Intercellular communication between vascular smooth muscle and endothelial cells mediated by heparin-binding epidermal growth factor-like growth factor and vascular endothelial growth factor

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Abstract Heparin-binding epidermal growth factor-like growth factor (HB-EGF), a potent mitogen and migration factor for vascular smooth muscle cells (SMC), promoted neovascularization in vivo in the rabbit cornea. MRI demonstrated quantitatively the angiogenic effect of HB-EGF when introduced subcutaneously into nude mice. HB-EGF is not directly mitogenic to endothelial cells but it induced the migration of bovine endothelial cells and release of endothelial cell mitogenic activity from bovine vascular SMC. This mitogenic activity was specifically blocked by neutralizing anti-vascular endothelial growth factor (VEGF) antibodies. In contrast, EGF or transforming growth factor-α (TGF-α) had almost no effect on release of endothelial mitogenicity from SMC. In addition, RT-PCR analysis demonstrated that $VEGF_{165}$ mRNA levels were increased in vascular SMC 4–10-fold by 0.35–2 nM of HB-EGF, respectively. Our data suggest that HB-EGF, as a mediator of intercellular communication, may play a new important role in supporting wound healing, tumor progression and atherosclerosis by stimulating angiogenesis.

Key words: Angiogenesis; Heparin-binding epidermal growth factor-like growth factor; Vascular endothelial growth factor; Vascular smooth muscle cell; Endothelial cell

1. Introduction

Angiogenesis, the growth of new capillary blood vessels by sprouting from established vessels, is a normal process inherent in embryogenesis, wound repair, ovulation and in various pathologies, such as psoriasis, atherosclerosis and tumor growth.

Angiogenesis is characterized by the migration and proliferation of vascular endothelial cells and vascular smooth muscle cells (SMC). The migration and proliferation of vascular SMC and endothelial cells during angiogenesis is mediated by specific growth factors. Some of the best characterized of these growth factors are platelet-derived growth factor (PDGF), transforming growth factor-α (TGF-α), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) [1,2]. VEGF is a particularly important endothelial growth factor associated with tumor progression, wound healing and development. VEGF is a secreted, 46-kDa dimeric protein active as a direct and specific mitogen for vascular endothelial cells and as an angiogenic growth factor [3,4]. VEGF appears to function as a key regulator of physiologic as well as pathologic angiogenesis [5,6]. $VEGF_{165}$ is the predominant isoform secreted by a variety of normal and transformed cells [7]. Unlike the endothelial cell mitogens, basic and acidic FGF [8,9] which lack a signal peptide, VEGF is a secreted protein [4]. Therefore, VEGF has the potential to be released by intact cells without the need for postulating cell death or specialized transport mechanisms. VEGF mRNA levels in SMC are induced by hypoxia, by PDGF-BB [10] and by interleukin-1b, but not by epidermal growth factor (EGF) [11].

More recently, heparin-binding EGF-like growth factor (HB-EGF), a novel member of the EGF family, has been shown to be associated with SMC migration and proliferation [12,13], atherosclerosis [14,15], pregnancy [16,17], wound healing [18,19], and tumor development [15]. HB-EGF is synthesized as a transmembrane precursor which is later processed to the mature 86-amino acid form. Mature bioactive HB-EGF is a well characterized 20–22-kDa glycoprotein that binds the EGF receptor with high affinity and stimulates its phosphorylation [12]. HB-EGF is a potent mitogen for fibroblasts, smooth muscle cells [12] and keratinocytes [18] but is not mitogenic for endothelial cells [12]. HB-EGF is as active as PDGF but considerably more potent than EGF and TGF-α in stimulating bovine aortic SMC proliferation and migration [12,13]. Since the formation of mature blood vessels requires a concerted growth and migration of vascular SMC and endothelial cells, the possibility was tested that HB-EGF, which is a potent mitogen and migration factor for vascular SMC and is present in atherosclerotic, tumor and injury sites, is also angiogenic. We report here for the first time that HB-EGF induces angiogenesis in vivo, that it induces endothelial cell migration and that it mediates endothelial cell proliferation by inducing VEGF expression and secretion by co-cultured vascular SMC.

