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Article *in* Journal of Bone and Mineral Research · April 2000

DOI: 10.1359/jbmr.2000.15.3.534 · Source: PubMed

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JOURNAL OF BONE AND MINERAL RESEARCH
Volume 15, Number 3, 2000

Vascular Endothelial Growth Factor Is Expressed in Human Fetal Growth Cartilage

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ABSTRACT

Angiogenesis is a crucial event in endochondral ossification. Chemoattractants and mitogens for endothelial cells (such as basic fibroblast growth factor [bFGF] and transforming growth factor β [TGF-β]), which act as local regulators of the process, are synthesized by chondrocytes under several stimuli and in relation to the differentiation stage of the cartilage. Vascular endothelial growth factor (VEGF) is a 44-kDa protein well known as a potent angiogenic molecule owing to its mitogenic and permeability-causing properties. In this work, VEGF was located by immunohistochemistry in growth plate cartilage of human fetuses (20–22 weeks old) and its expression was demonstrated by reverse-transcription polymerase chain reaction (RT-PCR). Primary culture of human fetal epiphyseal chondrocytes (HFEC) maintained VEGF expression at protein and messenger RNA (mRNA) levels and this expression was stimulated by cartilage-promoting growth factors incorporated into the culture media (rFGF-b, rTGF-β1, and insulin-like growth factor [rIGF-I] at 50 ng/ml). The conditioned medium (CM) of HFEC stimulated the proliferation of endothelial cells, and this was partially blocked by anti-VEGF antibody. These studies showed VEGF production by chondrocytes of the epiphyseal growth cartilage and suggested a role of this factor in cartilage physiology and the angiogenic process. (J Bone Miner Res 2000;15:534–540)

Key words: VEGF, growth cartilage, chondrocytes, fetal

INTRODUCTION

DURING ENDOCHONDRAL BONE FORMATION, capillaries from developing bone invade uncalcified and calcified cartilage, resulting in cartilage removal and replacement by bone.[1] Therefore, neovascularization is a key event in the ossification process that occurs in the growth plate at the epiphyses of long bones.

Generally, cartilage has been described as an avascular tissue, which, furthermore, produces angiogenic inhibitors; however, hypertrophic cartilage, which secretes a specific matrix with collagen type X, acts as a target for capillary invasion and angiogenesis with synthesis of angiogenic activators.[2,3] Among the latter, endothelial cell–stimulating angiogenic factor (ESAF) released by chondrocytes during calcification exerts its action by activating matrix metalloproteinases (MMPs), which, in turn, degrade extracellular matrix components.[4] In this respect, mice with homozygous gelatinase B null mutation present a delayed process of vascularization and ossification, which also indicates the mediation of MMPs and their inhibitors in the neovascularization process.[5] Additionally, extracellular matrix components participate in the initiation and maintenance of angiogenesis.[6]

A number of growth factors and hormones that modulate cartilage growth and differentiation may, in parallel, function as potential endothelial cell chemoattractants and mitogens: transforming growth factor β (TGF-β) has been partly implicated in the human chondrocyte-dependent inhibition of vascularity, colony-stimulating factor- 1 (CSF-1) directly or indirectly accelerates angiogenesis, and vitamin D_3 metabolites promote expression of an angiogenic mol-

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ecule.[3,7,8] Recent evidence that subchondral blood vessel cells are a source of factors that influence growth and differentiation of chondrocytes supports the idea that mutual control between chondrocyte differentiation and endothelial invasion exists and leads to normal endochondral ossification; however, an understanding of the angiogenic process in endochondral bone formation remains limited.[9]

Vascular endothelial growth factor (VEGF) is a potent angiogenic peptide with specific mitogenic and chemotactic actions on endothelial cells. The biological function of VEGF is mediated by its binding to specific tyrosine kinase receptors flt-1 and KDR, which are expressed exclusively by endothelial cells.[10,11] Alternative splicing of VEGF messenger RNA (mRNA) results in the generation of four protein species: 121, 165, 189, and 206 aminoacids. VEGF is secreted by many cell types such as osteoblasts and osteoblast-like cells, whereas its expression is regulated by several growth factors, hormones, and cytokines (insulin-like growth factor I [IGF-I], prostaglandin E_1 [PGE_1], PGE_2, 1,25-dihydroxyvitamin D_3, and parathyroid hormone [PTH]).[12–14] In addition to its reported effects on endothelial cells, VEGF induces indirectly proliferation and differentiation of osteoblasts by stimulating endothelial cells to produce osteoanabolic growth factors.[15] Nevertheless, a direct effect of VEGF on fetal bovine osteoblast differentiation has been described.[16]

In this study, evidence of VEGF at the human epiphyseal growth plate and its expression by primary chondrocyte cultures (human fetal epiphyseal chondrocytes [HFEC]) were obtained for the first time. Secretion of VEGF in these HFEC cultures appeared to be modulated by certain growth factors and hormones. Our data indicate possible VEGF involvement in the neovascularization process during endochondral ossification at the human growth plate.

