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Heparin-binding Epidermal Growth Factor-like Growth Factor: p91 Activation, Induction of Plasminogen Activator/Plasminogen Activator Inhibitor, and Tubular Morphogenesis in Human Microvascular Endothelial Cells

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Epidermal growth factor (EGF) or transforming growth factor-α (TGF-α) stimulates cell migration, proliferation and the formation of tube-like structures of human microvascular endothelial cells in culture. Heparin-binding EGF-like growth factor (HB-EGF), which shows 35% homology with EGF/TGF-α, is a member of the EGF family, and it is ubiquitous in many tissues and organs. We examined whether or not HB-EGF induced angiogenic responses in human microvascular endothelial cells. HB-EGF inhibited the binding of ^{125}I-EGF to the EGF receptor and induced autophosphorylation of the receptor on endothelial cells. Exogenous HB-EGF induced the loss of more than 70% of the EGF receptor from the cell surface within 30 min, with similar kinetics to that of EGF. The level of c-*fos* mRNA markedly increased at 30 min in response to HB-EGF as well as EGF. A gel shift assay demonstrated the activation of the transcription factor p91 by HB-EGF and EGF. This factor directly interacts with the EGF receptor and mediates the activation of c-*fos* gene promoter. HB-EGF enhanced the mRNA expression of tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) mRNA. However, the enhancement of t-PA and PAI-1 by HB-EGF was less than that by EGF. Heparitinase/chlorate, which digests the heparan sulfate proteoglycan of the endothelial cell surface, restored both t-PA and PAI-1 mRNA levels in response to HB-EGF in a manner similar to that by EGF. HB-EGF at 10 ng/ml developed tube-like structures in type I collagen gel at similar levels to that of EGF at 10 ng/ml, suggesting that HB-EGF is also a potent angiogenic factor in the model system for angiogenesis. The tubulogenesis activity of HB-EGF is discussed in relation to the expression of the t-PA and PAI-1 genes.

Key words: Angiogenesis — Human microvascular endothelial cell — HB-EGF — p91 — t-PA

Angiogenesis depends upon a balance of angiogenic factors and inhibitors. Angiogenesis proceeds in three sequential steps: endothelial cells degrade the vascular basement membrane and interstitial matrix, migrate and proliferate, then induce tubulogenesis and the formation of capillary networks. During this process, proteolytic enzymes produced by the endothelial cells play a critical role in the degradation of the perivascular extracellular matrix and tissue stroma, and a cascade of proteolytic events then leads to the retraction of the extracellular matrix.[1–3] Plasminogen activator (PA) produced by the endothelial cells converts plasminogen to plasmin, that degrades the extracellular matrix.[1,4,5] Among angiogenic factors such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), and vascular endothelial growth factor (VEGF), bFGF induces cell proliferation, migration, and the formation of tube-like structures and urokinase-type PA in bovine aortic endothelial and human umbilical vein endothelial cells, whereas transforming growth factor-beta (TGF-β) antagonizes the effects of bFGF.[6–11] VEGF, which has a potent angiogenic property *in vivo*,[12,13] also induces the formation of tube-like structures and urokinase-type PA (u-PA) in human umbilical vein endothelial cells.[14,15] The expression of u-PA in endothelial cells is thus closely coupled with angiogenesis in bovine endothelial and human umbilical vein endothelial cells in the presence of bFGF or VEGF.

EGF/TGF-α stimulates bovine or murine pulmonary endothelial cells *in vitro* and promotes angiogenesis in the hamster chick pouch *in vivo*.[16,17] In our laboratory, we established human microvascular endothelial cells from omental adipose tissue. In this microvascular endothelial cell system, EGF/TGF-α among various angiogenic factors examined is the most potent in stimulating cell migration and tissue-type plasminogen activator (t-PA) gene expression,[18–20] and also the formation of the tube-like structures in a type I collagen gel.[21–24] This EGF/TGF-α-dependent formation of the tube-like structures is blocked by the co-administration of TGF-β or by co-