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Abbreviations: AVD, apparent vessel density; MRI, magnetic resonance imaging; RT-PCR, reverse transcriptase polymerase chain reaction

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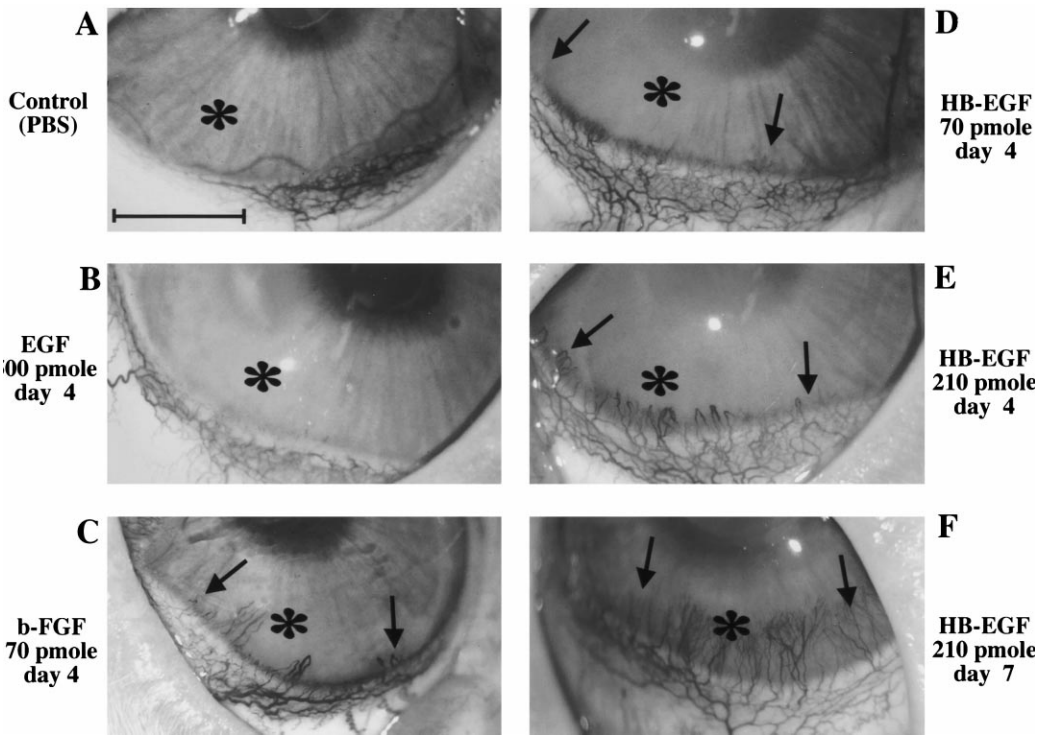

Fig. 1. HB-EGF promotes neovascularization in vivo in the rabbit cornea assay in a time- and dose-dependent manner. Photographs were taken 4 days after injection (A–E), and 7 days after injection (F). The injection site is marked by asterisks. Corneal angiogenesis (between arrows) is prominent in corneas injected with HB-EGF (D–F). PBS (A) served as a negative control. Basic FGF served as a positive control (C). EGF (500 pmol) did not induce angiogenesis (B). $n = 6$. Bar length 0.5 cm.

2. Materials and methods

2.1. Materials

Neutralizing anti-HB-EGF polyclonal antibody 197 was raised in goats by injection of a recombinant 77-amino acid form of human HB-EGF. The antibody is specific and does not neutralize EGF, TGF-α or amphiregulin. Goat anti-human VEGF neutralizing antibodies were purchased from R&D systems (Minneapolis, MN). Recombinant human HB-EGF consisted of residues 73–148 as predicted from the nucleotide sequence of the HB-EGF precursor's 208-codon open reading frame, and was produced using an *Escherichia coli* expression system [20,21]. Human recombinant HB-EGF and neutralizing anti-human HB-EGF polyclonal antibody 197 were received from Scios Inc. (Sunnyvale, CA). EGF and TGF-α were purchased from Collaborative Biomedical Products (Bedford, MA). 77R is an anti-bFGF polyclonal antibody raised in sheep and directed against the recombinant 154-amino acid human basic FGF (bFGF) produced in *E. coli*.

2.2. Cell lines

Brain bovine capillary endothelial cells (BCE), bovine aortic endothelial cells (BAE) and vascular SMC were kindly provided by Prof. Israel Vlodavsky (Hadassah Medical center, Jerusalem). BCE and vascular SMC were cultured at 37°C in low-glucose DMEM (1 g/l) supplemented with 10% calf serum (HyClone, Logan, UT), a serum-free supplement: biogro-1 (Beth Haemek, Israel), penicillin (100 U/ml), streptomycin (0.1 mg/ml) (Biological Industries, Israel) and 2 mM glutamine (Biolab Ltd. Israel) (GPS). Bovine aortic vascular smooth muscle cells (SMC) were cultured in low-glucose DMEM (1 g/l) supplemented with 10% fetal calf serum (HyClone) and GPS. For RNA experiments, SMC were seeded in 100 mm diameter dishes at a density of 1.6×10^5 cells/dish (approximately 20% confluence). Medium was changed after 24 h to starvation medium (DMEM with GPS) and 48 h later HB-EGF was added at different concentrations for 24 h.

