 4, medium was replaced by differentiation medium containing ascorbic acid (50 μg/ml) and various concentrations of strBMPR-1A (A) or noggin (B). After 72 h of culture (d 7), VEGF-A production was measured in the conditioned medium of KS483 cells by ELISA. Cultures were performed in triplicate. Values were significantly different from control as indicated (*, $P < 0.05$). Co, Untreated cultures; strBR-1A, strBMPR-1A; B, BMP-4.

125 and 500 ng/ml noggin (Fig. 5B), illustrating that endogenous BMPs are involved in the regulation of VEGF-A production by KS483 cells. In addition, the addition of noggin to BMP-4-treated cultures blocked BMP-induced VEGF-A production (Fig. 5). This action of noggin was specific for BMP-2 and –4, as treatment of KS483 cells with noggin in the presence of BMP-6 (100 ng/ml) had no effect on VEGF-A production, (1072 ± 33 pg/ml VEGF-A in BMP-6-treated cultures to 921 ± 55.91 pg/ml VEGF-A in BMP-6- and noggin-treated cultures).

BMP-induced osteoblast differentiation is independent of VEGF-A production

To study whether BMP-induced osteoblast differentiation requires VEGF-A production, KS483 cells were treated with BMP-4 or BMP-6 for 72 h (d 4–7) and in the presence or absence of amVA during the whole culture period. Treatment of KS483 cells with amVA had no effect on calcium deposition by BMP-4-stimulated KS483 cells or on calcium deposition by unstimulated cells (Fig. 6). Similar results were obtained using BMP-6 (data not shown). To block VEGF-A activity at a different level, a tyrosine kinase inhibitor was used that specifically blocks VEGF receptor 2 signaling and is effective in blocking angiogenesis at concentrations of 10^{-8} and 10^{-6} M in the fetal mouse metatarsal assay (data not shown). Again, this agent had no effect on differentiation of untreated and BMP-stimulated cells when concentrations were used between 10^{-12} and 5×10^{-6} M. Treatment of KS483 cells with a concentration of 10^{-6} M or higher was toxic to the cells (data not shown). Together these data demonstrate that both nonstimulated and BMP-stimulated osteoblast differentiation are independent of VEGF-A production.

BMPs stimulate angiogenesis via stimulation of VEGF-A

Given the crucial role of VEGF-A in angiogenesis, we tested whether BMPs induce angiogenesis through VEGF-A. BMPs were assayed for their capacity to induce angiogenesis in an assay comprised of fetal bone explants, previously developed in our laboratory (24). Treatment of bone explants with BMP-2 dose-dependently increased angiogenesis, as shown by increased formation of PECAM-1-positive tube-like structures, whereas treatment with TGFβ dose-dependently inhibited this (Fig. 7A). Interestingly, in both BMP- and TGFβ-treated cultures VEGF-A levels increased from 353 ± 67 pg/ml in control cultures to 587 ± 27 pg/ml in BMP-2 (100 ng/ml)-treated cultures and 840 ± 9.3 pg/ml in TGFβ (25 ng/ml)-treated cultures after 10 d of culture. BMP-induced angiogenesis was blocked by amVA (Fig. 7B). In agreement with our previous observations (24), amVA also inhibited angiogenesis in untreated metatarsals (Fig. 7B).

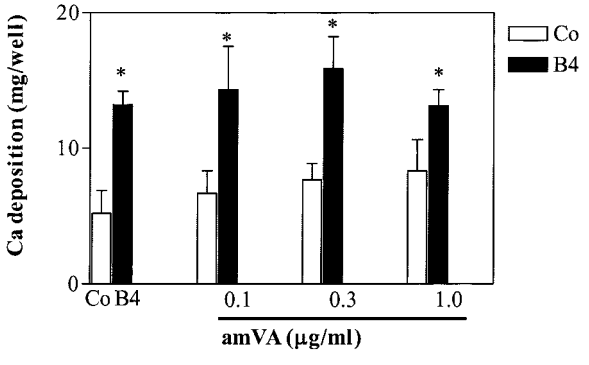

FIG. 6. BMPs stimulate osteoblast differentiation independently of VEGF-A. KS483 cells were plated at a density of 15,000 cells/cm^2 and cultured in phenol red-free αMEM containing 10% FBS and P/S until confluence. On d 4 medium was replaced by differentiation medium containing ascorbic acid (50 μg/ml) in the presence or absence of BMP-4 (50 ng/ml) and various doses of amVA. BMP-4 treatment was only present from d 4–7, whereas amVA was present from d 4–18. After 18 d of culture, calcium deposition was measured. Values were significantly different from control (Co) as indicated (*, $P < 0.05$). B4, BMP-4.