MATERIALS AND METHODS

Cartilage processing and cell culture

Epiphyseal cartilage from six human fetuses 20–22 weeks old, products of therapeutic abortions, was collected with parental consent and processed. Samples were fixed in 10% formaldehyde and paraffin-embedded according to Emery and Kalpaktsoglou.[17] Alternatively, cartilage was digested enzymatically using sequential trypsin and collagenase 0.2% treatment and isolated chondrocytes, mainly from resting and proliferative zones, were seeded in Petri dishes and cultured with Ham F-12 supplemented with 10% fetal bovine serum (FBS) and glutamine 1 mM until confluency.[18] Medium was changed every 48 h. Chondrocyte culture also was established in 96-well plates at a density of 20,000 cells/well. Spontaneously transformed human umbilical vein endothelial cell line ECV-304 was kindly provided by Dr M. Jardí from the Oncologic Research Institute (Barcelona, Spain). Growth factors such as fibroblast growth factor a (rFGFR-a), rFGF-b, epidermal growth factor (rEGF), rTGF-β1, and retinoic acid were purchased from Sigma (Santa Cruz, CA, U.S.A.); rVEGF was purchased from Santa Cruz Biochemicals (CA, U.S.A.), insulin-like growth factor I (rIGF-I) and rIGF-II were kindly supplied by Pharmacia (Uppsala, Sweden); and 1,25 OH Vit D by Solvay Duphar (Weesp, U.K.). RPMI 1640, Ham F-12, and FBS 10% were supplied from CanSera (Ontario, Canada).

Immunohistochemistry

Cartilage sections were deparaffinized, heated at 60°C for 1 h, immersed in 1% H_2O_2 in methanol for 5 minutes, washed in phosphate-buffered saline (PBS), treated for nonspecific blocking, and incubated with a specific rabbit antibody (Santa Cruz Biochemicals, Santa Cruz, CA, U.S.A.) at 1 μg/ml capable of recognizing the three variants (189, 165, and 121 aa) of VEGF. Chondrocytes in culture slides also were processed by immunohistochemistry. Briefly, cells were washed in cold PBS and fixed in formaldehyde. Type II collagen immunostaining was obtained with the polyclonal purified antibody of Calbiochem, 1/100, with 0.5% hyaluronidase (Sigma, St. Louis, MO, U.S.A., type IS) for 10 minutes at 37°C pretreatment.[19] Negative controls were obtained in parallel avoiding the first antibody. Standard avidin-biotin peroxidase complex (ABC) methodology was applied and antibody binding was visualized using diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC).

Effects of conditioned medium of HFEC on [^{3}H]thymidine incorporation into endothelial cells

To investigate the effect on endothelial proliferation of chondrocyte-conditioned media, we used the spontaneously transformed human umbilical vein endothelial cell line ECV-304. Endothelial cells were grown in monolayers in RPMI 1640 medium, plated at 20,000 cells/well in 96-multiwell dishes and incubated at different concentrations of HFEC-CM for 24 h. [^{3}H]Thymidine was added for an additional 24 h and [^{3}H]thymidine incorporation was determined in a liquid scillintation counter. Rabbit anti-VEGF antibody (Santa Cruz Biochemicals, CA, U.S.A.) was used to study whether VEGF was involved in the HFEC-CM stimulatory effect.

VEGF assay

Conditioned media (CM) of chondrocyte cultures obtained after 24 h of serum deprivation in different culture phases or under growth factor stimulation for an additional 48 h were preserved at −80°C until assayed. ELISA, designed to detect $VEGF_{121}$ and $VEGF_{165}$ with a sensitivity of 9.0 pg/ml, was performed according to the manufacturer's instructions (R & D Systems, Abingdon, U.K.).