2.3. Reverse transcriptase-PCR analysis

RNA was isolated from SMC cells using TRI reagent [22] (Molecular Research Center Inc., Cincinnati, OH). Complementary DNA was synthesized from 300 ng of total RNA using oligo(dT) as a primer and AMV reverse transcriptase. PCR amplification was carried out in the presence of [^{32}P]dCTP tracer (2 μCi in 100 μl reaction volume), 1 mM of each dNTP, 2.5 mM $MgCl_2$, and 2.5 units of Taq polymerase. Twenty-five amplification cycles were used, of 1 min at 94°C, 2 min at 65°C and 3 min at 72°C. The cDNAs were co-amplified with oligonucleotides of VEGF (GGAGAGATGAGCTTCCTACAG and TCACCGCCTTGGCTTGTCACA, corresponding to the coding region for amino acids 92–98 and the six carboxy-terminal amino acids of VEGF, respectively); and L19 (serving as an internal standard, CTGAAGGTCAAAGGGAATGTG and GGACAGAGTCTTGATGATCTC, corresponding to forward and reverse sequences). Amplified fragments were resolved in 6% non-denaturing polyacrylamide gel and visualized by autoradiography. Radioactivity of the hybridization signal on the filter was measured by using a Fujix Bas 1000 Bioimaging Analyzer (Fuji, Japan).

2.4. Co-culture assay for SMC and BCE cells

SMC were seeded in 6-well plates (3×10^4 cells/well), where each well contained a sterile round cover glass (18 mm diameter). BCE cells were seeded in 24-well plates (5×10^3 cells/well), on sterile round cover glass (13 mm diameter). Twenty-four hours following seeding, both cell lines were starved for 48 h (DMEM with only GPS for SMC and DMEM with GPS and 0.5% bovine serum albumin (BSA) for BCE). After 48 h of starvation, the 18-mm cover glasses in 6-well plates were replaced by new ones (exactly on the bald spot created by removing the original cover glass). Smaller (13-mm) cover glasses with their adherent BCE were removed from the 24-well plates and were placed on top of the fresh 18-mm cover glasses in the 6-well plates together but separated from the vascular SMC. Growth factors and neutralizing antibodies were then added in fresh medium for 30 h (in triplicate). Neutralizing antibodies were added at concentrations of 10–13 μg/ml. [^{3}H]Methylthymidine 5 μCi/ml (ROTEM Ind. Ltd., Israel) was added to the cells for the last 6 h of the 30 h co-culture period. The cells were rinsed with 2 ml methanol for 10 min, followed by 2 ml 5% cold trichloroacetic acid (TCA). Afterwards cells were washed with water and glasses with BCE were removed to separate 24-well plates.

Cells were lysed with 0.5 M NaOH. Radioactive thymidine incorporation into DNA was determined in a β counter (LKB, WALLAC, Finland). Experiments were performed at least twice. Control experiments in which vascular SMC or BCE cells were incubated separately in the presence of HB-EGF demonstrated that SMC were not found on the cover glass and that BCE from the cover glass were not seen in the well.

2.5. Migration assay for endothelial cells

Boyden chamber migration assays were performed as previously described [23]. Collagen IV prepared from EHS tumors was dried on a polycarbonate filter (5 mg/filter) (PVP-free, Nucleopore, Pleasanton, CA). Fibroblast conditioned medium (obtained from confluent NIH-3T3 cells cultured in serum-free DMEM) was used as a positive chemoattractant control. BCE or BAE cells were harvested by brief exposure to 1 mM EDTA, washed with DMEM containing 0.1% BSA and added to the Boyden chamber (2×10^5 cells). The chambers were incubated in a humidified incubator at 37°C in 5% CO_2/95% air atmosphere for 6 h. The cells which traversed the collagen layer to the side to which chemoattractants were added, and attached to the lower surface of the filter, were stained with Diff Quick (American Scientific Products) and counted. The results are the average of two independent experiments, each performed in duplicate.

2.6. Rabbit cornea assay for angiogenesis

New Zealand albino rabbits (weight 3 kg, males and females in equal numbers) were used in our study. The animals were deeply anaesthetized with 5 mg/kg xylasine and 35 mg/kg ketamine-HCl injected intramuscularly. The rabbit eyes were treated with Localin, an ophthalmic solution for surface anesthesia (Benoxinate HCl 0.4%, Fisher, Pittsburgh, PA). Using a surgical microscope (Inami, Japan), 5 μl of HB-EGF, bFGF or EGF in phosphate-buffered saline (PBS) were injected subepithelially into the cornea by using a 10-μl Hamilton syringe. The injection was done 3–4 mm away from the limbal margin of the cornea towards its center. The area chosen was the midline between the insertions of the recti muscles, superior and lateral or medial, right or left eye respectively. After the injection a white opaque elevation of epithelium could be observed. Experiments were done in duplicate and repeated three times (each treatment consisted of six animals: three males and three females).