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cultured chondrocytes which produce TGF-β1 or β2.[21,25] Microvascular endothelial cells develop capillary networks on Matrigel,[26] and in this system, the PA activity is also closely correlated with the development of capillary networks. The expression of the bFGF gene is enhanced in microvascular endothelial cells treated with tumor necrosis factor-α,[27] suggesting an autocrine loop in this endothelial cell system. We also demonstrated that EGF shares a common signal transduction pathway with tumor necrosis factor-α in human microvascular endothelial cells.[28]

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) which is a member of the EGF family with 35% homology to EGF, binds to the EGF receptor and triggers its autophosphorylation.[29–31] HB-EGF is a potent mitogen for fibroblasts, smooth muscle cells, hepatocytes and keratinocytes, but not for bovine vascular endothelial cells.[31–33] HB-EGF is distributed in a wide variety of normal tissues,[34–37] and appears in wound fluid as a response to injury.[37] Tumor necrosis factor-α increases expression of the HB-EGF gene in human umbilical endothelial cells.[38] Furthermore, phorbol ester also induces the expression of HB-EGF mRNA in rat aortic smooth muscle cells,[39,40] suggesting that HB-EGF is a potent autocrine factor for smooth muscle cells. However, there is no evidence regarding the stimulatory effect of HB-EGF on vascular endothelial cells. In this study, we first studied the potency of HB-EGF to induce tubular morphogenesis and PA gene expression in an angiogenesis model system with microvascular endothelial cells. Tubular morphogenesis and related biochemical reactions induced by HB-EGF are discussed in comparison with EGF-induced effects.

MATERIALS AND METHODS

Human microvascular endothelial cells Microvascular endothelial cells were isolated from human omental tissues[41] and endothelial cells were identified as described.[18,19,23,26,28] Endothelial cells were cultured in M-199 supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 60 μg/ml kanamycin as reported. Since endothelial cells decreased in responsiveness to growth factors such as EGF during serial cultivation, we used cells at 3 to 5 passages.[20]

Materials Recombinant HB-EGF was prepared in an *Escherichia coli* expression system.[42] EGF was purchased from Toyobo Co., Osaka. Human t-PA cDNA obtained from W-D. Schleuning (Schering Aktiengesellshaft Pharma Forschung, Berlin, Germany)[43]; plasminogen activator inhibitor-1 (PAI-1) cDNA was from D. J. Loskutoff (The Research Institute of Scripps Clinic, La Jolla, CA)[44]; a 72 kD type IV collagenase cDNA was obtained from G. Goldberg (Washington Univ., School of Med.)[45]; a c-*fos* cDNA probe consisting of c-*fos* human cDNA inserted into plasmid pSPT18 was obtained from the Japanese Cancer Research Resources Bank.

Iodination of EGF and binding assay EGF was iodinated using chloramine T as described.[46,47] The specific activity was 250,156 cpm/ng. Confluent cells in monolayers were washed twice with phosphate-buffered saline (PBS), and incubated in binding buffer containing 20 m*M* Hepes, 0.2% bovine serum albumin, and 2.5 ng/ml of ^{125}I-EGF, with or without excess unlabeled EGF or HB-EGF. After incubation for 2 h at 4°C, the binding buffer was aspirated. The monolayers were washed three times with PBS, then detached with 1 *N* NaOH, and the radioactivity in bound ^{125}I-EGF was measured in a gamma-counter as described.[20,28]