Western blot

CM were recovered at 4°C, centrifuged to eliminate cells and debris, and concentrated (Ultrafree-CL 10,000 nmwl, Millipore, Bedford, MA, U.S.A.). The samples were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto an 0.2 mm nitrocellulose sheet (Bio-Rad, CA, U.S.A.). VEGF was probed with the primary antibody (1:200 dilution of polyclonal rabbit anti-human VEGF) (Santa Cruz Biochemicals, CA, U.S.A.). The membrane was washed and then incubated with a biotinylated secondary antibody. Protein bands were visualized with DAB.

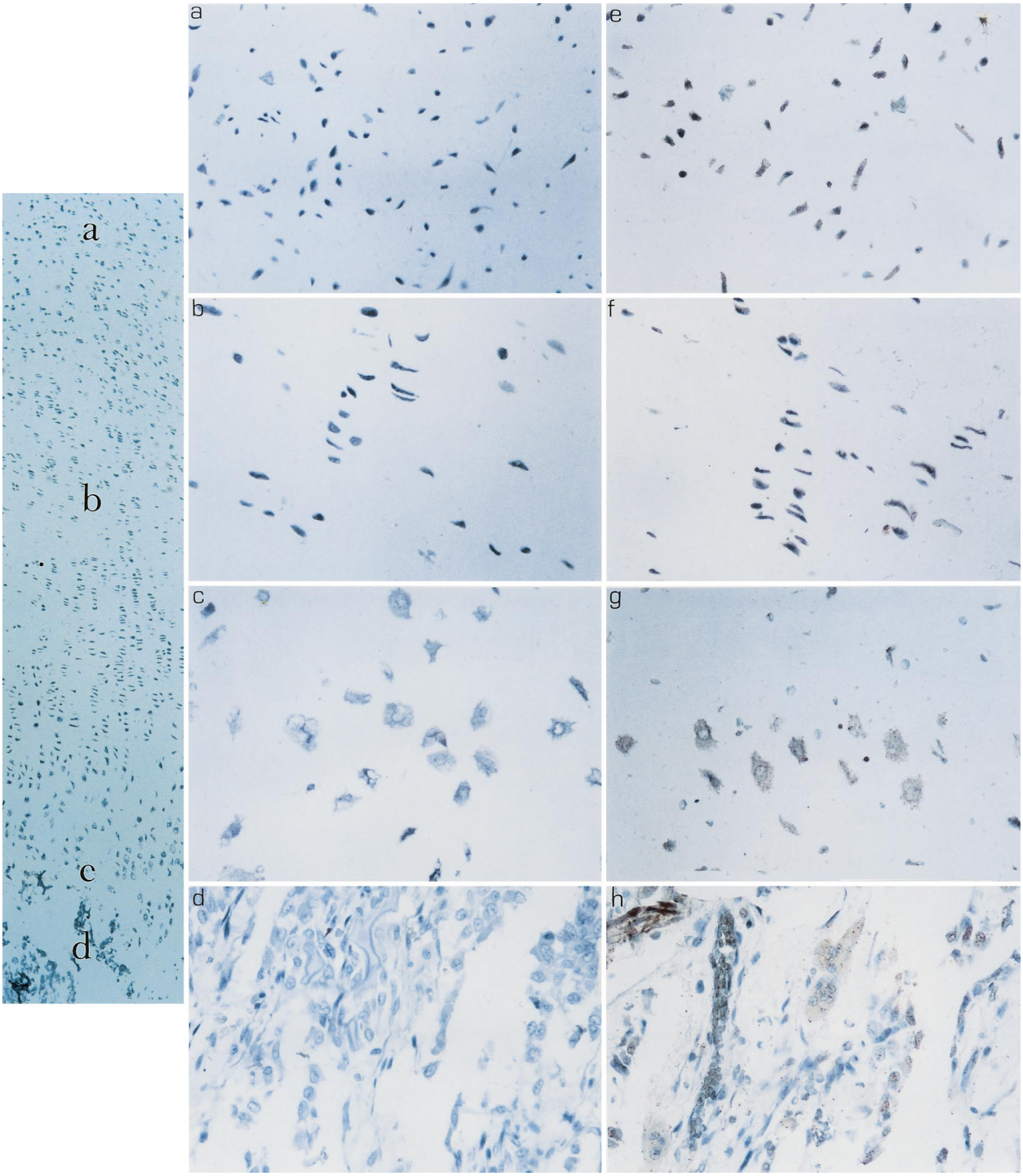

FIG. 1. Immunohistochemistry in paraffined section of a human epiphyseal growth plate from a 20-week-old fetus. (A and E) Resting zone; (B and F) proliferating zone; (C and G) hypertrophic zone of cartilage portion, and (D and H) ossification line and adjacent trabecular bone portion. (A–D) negative control and (E–H) VEGF staining. Magnification × 250; (A–D) low magnification ×100.