2.7. MRI analysis of subcutaneous angiogenesis in nude mice

Spherical agarose beads of approximately 1 mm in diameter were formed from 4% low gelling temperature agarose (Sigma) in PBS containing HB-EGF or b-FGF. The candidate angiogenic compound (5 μl/bead) was warmed in sterile micro test tubes to 40°C in a dry bath for a few seconds. 10 μl of agarose solution (6% in saline, 45°C) was then added to the 5 μl of the angiogenic compound and beads were formed above ice using a 20-μl pipette tip. Mice were anesthetized with a single dose of 75 mg/kg ketamine+3 mg/kg xylazine (i.p.) and beads were implanted 1 cm away from the incision site as reported previously for multicellular spheroids [24]. Experiments were done in duplicate and repeated three times. MRI experiments were performed on a horizontal 4.7-T Bruker-Biospec spectrometer using a 2-cm surface coil as reported previously [24,25]. Mice were anesthetized as described above and placed supine with the bead located at the center of the surface coil. Gradient echo images (slice thickness of 0.5–0.6 mm, repetition time of 100 ms, 256×256 pixels, in plane resolution of 110 μm) were acquired with echo times of 10.5 and 20 ms. Growth of the capillary bed was reflected by reduction of the mean intensity at a region of interest of 1 mm surrounding the agarose bead. Angiogenic capacity of the implanted beads was evaluated from the difference between the apparent vessel density determined by MRI (AVDMRI) of day 4 and that of day 1. Validation of the method and derivation of the physical source of contrast have been reported elsewhere [25].

2.8. Data processing

MRI data was analyzed on a Personal Iris work station (Silicon Graphics, USA) with software from NMRi (TRIPOS). Statistical significance of treatments was determined using Student's *t*-test or ANOVA.

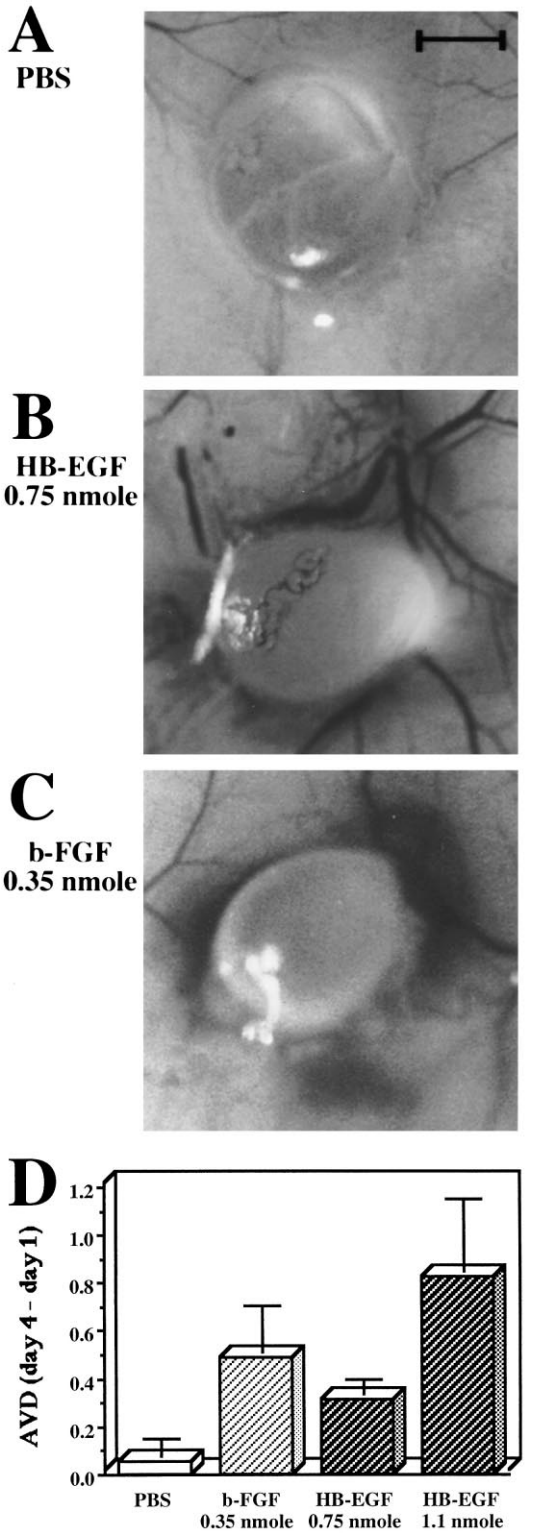

Fig. 2. HB-EGF promotes neovascularization in vivo in beads implanted subcutaneously in nude mice in a dose-dependent manner. The angiogenic potential of HB-EGF in vivo is demonstrated 4 days after implantation, in skin specimens (A–C) and quantitatively by MRI (D). PBS served as a negative control (A,D), while bFGF (0.35 nmol/bead), as a positive control (C,D). HB-EGF at 0.75–1.1 nmol/bead induced a significant dose-dependent neovascularization around the beads (B,D). $n=8$. Bar length 0.5 mm.