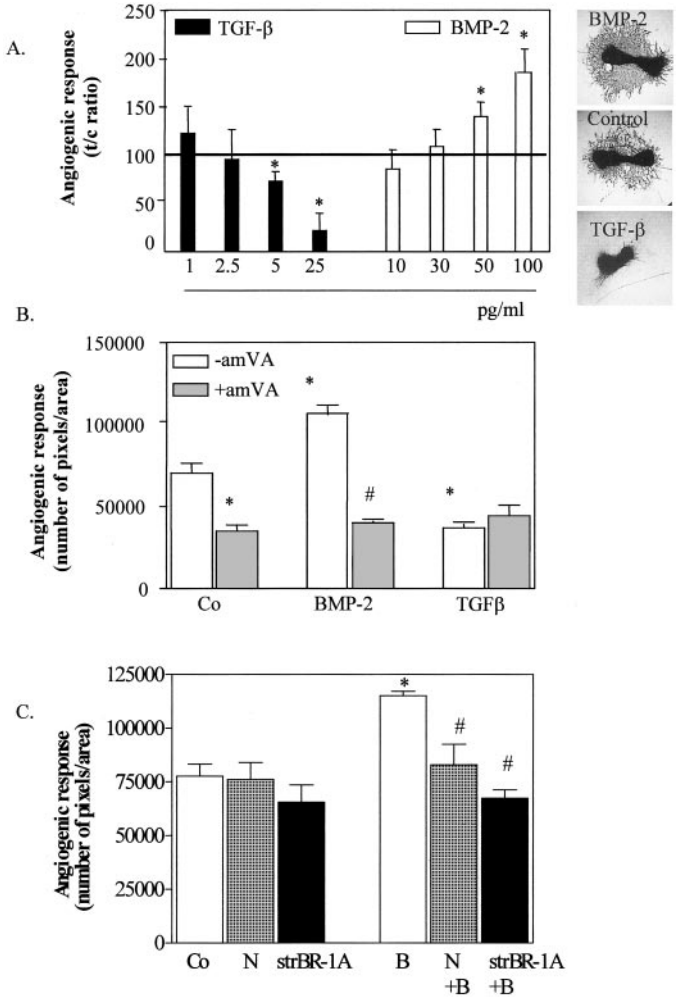

FIG. 7. BMPs stimulate angiogenesis in the fetal metatarsal angiogenesis assay. Seventeen-day-old fetal mouse bone explants were dissected and cultured in 24-well plates containing 150 μl αMEM with 10% heat-inactivated FBS and P/S. A, After 72 h medium was replaced by fresh medium containing BMP-2 (10–100 ng/ml) or TGFβ (0.5–25 ng/ml). Medium was refreshed after 7 d, and cultures were fixed after 14 d of culture. PECAM-1 staining was performed, and the area of PECAM-1-positive tubular structures was quantitated and expressed as a percentage of the control. In the *right panel*, representative images of BMP-treated (100 ng/ml), control, and TGFβ-treated (25 ng/ml) cultures are depicted. B, Quantification of vascular outgrowth in the presence or absence of BMP-2 (100 ng/ml) or TGFβ (25 ng/ml) and amVA (1 μg/ml). Results are depicted as the number of pixels per area. C, Quantification of vascular outgrowth in the presence or absence of BMP-2 (100 ng/ml) (□), in the presence or absence of noggin (250 ng/ml; ▨) or strBMPR-1A (strBR-1A; 1 μg/ml; ■) after 14 d of culture. Values were significantly different from control (Co) or BMP-2-stimulated metatarsals as indicated, respectively (* and #, $P < 0.05$). Cultures were performed six times, and experiments were repeated at least twice. n, Noggin; strBR-1A, strBMPR-1A; B, BMP-2.

To test whether endogenous BMPs play a role in the regulation of angiogenic response under control conditions, metatarsals were treated with noggin (250 ng/ml) or strBMPR-1A (1 μg/ml). Although both agents were able to revert BMP-induced angiogenesis to control levels, angio-

genesis of unstimulated metatarsals was not changed (Fig. 7C), suggesting that VEGF-A in unstimulated cultures is derived from BMP-independent sources. As BMPs stimulate VEGF-A production in KS483 cells, the conditioned medium of either nonstimulated or BMP-treated cells was assayed for their angiogenic capacity. Conditioned medium of BMP-stimulated KS483 cells significantly increased the formation of tube-like structures compared with conditioned media derived from nontreated cells (Fig. 8). Unconditioned medium and medium from untreated cells exerted a similar angiogenic response. In the presence of amVA (1 μg/ml), the angiogenic response of cultures treated with conditioned medium of BMP-treated KS483 cells was inhibited. The angiogenic response of metatarsals treated with conditioned medium of control cells was also inhibited by amVA.