mRNA extraction and Northern blot analysis

Ten micrograms of total mRNA, obtained by a standard technique (Promega Corp., Madison, WI, U.S.A.), were fractionated on formaldehyde denaturing gels and transferred to Hybond nylon membrane (Amersham Corp., Amersham, U.K.). Hybridization was performed with a VEGF polymerase chain reaction (PCR) product common for all isoforms previously purified (Promega, Madison, WI, U.S.A.) and sequenced (Applied Biosystems, Foster City, CA, U.S.A.) to confirm its sequence.[20]

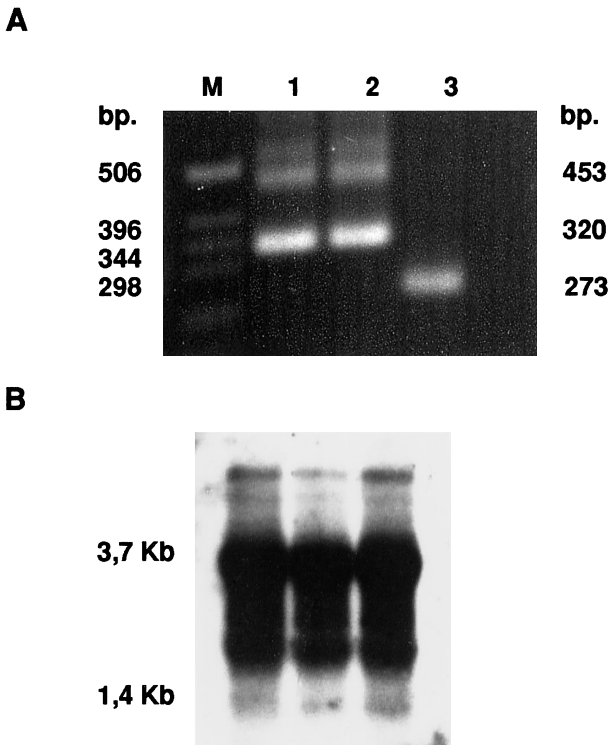

FIG. 2. (A) RT-PCR products of VEGF analysis in 2% agarose: M (1-kb marker), 1, 2 (alternative splicing results show two abundant isoforms in human fetal epiphyseal cartilage; 1 = HFEC, 2 = cartilage), and 3 (VEGF product obtained with a pair of primers for a common sequence for all VEGF forms). (B) Northern blot shows VEGF expression in HFEC cultures.

Reverse-transcription PCR

Complementary DNA (cDNA) was obtained using M-MLV reverse-transcriptase enzyme (Gibco, BRL, Paisley, Scotland) and PCR was performed according to Morii et al. with two pairs of primers: the first 5′-TCCAGGAGTACCCTGATGAG-3′ and 5′-ATTCACATTTGTTGTGCTGT-3′ gave a common sequence for all VEGF forms, and the second 5′-CGAAGTGGTGAAGTTCATGGATG-3′ and 5′-TTCTGTTCAGTCTTTCCTGGTGAG-3′ permitted the alternative splicing forms to be distinguished.[20]

Statistical analysis

Each value represents the mean of one experimental condition performed in quadruplicate and was expressed as M ± SD. Each experiment was repeated three times. Statistical analysis was made using the StatView (4.5) program (Abacus Concepts, Inc., Berkerely, CA, U.S.A.) for Macintosh. Differences between groups of data were evaluated using a Bonferroni/Dunn test for nonparametric variables. Statistically significant differences were considered between groups at $p < 0.05$.

RESULTS

VEGF located in fetal epiphyseal cartilage

Immunohistochemical study of paraffin sections revealed widespread VEGF protein in cytoplasm of chondrocytes located in the distinct differentiation zones (resting, proliferative, and hypertrophic) of the human fetal epiphyseal growth plate (Fig. 1). VEGF positivity of osteoclasts considered as an internal control in the trabecular bone portion adjacent to the junctional line was obtained in all sections studied. VEGF-specific RNA transcripts also were shown by reverse-transcription PCR (RT-PCR) in total RNAs isolated from fetal epiphyseal cartilage (Fig. 2A).