3. Results

3.1. HB-EGF induces angiogenesis in the rabbit cornea

The angiogenic capacity of HB-EGF was demonstrated in vivo in the rabbit cornea assay. Human recombinant HB-EGF (70–210 pmol) was introduced subepithelially into the rabbit cornea with minimal damage to the cornea. Two to three days following introduction of HB-EGF into the cornea, new blood vessels began to grow, in a dose-dependent manner, from the limbal margin of the cornea towards the injection site. HB-EGF at low doses (70 pmol) was able to induce neovascularization in the cornea within 4 days after introduction (Fig. 1D). A higher dose of HB-EGF (210 pmol) induced an even more rapid and intense neovascularization 4 days after introduction (Fig. 1E). Blood vessels continued to grow and to develop and became larger and longer at 7 days after HB-EGF introduction (Fig. 1F). The rabbit eyes were clear with no evidence of non-specific inflammation. PBS alone as a negative control or even 500 pmol of EGF did not induce any new blood vessel formation (Fig. 1A,B). To eliminate the possibility that the angiogenic reaction was due to traces of endotoxin or reaction to a foreign protein, we introduced boiled HB-EGF (210 pmol) and found no angiogenic response (not shown). The angiogenic response of 210 pmol of HB-EGF was comparable with that of 70 pmol of bFGF (Fig. 1C). Interestingly, when HB-EGF was repeatedly introduced into the cornea with a 1-month interval between the introductions, even doses as low as 6–20 pmol of HB-EGF induced new blood vessel formation (not shown).

3.2. HB-EGF induces angiogenesis in the skin

The angiogenic capacity of HB-EGF was also examined in nude mice by subcutaneous implantation of agarose beads containing HB-EGF, bFGF or PBS. The beads were implanted at a distance of 1 cm from the incision through which they were introduced, to avoid an effect resulting from injury. Neovascularization around the beads was evaluated by subtraction of the apparent vessel density (AVDMRI) measured on day 1 from that measured on day 4 by MRI. PBS, a negative control, did not cause neovascularization around the bead (Fig. 2A,D), while bFGF (0.35 nmol), a known and potent angiogenic factor, caused significant neovascularization around the beads (Fig. 2C,D). In comparison, HB-EGF also caused significant dose-dependent neovascularization around the beads at 0.75–1.1 nmol doses (Fig. 2D). HB-EGF induced a comparable angiogenic effect to bFGF. Both HB-EGF and bFGF induced an apparent yellowish color inside and around the bead (Fig. 2B,C) which may be related to increased leakage of plasma substances from the nearby vessels.

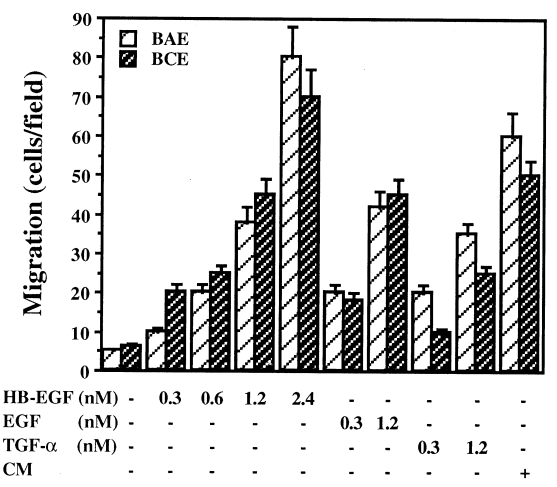

Fig. 3. HB-EGF induces endothelial cell migration. The migration response of BCE and BAE cells to HB-EGF, EGF and TGF-α was examined in Boyden chambers for 6 h. Conditioned medium (CM), obtained from confluent NIH-3T3 cells cultured in serum-free DMEM, served as a positive control.

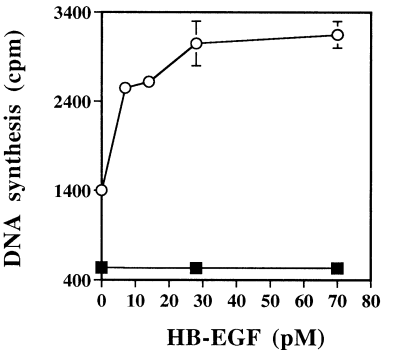

Fig. 4. HB-EGF induces secretion of an endothelial cell mitogen by vascular SMC. DNA synthesis was measured in BCE co-cultured with (empty circles) or without (full squares) vascular SMC in presence of various concentrations of HB-EGF as described in Section 2.