Assay of EGF receptor phosphorylation with antiphosphotyrosine antibody Confluent cells were incubated with M-199 containing 1% dialyzed FBS and with EGF or HB-EGF for various periods. Thereafter, cells were solubilized with 1 ml of PBS-TDS (10 m*M* NaH_2PO_4, 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.2% NaN_3 and 0.004% sodium fluoride) including protease inhibitors (1 m*M* PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and a phosphatase inhibitor (0.2 m*M* Na-orthovanadate). Cell lysates were centrifuged at 15,000 rpm for 10 min, the supernatant was immunoprecipitated with 3 μl of anti-EGF receptor antibody (Ab-1, Oncogene Science, New York) for 9 h, and protein A Sepharose pellets were washed with ice-cold PBS-TDS five times at 4°C by centrifugation at 12,000 rpm for 2 min. The resulting precipitates were dissolved in SDS sample buffer, analyzed by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE), then blotted onto a nitrocellulose membrane in 25 m*M* Tris-HCl (pH 8.3), 92 m*M* glycine, and 20% methanol for 2 h at 200 V. Nitrocellulose membranes were incubated with anti-phosphotyrosine antibody (PY20) (ICN, New York) for 1 h at room temperature, then with anti-mouse immunoglobin G fragment.[46,47] Antibody bound to phosphotyrosine was detected using the ECL-Western blotting detection system (Amersham, Buckinghamshire, UK) after a 2 h exposure to Hyperfilm-ECL X-ray film (Amersham) at room temperature.[22,28]

Northern blots Northern blotting was performed as described.[19,48] Total RNAs were isolated from cells incubated with or without growth factors as described above, resolved by electrophoresis in a 1% agarose gel containing 2.2 *M* formaldehyde, then blotted onto Hybond-N^+ (Amersham). The membranes were hybridized with ^{32}P-labeled probes, then visualized by autoradiography. Densitometric analysis was performed using a BAS 2000 FUJIX-imaging analyzer (FUJIX, Tokyo).

Gel shift assay Gel shift assays were performed as

described.[28, 48] Nuclear extracts were isolated from cells incubated with or without growth factors, and mixed with the p91 consensus sequence. Samples containing 4 μg protein were resolved by electrophoresis on a 4% polyacrylamide gel (polyacrylamide/bisacrylamide, 80: 1) at 4°C using 1× TGE (25 m*M* Tris base, 190 m*M* glycine, 1 m*M* EDTA, pH 8.5). The gels were dried and analyzed by autoradiography. The p91 consensus sequences were synthesized.[28, 48] The sequence of the oligonucleotides included a c-*sis* inducible element: AGTTCCCGTCAAATCCT.

EGF receptor down-regulation Confluent cells in 24-well dishes containing M-199 and 1% dialyzed FBS were incubated with or without growth factors for up to 30 min at 37°C. The cells were then washed three times with PBS to remove growth factors and incubated for 2 h at 4°C with a 1:100 dilution of anti-EGF receptor monoclonal antibody which specifically recognizes the extracellular domain of the human EGF receptor. After having been washed twice, the cells were incubated with 191,758 cpm/ml of ^{125}I-protein A for 1 h at 4°C.[47] The cells were again washed twice with PBS and lysed with 1 *N* NaOH to determine the radioactivity level as described.[47]

Tube formation in type I collagen gel and quantitation Eight volumes of type I collagen (Nitta Gelatin, Osaka), one volume of 10× M-199 or 10× Dulbecco's modified Eagle's medium, and one volume of 0.05 *N* NaOH containing 200 m*M* Hepes and 260 m*M* $NaHCO_3$ were mixed on ice, poured into 35 mm plastic dishes (Corning, New York), and allowed to gel at 37°C. Endothelial cells were plated onto the surface of the type I collagen gel in M-199 containing 10% FBS. When cells reached confluence on the gel, the medium was replaced with M-199 containing 0.2% FBS, then various factors were added and the gel was incubated for 3 days.[21–23, 25] On the third day, phase contrast microscopic images of each dish were recorded on a still video camera recorder (R5000H; Fuji, Tokyo), and the total length of the tube-like structures per field was measured using a Cosmozone IS image analyzer (Nikon, Tokyo) as described.[12, 21]