The conditioned medium of BMP-stimulated KS483 cells was used in a 1:10 dilution. This suggests that the observed angiogenic response was primarily due to osteoblast-derived VEGF-A and not to exogenous BMP present in the conditioned medium, because BMP concentrations below 50 ng/ml did not affect angiogenesis. To test this further, metatarsals were cotreated with conditioned medium of BMP-2-stimulated KS483 cells and noggin (250 ng/ml). This concentration of noggin is sufficient to inhibit BMP-induced VEGF-A production in KS483 cells treated with 50 ng/ml BMP-2. The angiogenic response induced by conditioned medium of BMP-2-treated KS483 cells was again decreased by amVA from 86,311 ± 7,879 pixels/area in BMP-stimulated cultures to 30,948 ± 5,167 pixels/area ($P < 0.05$), but not by noggin (81,233 ± 1,089 pixels/area). These data indicate that osteoblast-derived VEGF-A is biologically active and that BMPs stimulate angiogenesis indirectly through the production of VEGF-A.

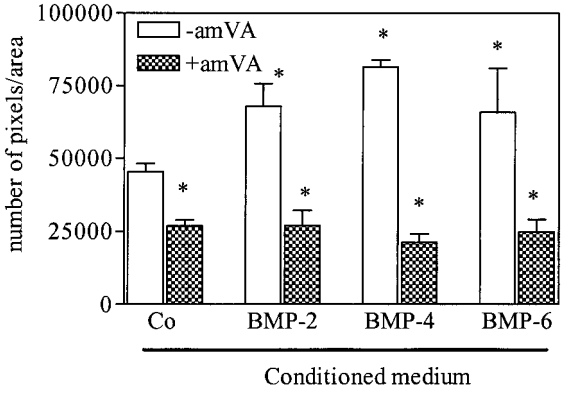

FIG. 8. Conditioned medium of BMP-stimulated KS483 cells induces angiogenesis. KS483 cells were plated at a density of 15,000 cells/cm^2 and cultured in phenol red-free αMEM containing 10% FBS and P/S until confluence. On d 4 medium was replaced by differentiation medium containing ascorbic acid and BMPs (100 ng/ml) for 72 h. After 72 h, conditioned medium was aspirated and stored until further use. Seventeen-day-old fetal mouse bone explants were dissected and cultured in 24-well plates containing 150 μl αMEM with 10% heat-inactivated FBS and P/S. After 72 h, medium was replaced by fresh medium containing conditioned medium of KS483 cells (Co) or conditioned medium of KS483 cells stimulated with BMP-2, -4, or -6 (10%, vol/vol) in the presence or absence of amVA (1 μg/ml). Medium was refreshed after 7 d, and cultures were fixed after 14 d of culture. PECAM-1 staining was performed, and the area of PECAM-1-positive tubular structures was determined by image analysis. Cultures were performed six times, and experiments were repeated at least twice. Values were significantly different from metatarsals treated with conditioned of untreated KS483 cells (Co) as indicated (*, $P < 0.05$).

Discussion

Vascularization is observed at the transition of preosteoblasts to mature osteoblasts during both development and fracture healing. This suggests that bone cells may interact with endothelial cells, probably through the secretion of angiogenic factors (27–29). In cocultures of endothelial cells with osteoblasts, proliferation of endothelial cells was enhanced by the production of osteoblast-derived angiogenic factors such as VEGF-A (5). Osteoblast-derived VEGF-A production was stimulated in the presence of anabolic factors such as IGF-I or BMPs (13, 15, 30). BMP-stimulated VEGF-A production may, therefore, contribute to the anabolic effect of BMPs *in vivo*, as we have previously reported that exogenous VEGF-A stimulated osteoblastic differentiation (13). In addition, BMPs have also been shown to stimulate angiogenesis in fracture-healing models *in vivo* (22, 31, 32). BMP-induced angiogenesis may thus be the result of BMP-induced VEGF-A production by osteoblasts. Alternatively, BMPs have been shown to stimulate angiogenesis in *in vivo* angiogenesis models independently of osteoblasts, such as in the corneal assay and chick chorioallantoic membrane (32, 33). In this study we tested whether BMPs stimulate osteoblast differentiation and angiogenesis through VEGF-A.