3.3. HB-EGF induces endothelial cell migration

EGF, TGF-α and HB-EGF share 35% sequence homology and bind to the EGF receptor. EGF and TGF-α were previously shown to be potent migration stimulating factors for endothelial cells [26]. We have examined in Boyden chambers the migration-stimulating capacity of HB-EGF and compared it with that of EGF and TGF-α. Capillary as well as aortic endothelial cells responded similarly and migrated in response to HB-EGF in a dose-dependent manner (Fig. 3). At concentrations of 0.3–2.4 nM, HB-EGF induced a 3–13-fold increase in endothelial cell migration, respectively (Fig. 3). At equimolar concentrations, the migration stimulated by HB-EGF was similar to that induced by EGF and TGF-α.

3.4. HB-EGF induces VEGF secretion by SMC, leading to enhanced DNA synthesis in endothelial cells

It was previously shown that HB-EGF is a potent mitogen for vascular SMC fibroblasts and keratinocytes, but not for endothelial cells. To reconcile the different activities of HB-EGF in vivo and in vitro with respect to angiogenesis, we have postulated that vascular SMC might be involved in HB-EGF's angiogenic effect in vivo by inducing the proliferation of endothelial cells. We developed an assay in which serum-starved BCE were co-cultured with serum-starved, HB-EGF-treated SMC without contact between the two cell types, and could thus measure whether there was a release of an endothelial mitogenic activity from the induced SMC. Indeed, when vascular SMC were co-cultured with endothelial cells, HB-EGF induced an increase in DNA synthesis in BCE cells in a dose-dependent manner, compared with non-induced SMC (Fig. 4) as well as in BAE (not shown). At a concentration as low as 7 pM, HB-EGF increased DNA synthesis in BCE approximately 1.9-fold compared with non-treated con-

trol (Fig. 4). HB-EGF alone had no effect on DNA synthesis in BCE (Fig. 4). The effect of HB-EGF is specific, since most of it (90%) is blocked in the presence of neutralizing anti-HB-EGF antibodies both for SMC and BCE in the co-culture assay (Fig. 5A,B). At 70 pM HB-EGF increased DNA synthesis in co-cultured BCE 3-fold, compared with BCE co-cultured with non-treated SMC. EGF or TGF-α had almost no effect on DNA synthesis in the co-cultured BCE (Fig. 5A). In contrast, the effect of these three growth factors on DNA synthesis in SMC themselves was similar under these conditions: HB-EGF (70 pM) increased DNA synthesis 2.5-fold, while TGF-α and EGF (140 pM) increased DNA synthesis 2.5- and 2.1-fold respectively (Fig. 5B). In order to identify the secreted mitogenic activity for BCE which is induced in HB-EGF-stimulated SMC we used neutralizing anti-VEGF and neutralizing anti-bFGF antibodies. Neutralizing antibody against bFGF suppressed only 25% of the endothelial cell mitogenic effect induced by HB-EGF, while neutralizing anti-VEGF antibodies suppressed almost all the mitogenic effect of HB-EGF on BCE co-cultured with vascular SMC