Heparitinase and chlorate treatment To digest cell surface heparan sulfate, cells were exposed to heparitinase and chlorate, an inhibitor of sulfation. For heparitinase digestion, cells were washed twice with M-199 without serum and incubated for 1 h at 37°C with M-199 containing 0.01 U/ml of heparitinase (Seikagaku Co., Tokyo). A second aliquot of heparitinase was then added to each dish to bring the concentration to 0.02 U/ml and the incubation proceeded for another 2 h. To inhibit sulfation, endothelial cells were incubated with M-199/10% dialyzed FBS supplemented with 30 m*M* Na-chlorate for 48 h. Dialyzed FBS was used to reduce the concentration of cysteine to 50 m*M*.[31, 49, 50]

RESULTS

Interactions of HB-EGF with cell surface EGF receptors of human microvascular endothelial cells HB-EGF binds to the EGF receptor and is mitogenic for mouse Balb/3T3 fibroblasts and bovine aortic smooth muscle cells, but not for bovine adrenal capillary endothelial cells.[29] HB-EGF almost completely competed out EGF binding to EGF receptors in human cancer A431 cells as well as in bovine smooth muscle cells.[20, 30] We examined whether HB-EGF had similar affinity to that of EGF for EGF receptors on human microvascular endothelial cells. As shown in Fig. 1, HB-EGF competed with ^{125}I-EGF for binding to the EGF receptor with similar kinetics to those of EGF. Thus, the competition assays demonstrated that HB-EGF and EGF were equipotent in binding to the EGF receptors of microvascular endothelial cells.

The EGF receptor is typical of receptors with tyrosine kinase activity, and tyrosine is rapidly phosphorylated in response to EGF. The EGF receptor of microvascular endothelial cells is autophosphorylated by exogenous EGF/TGF-α.[24, 28] HB-EGF as well as EGF at 10 ng/ml significantly enhanced the autophosphorylation of the

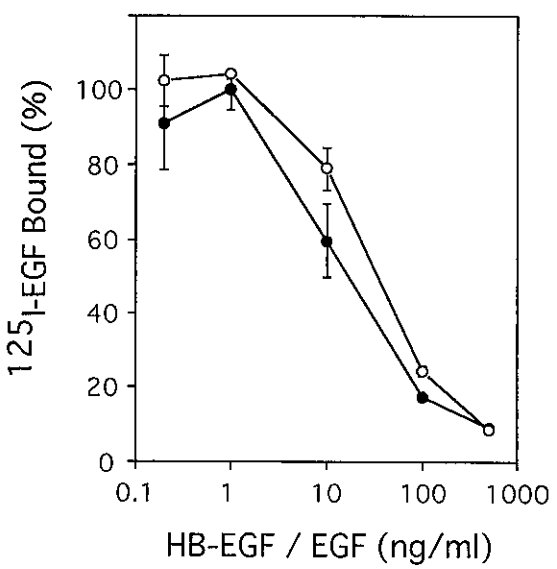

Fig. 1. Binding of EGF or HB-EGF to the EGF receptor. Confluent microvascular endothelial cells on 24-well dishes were incubated with 2.5 ng/ml ^{125}I-EGF and various concentrations of unlabeled EGF (●) or HB-EGF (○) at 4°C for 2 h. The endothelial cells were washed twice with chilled PBS and lysed with 1 *N* NaOH for radioactivity determination. The maximal binding (100%; 1.2×10^4 cpm/well) was determined in the absence of unlabeled EGF and HB-EGF. Data points are expressed as the mean±SD of duplicate experiments.

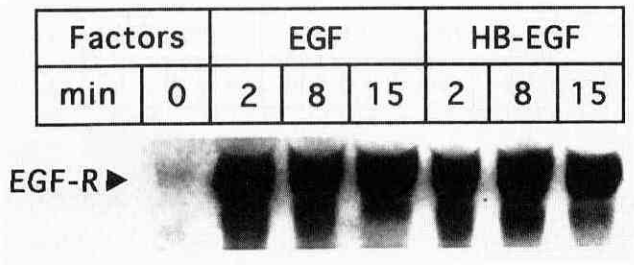

Fig. 2. EGF receptor autophosphorylation by EGF or HB-EGF. Confluent endothelial cells were incubated with 10 ng/ml EGF or HB-EGF at 37°C for the indicated periods. Cell lysates were immunoprecipitated with anti-EGF receptor antibody, and resolved by 10% SDS-PAGE. The arrow indicates the EGF receptor (EGF-R).