VEGF production by primary culture of human epiphyseal chondrocytes

VEGF protein and mRNA were present in isolated chondrocytes in culture as shown by immunoassay, Western blot, Northern blot, and RT-PCR. Immunohistochemistry of semiconfluent HFEC cells cultured in serum showed VEGF to be located in cytoplasm where intense staining was observed (Fig. 3A). HFEC primary culture was able to secrete VEGF into the culture media in the presence or absence of serum (10% FBS) (Fig. 4A). When protein extracts from the culture media were analyzed by Western blot, a set of immunoreactive bands was obtained with apparent molecular masses in the range of 31 kDa and 45 kDa consistent with small molecular forms of VEGF (Fig. 4B). In addition to protein demonstration, VEGF-specific mRNA was detected by Northern blot and RT-PCR using the two pairs of primers (Fig. 2).

Alternative splicing of VEGF in human epiphyseal chondrocytes cultures

Total RNAs isolated from HFEC cultures in FBS (10%) were processed by Northern blot. Overexposure of hybridized filters with VEGF probe yielded an array of different-length RNAs in addition to predominant 3.7-kilobase (kb) mRNA. Two alternative splicing products were obtained by RT-PCR of lengths consistent with 121 and 165 aa VEGF isoforms (Fig. 2).

Modulation of VEGF production by FBS, IGF-I, IGF-II, FGF-b, FGF-a, EGF, TGF-β1, retinoic acid, and 1,25 OH Vit D in primary culture of human epiphyseal chondrocytes

VEGF secretion by HFEC into the culture media was up-regulated by FBS, rIGF-I, rIGF-II, rFGF-b, and rTGF-β1 (50 ng/ml; 702, 184, 135, 552 and 338% increase, respectively) after 48 h of treatment, whereas no clear effect on VEGF production was observed for other well-characterized osteogenesis regulators (EGF, FGF-a, retinoic acid, and vitamin D) (Fig. 5).

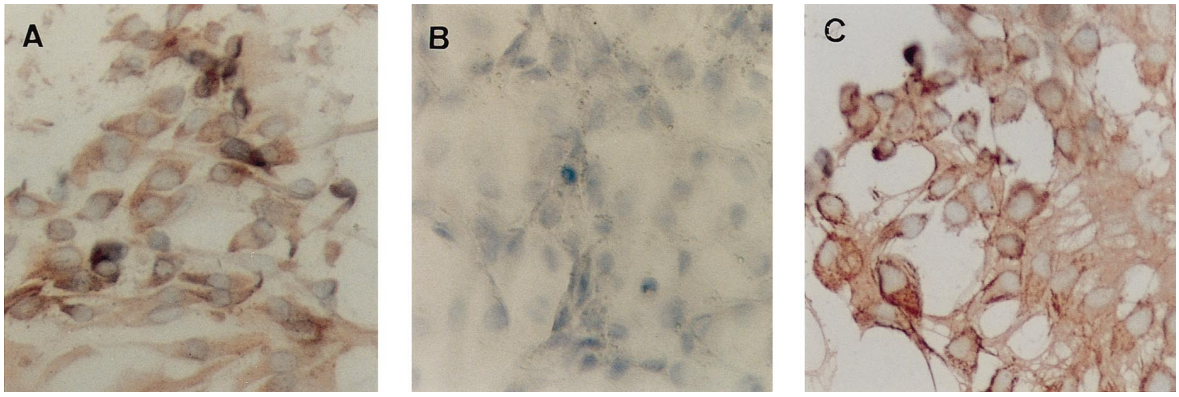

FIG. 3. (A) Primary culture of isolated chondrocytes of the human fetal epiphyseal growth plate immunolabeled with VEGF antibody. (B) negative control. (C) Col II. Magnification ×400.