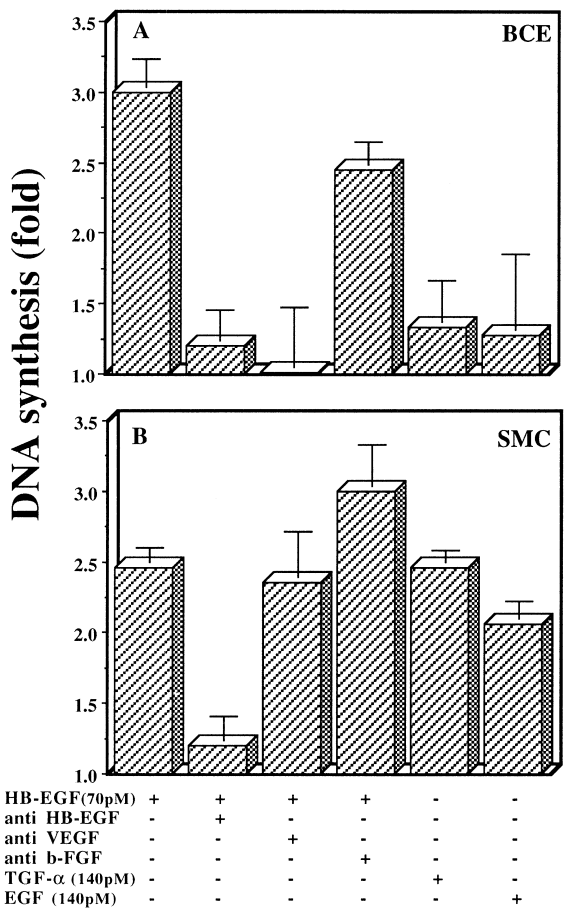

Fig. 5. HB-EGF induces secretion of VEGF by vascular SMC. Vascular SMC were co-cultured with BCE. Cell proliferation was monitored by [^{3}H]thymidine incorporation into BCE cells (A) or SMC (B) in comparison with non-treated cells. The effect of neutralizing antibodies against HB-EGF, VEGF or bFGF on the enhancing effect of HB-EGF on co-cultured BCE cells (A) or SMC (B) was examined. In comparison to HB-EGF, TGF-α and EGF had almost no effect on the co-cultured BCE cells (A). Actual [^{3}H]thymidine incorporation into BCE cells was 5500 cpm when incubated with control vascular SMC and 16 600 cpm when incubated with vascular SMC primed with 70 pM HB-EGF.

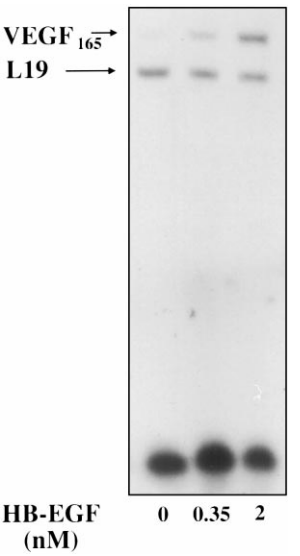

Fig. 6. HB-EGF induces the level of VEGF mRNA in vascular smooth muscle cells. RNA was prepared from control non-treated SMC and SMC treated for 24 h with 0.35 nM and 2 nM HB-EGF. VEGF mRNA levels were compared by RT-PCR analysis. Amplified VEGF cDNA fragment corresponds to mRNA of $VEGF_{165}$. Co-amplified ribosomal L19 served as an internal standard.

(Fig. 5A). Neutralizing anti-VEGF antibodies had no effect, however, on the mitogenicity of HB-EGF for SMC (Fig. 5B). This demonstrates that the major endothelial cell mitogen secreted from HB-EGF-induced vascular SMC is VEGF. The specificity of the anti-VEGF antibodies was assessed by measuring DNA synthesis in BCE cells incubated with 5 ng/ml hVEGF or 1 ng/ml of bFGF in presence of medium derived from control non-treated vascular SMC. Neutralizing anti-VEGF antibodies inhibited most (75%) of the 3-fold increase in DNA synthesis induced in BCE cells incubated with hVEGF while they had almost no effect (15%) on the 3-fold increase in DNA synthesis induced in BCE by bFGF (not shown).

3.5. Induction of VEGF mRNA level in SMC by HB-EGF

To examine whether HB-EGF affects VEGF expression in vascular SMC, RNA was extracted from SMC exposed to HB-EGF for 24 h and steady-state levels of VEGF mRNA were compared with non-induced cells by RT-PCR analysis. $VEGF_{165}$ mRNA levels were markedly increased in vascular SMC by HB-EGF. $VEGF_{165}$ was the predominant form detected in this assay. Quantitation by Bioimaging Analyzer of the $VEGF_{165}$ mRNA, normalized versus the internal standard ribosomal L19, demonstrated a 4–10-fold increase by 0.35–2 nM of HB-EGF, respectively (Fig. 6). Interestingly, VEGF is also expressed, although at low levels, in non-induced vascular SMC, reinforcing our observation that non-induced SMC release endothelial cell mitogenic activity (Fig. 4), possibly through an HB-EGF autocrine loop.

4. Discussion

Angiogenesis plays an important role in wound healing, pregnancy, tumor progression and atherosclerosis [1,2,27]. Activated monocyte-macrophages are key angiogenesis effectors in these settings [1,27]. HB-EGF, a recently described member

of the EGF family, is synthesized by inflammatory cells, including monocytes, macrophages [12,28] and T lymphocytes [15,29], and has been shown to be associated with atherosclerosis [14,15], tumor development [15], pregnancy [17] and with wound healing as the major heparin-binding growth factor for fibroblasts and keratinocytes present in wound fluid [18,19].

In this study we show for the first time that HB-EGF promotes neovascularization in vivo in the rabbit cornea assay in a time- and dose-dependent manner, at doses as low as 70 pmol, and can thus be considered a novel angiogenic factor. At 210 pmol HB-EGF induced a comparable angiogenic activity in our rabbit cornea assay as bFGF at a dose of 70 pmol. On the other hand, EGF, which has weak angiogenic properties, did not induce angiogenesis in our rabbit cornea assay even at a dose of 500 pmol. In comparison, tumor necrosis factor type α (TNF-α), a known angiogenic factor, was previously shown to stimulate neovascularization in the rabbit cornea assay at doses of 300 pmol, while bFGF produced neovascularization in that report at 30 pmol [30]. In addition, by using MRI we have been able to demonstrate quantitatively the angiogenic capacity of HB-EGF introduced subcutaneously into nude mice. Similar to the rabbit cornea assay, in the MRI assay bFGF had an angiogenic activity comparable to that of HB-EGF. To our knowledge, this is the first time MRI has been used to study a new angiogenic factor in vivo in a non-invasive way.