Treatment of the murine osteoblast-like cell line KS483 with BMP-2, -4, and -6 resulted in an increase in calcium deposition and VEGF-A production. Moreover, a short exposure of KS483 cells to BMPs was sufficient to stimulate osteoblast differentiation during the whole culture period, as shown by enhanced osteocalcin mRNA expression and calcium deposition. The enhanced differentiation caused by BMP treatment was associated with a dose-dependent increase in VEGF-A protein levels in the supernatants. There was no apparent difference in the ability of various BMP members to stimulate osteoblast differentiation or VEGF-A production. Induction of differentiation and VEGF-A production was specific, as BMP-4-induced responses, but not BMP-6-induced responses, were blocked by noggin. This response was specific for BMPs, as TGFβ2 inhibited osteoblast differentiation. KS483 cells express BMP-2, -4, and -6 and their corresponding receptors (Ter Horst, G., personal communication). We, therefore, performed BMP-blocking experiments to test whether endogenous BMPs are involved in VEGF-A production in KS483 cells. Treatment of KS483 cells with noggin or strBMPR-1A showed that BMP antagonists dose-dependently inhibited VEGF-A production. These data indicated that endogenous BMPs regulate not only osteoblast differentiation but also VEGF-A production in KS483 cells.

Treatment with TGFβ or BMP-2 enhanced VEGF-A mRNA levels within 90 min, which was independent of protein synthesis and dependent on transcription. These data are in agreement with recent studies (30, 34), in which BMP-induced VEGF-A transcription in primary osteoblasts and MC3T3-E1 cells, respectively, was reported. Similarly, analysis by semiquantitative RT-PCR showed basal expression of

VEGF-A_{120} and VEGF-A_{164} mRNA levels in undifferentiated KS483 cells, which was further increased after treatment with BMP-2, -4, or -6. In differentiated KS483 cells, however, the response was much weaker, suggesting that at this stage of differentiation KS483 cells were less responsive to BMP treatment, at least as far as VEGF-A mRNA levels are concerned. BMP-induced VEGF-A protein production was first noted after 24 h of treatment, in agreement with recent studies in murine MC3T3E1 osteoblast-like cells (30). In the latter study BMP-induced VEGF-A production was shown after 12 h of treatment and not before that time point. The lack of increased VEGF-A levels in the supernatants may be explained by binding to the extracellular matrix and/or a difference in the sensitivity of RT-PCR and ELISA techniques.

A single treatment of KS483 cells with BMPs for 72 h was sufficient to stimulate osteoblast differentiation during the whole culture period. It is known that BMPs stimulate expression of master genes, such as CBFA1, in osteoblast precursors to induce commitment to the osteoblast lineage (35). This indicates that BMPs exert their effects early in osteoblast differentiation. Exogenously added VEGF-A, however, only stimulated osteoblast differentiation at a late stage of osteoblast differentiation (13). It is possible that in a BMP-stimulated condition VEGF-A may act on early osteoblasts and stimulate their differentiation. In our study, however, we did not find evidence for that, as neither unstimulated nor stimulated osteoblast differentiation was affected after blocking VEGF-A activity, confirming previous observations that unstimulated osteoblast differentiation is independent of VEGF-A production (13, 15).

In addition, we demonstrated that BMP-induced osteoblast differentiation is also VEGF-A independent. Blocking VEGF-A activity with either a VEGF-A antibody or a tyrosine kinase inhibitor had no effect on osteoblast differentiation, but was effective in blocking both nonstimulated and VEGF-stimulated vascular outgrowth in the fetal metatarsal angiogenesis assay. In contrast, Yeh *et al.* (15) showed that BMP-7-induced, but not unstimulated, osteoblast differentiation was partially blocked by VEGF-A antisense treatment. This difference might be explained by the type and concentration of BMP used, although we could not find a difference in the ability of the BMP-2, -4, or -6 to induce VEGF-A levels in KS483 cells. It might also be due to sensitivity of the cells for VEGF-A; KS483 respond to exogenous VEGF-A by enhanced nodule formation, whereas the cells used by Yeh *et al.* were not responsive to exogenous VEGF-A.