HFEC-CM stimulates [^{3}H]thymidine incorporation into endothelial cells

The HFEC-CM significantly stimulated, in a concentration-dependent manner, [^{3}H]thymidine incorporation into endothelial cells EVC-304 (Fig. 6). It has been reported that EVC-304 cells have a functional VEGF/VEGF receptor system.[21] Recombinant VEGF added to the EVC-304 in a similar dose as VEGF-HFEC-CM levels (determined by ELISA) produced a similar proportion of [^{3}H]thymidine incorporation. In addition, anti-VEGF antibody added at the same time as HFEC-CM to the EVC-304 cells partly inhibited CM-stimulating effect. Mitogens, other than VEGF, such FGF and TGF-β, are known to be present in HEFC-CM; for this reason, even the presence of a high concentration of antibody (up to 1 μg/ml) was not able to block completely (43%) [^{3}H]thymidine incorporation.

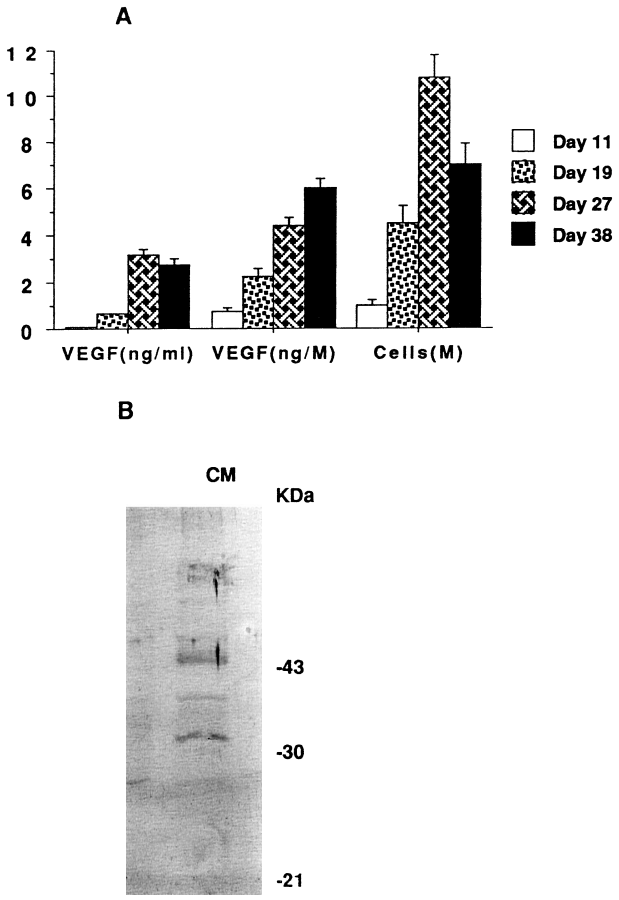

FIG. 4. (A) VEGF production in the culture media of primary chondrocytes during culture development expressed as concentration in the culture medium (VEGF; ng/ml), as production (VEGF; ng/M = ng/10^6 cells) with respect to cell number (M = 10^6 cells); mean ± SD of four petri dishes. (B) Western blot of VEGF in CM. Standard molecular weight markers are indicated in kilodaltons.

DISCUSSION

The neovascularization process is a crucial event in endochondral ossification at epiphyseal growth plates responsible for longitudinal bone growth. Recent advances in this field have indicated a close relationship between cartilage differentiation and neovascularization; endothelial cells express cytokines and growth factors that affect the progress of cartilage differentiation and several factors produced by chondrocytes (such as FGF-b and TGF-β1) function as endothelial chemoattractants and mitogens.[9,22,23] In fact, many angiogenic factors that probably constitute a redundant system, as in nearly all biological systems, are implicated in the progressive cartilage invasion by endothelial cells and vessel formation; however, a predominant factor cannot be ruled out.

Although various agents are known to be potentially angiogenic, VEGF is one of the most potent mitogens and mobility-promoting molecules. It exerts its action specifically on endothelial cells and increasing reports show its essential role in human fetal development because the loss of a single VEGF allele is lethal for the embryo.[24,25] Our results provide the first evidence of VEGF protein location

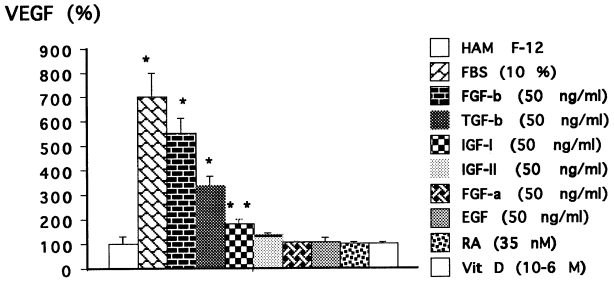

FIG. 5. Quantification of VEGF in culture media after 48 h of serum deprivation and treatment for additional 48 h with several factors. Results expressed as percent of control (Ham F-12). Mean ± SD of four wells. *$p < 0.0001$ versus Ham F-12; **$p < 0.001$ versus Ham F-12.