Ushiro et al. recently reported that HB-EGF binds to the EGF receptor on human microvascular endothelial cells and induces the formation of tube-like structures in type I collagen gel in vitro [31]. These findings in human microvascular endothelial cells demonstrate another property of HB-EGF which may be important for its activity as an angiogenic factor in vivo.

HB-EGF does not induce proliferation of endothelial cells [12]. The question was raised as to what may then be the mechanism of the angiogenic activity of HB-EGF that we observed in vivo. The factors EGF and TGF-α were previously shown to be angiogenic in vivo [32] and to be among the most potent migration factors to endothelial cells [26,33]. Examination of the migration-stimulating potential of HB-EGF for endothelial cells demonstrates, for the first time, that it is as potent as EGF and TGF-α.

Formation of mature blood vessels requires a concerted growth and migration of vascular SMC and endothelial cells. It was recently shown that the reciprocal interactions between endothelium and surrounding mesenchyme and smooth muscle cells is crucial for the normal development of veins mainly through angiopoietin-1 and the TIE2 receptor [34–36]. The ability of HB-EGF to stimulate vascular SMC growth and migration [12,13] suggests a possible role in blood vessel development. We have demonstrated that HB-EGF is an indirect endothelial cell mitogen, via its effect on SMC.

It was shown previously that aortic SMC express and secrete $VEGF_{165}$ [37,38]. In the present study we demonstrate that HB-EGF induces a marked concentration-dependent increase (4–10-fold) in the $VEGF_{165}$ mRNA level in vascular SMC. In addition we show that neutralizing anti-VEGF antibodies block most of the endothelial mitogenic activity secreted by HB-EGF-stimulated vascular SMC in vitro. These results suggest that the angiogenic effect of HB-EGF in vivo is mediated by inducing the migration of both endothelial and vascular smooth muscle cells without contact between the two cell types, as well as by the induction of VEGF in vascular SMC, and that this VEGF then acts on neighboring endothelial cells in a paracrine manner to induce proliferation and blood vessel formation. Since endothelial cell proliferation can be inhibited by contact with SMC and pericytes [39] it is possible that the effect of HB-EGF on the interaction between these two cell types when direct contact between them is made, may be different. EGF and TGF-α were as potent as HB-EGF in inducing endothelial cell migration, but had almost no effect on inducing release of endothelial mitogenic activity from vascular SMC. This is in agreement with our data that EGF, which is known to be a weak angiogenic factor, was not angiogenic in the rabbit cornea assay at a dose of 500 pmol, as well as with a previous finding that EGF does not induce VEGF mRNA in vascular SMC [11]. HB-EGF is a much more potent angiogenic factor than EGF in vivo. This potency may reflect the fact that unlike EGF, HB-EGF induces VEGF in vascular SMC.

The expression of HB-EGF at tumor and atherosclerosis sites in macrophages and T lymphocytes [14,15] suggests that these inflammatory cells may be the source for HB-EGF in these sites of intense angiogenesis. However, HB-EGF is also induced in several other cell types and other processes in which angiogenesis is involved, such as in the tubular epithelial cells in kidneys after ischemic injury [40], in cancer cells [41–45], in vascular SMC [46–48], and in vascular endothelial cells [49]. The source for HB-EGF as an angiogenic mediator may thus vary for the different tissues and processes.

VEGF was suggested to be involved in the maintenance of vascular endothelial cells by being released from vascular SMC [37,50] and was shown to be a survival factor in vivo [51]. HB-EGF is produced by both vascular endothelial cells and vascular SMC [46–49]. In accordance, HB-EGF is produced in normal arteries mainly in the endothelium and only faintly in medial SMC [52]. Being expressed by the two cell types constituting the blood vessel wall, HB-EGF may induce the expression of VEGF in vascular SMC in either a paracrine or autocrine manner and contribute to the maintenance of vascular endothelial cells in the blood vessel wall.

In conclusion, our study demonstrates that HB-EGF is a potent angiogenic factor in vivo and that it induces both migration of endothelial cells and the expression and secretion of VEGF in cultured SMC. This suggests a new role for HB-EGF in normal and pathological neovascularization and in vascular maintenance. Since HB-EGF has been shown to be associated with wound healing [18,19], liver regeneration [53], atherosclerosis [14,15] and tumor development [15], we suggest that HB-EGF may contribute to angiogenesis in these processes.

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