VEGF-A has been identified as the most crucial angiogenic factor during development of the cardiovascular system (for a review, see Ref. 36). It is well appreciated that angiogenesis precedes bone formation and that it is necessary for bone formation to occur (reviewed in Ref. 37). We, therefore, investigated whether BMPs have an effect on angiogenesis through the production of VEGF-A. BMPs stimulated vascular outgrowth in an angiogenesis assay consisting of fetal mouse metatarsals (24). This was accompanied by enhanced VEGF-A production in metatarsals and was abrogated by treatment with a VEGF-A antibody, suggesting that BMP-induced angiogenesis is mediated by VEGF-A. Although these data demonstrate that VEGF-A production is essential for BMP-induced angiogenesis, we cannot exclude that other angiogenic factors, such as fibroblast growth factor, may contribute to the effect. It should be mentioned that stimulation of fibroblast growth factor expression by BMPs in osteoblasts, however, has not been reported in the literature. To test whether endogenous BMPs are involved in angiogenesis under control conditions, metatarsals were treated with noggin or strBMPR-1A. Neither BMP antagonist affected the outgrowth of tube-like structures under control conditions, whereas they effectively reverted BMP-stimulated angiogenesis to control levels. This indicates that in unstimulated cultures, angiogenesis is mediated by BMP-independent VEGF-A sources.

To test whether osteoblast-derived VEGF-A was biologically active, metatarsals were treated with conditioned medium of KS483 cells either treated or untreated with BMPs. Conditioned medium of BMP-treated KS483 cells stimulated vascular outgrowth, which was repressed by a VEGF-A antibody. We could exclude that exogenous BMPs present in the conditioned medium of BMP-stimulated KS483 cells were responsible for the angiogenic response, as vascular outgrowth of metatarsals treated with conditioned medium of KS483 cells stimulated with BMPs was not affected by cotreatment with BMP blockers. This demonstrates that angiogenesis induced by conditioned medium of BMP-stimulated KS483 cells was mediated by osteoblast-derived VEGF-A. Moreover, and in agreement with our previous data (24), angiogenesis in both stimulated and unstimulated cultures was inhibited by the VEGF-A antibody.

In the osteoblast differentiation model as well as the angiogenesis model, the effects of BMP were distinct from those observed after treatment with TGFβ. Although both BMPs and TGFβ were potent stimulators of VEGF-A production, treatment with BMPs stimulated angiogenesis and osteoblast differentiation, whereas TGFβ inhibited these parameters. The different effects of BMPs and TGFβ on osteoblast differentiation are probably explained by the well known stimulation of CBFA1 by BMPs (35), whereas Alliston *et al.* (38) recently reported that repression of CBFA1 by TGFβ resulted in inhibition of osteoblast differentiation. TGFβ is a potent stimulator of matrix synthesis (39), which is an important mediator of angiogenesis. This combined with the fact that TGFβ inhibits endothelial cell proliferation (33) may result in an inhibition of the angiogenic response despite its stimulatory effect on VEGF-A synthesis.

Although BMP-induced angiogenesis in our study was dependent on VEGF-A production, BMPs may also stimulate angiogenesis through VEGF-independent pathways via indirect or direct effects on endothelial cells. Firstly, BMP receptors IA, IB, and II have recently been identified on endothelial cells during tube formation (40). Secondly, the endothelial-specific TGFβ type III receptor, endoglin, facilitates binding of BMPs to BMP receptors (41). Thirdly, BMPs have been shown to elicit an angiogenic response in angiogenesis assays devoid of bone cells, such as in the chick chorioallantoic membrane assay, and in endothelial cell migration assays (32, 33, 39). The BMP-stimulated migration of endothelial cells is a direct effect on endothelial cells, as only endothelial cells were present. It should be mentioned that these studies, however, were performed with GDF-5 (BMP-14), which is only distantly related to the BMPs used in the

present study. The chick chorioallantoic membrane assay and corneal pocket assay are, like the assay used in this manuscript, also multicellular assays, and direct or indirect actions of BMPs on endothelial cells thus cannot be specified. Moreover, in these latter assays VEGF-A levels were not measured, and a VEGF-dependent pathway can thus not be excluded.

In conclusion, we provide evidence that BMP-2, -4, and -6 induce differentiation of osteoblast-like cells, which is paralleled by enhanced VEGF-A production. This VEGF-A production does not contribute to osteoblast differentiation, but stimulates angiogenesis. We, therefore, hypothesize that BMP-induced VEGF-A production in osteoblast-like cells plays an important role in the coupling of bone formation and angiogenesis by acting as a chemoattractant for neighboring endothelial cells.

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