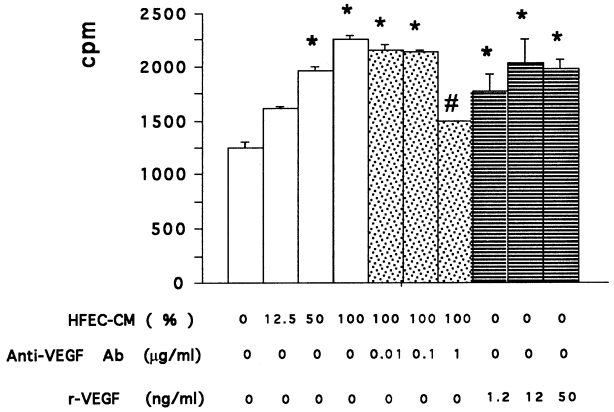

FIG. 6. Effect of HFEC-CM and rVEGF on [^{3}H]thymidine incorporation into ECV-304 and partial inhibition of HFEC-CM effect by anti-VEGF polyclonal antibody. After 24 h of incubation, [^{3}H]thymidine was added for an additional 24 h and the incorporation was determined. Results expressed as counts per minute (cpm) represent the mean ± SD of quadruplicates. *$p < 0.05$ versus HFEC-CM (0); #$p < 0.05$ versus HFEC-CM (100).

in human fetal epiphyseal cartilage and the expression of two different transcripts, encoding $VEGF_{121}$ and $VEGF_{165}$, as a consequence of the alternative splicing process.[26] The majority of cells and tissues that express VEGF present these same isoforms, which are secreted proteins: $VEGF_{121}$ is a soluble acidic polypeptide and $VEGF_{165}$ a basic, heparin-binding polypeptide that remains partly bound to the cell surface and the extracellular matrix. Availability of VEGF under several stimuli has been shown to induce complex mechanisms in endothelial cells, sprouting, induction of interstitial collagenase, plasminogen activators (PA), plasminogen activator inhibitor 1 (PAI-1), and extravasation of plasma proteins.[27] Although we showed VEGF at the fetal epiphyseal growth plate, its possible influence on the neovascularization process remains to be elucidated. A widely extended idea supports the discrete onset of angiogenesis in the cartilage hypertrophic zone (e.g., see Ref. 2); however, we found VEGF protein in the different zones of epiphyseal cartilage (resting, proliferative, and hypertrophic). In this respect, experimental evidence in avian embryos has indicated that the extent of tissue vascularization induced by VEGF implies positive ligand feedback to synthesis of its receptor in a permissive environment.[28]

Under specific conditions VEGF expression was maintained constitutively in primary culture of chondrocytes, which retain phenotypic characteristics of proliferative cartilage (Fig. 3B).[18] VEGF levels on cultured media were up-regulated by chondrocyte treatment with several well-described bone-forming growth factors such as TGF-β, FGF-b, and IGFs. In addition, the anti-VEGF antibody partially inhibited the proliferative effect of HFEC-CM on endothelial cells. These results support the idea that factors that promote bone formation may act in vivo by enhancing VEGF production and subsequently stimulating angiogenesis, as other authors have suggested (e.g., Refs. 9 and 15).

In summary, we obtained VEGF expression by chondrocytes of human fetal epiphyseal cartilage and modulated VEGF production by several growth factors crucial for cartilage and bone development. Thus, it may be speculated that VEGF is implicated in the neovascularization process at the fetal epiphyseal growth plate.

ACKNOWLEDGMENTS

We thank Christine O'Hara for useful help with the English version of the manuscript. Pharmacia-Upjohn is acknowledged for rIGF-I and rIGF-II supply, Solvay Duphar for 1,25-vitamin D, and Dr. Merce Jardí (I.R.O., Barcelona) who kindly supplied ECV-340 cells. This study was supported by Spanish grant no. PB-94-1250 and Pharmacia-Upjohn (Convenio I + D).

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Received in original form February 3, 1999; in revised form August 27, 1999; accepted October 12, 1999.