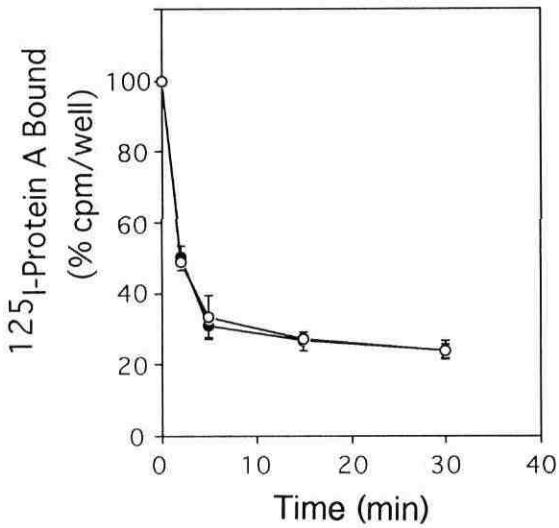

Fig. 3. EGF or HB-EGF induces down-regulation of the EGF receptor on microvascular endothelial cells. Confluent endothelial cells were incubated with 200 ng/ml EGF(●) and HB-EGF(○) at 37°C for the indicated periods, then for 2 h at 4°C with monoclonal antibody against human EGF receptor and for 1 h with ^{125}I-protein A. The relative amount of ^{125}I-protein A bound to the cells is plotted against the incubation period with EGF or HB-EGF.

EGF receptor of microvascular endothelial cells, and the receptor was similarly autophosphorylated by HB-EGF and EGF (Fig. 2).

Membrane traffic of EGF receptors under down-regulation by EGF or HB-EGF EGF receptor down-regulation by EGF is an obligatory pathway that regulates the number of receptor molecules on the cell surface.[46, 47, 51] We examined whether HB-EGF and EGF induced the down-regulation of cell surface EGF receptors in microvascular endothelial cells at similar rates. The cellular

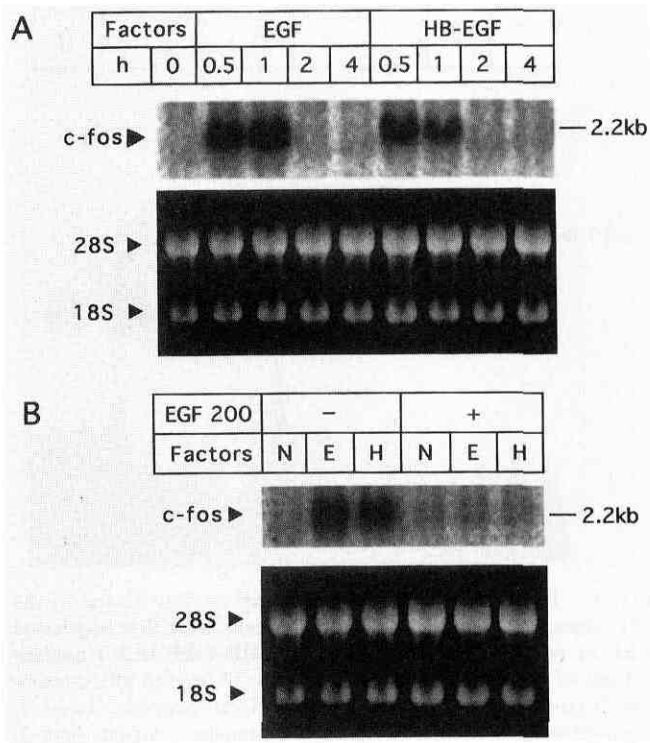

Fig. 4. Effect of EGF or HB-EGF on expression of c-*fos* gene with or without down-regulation of EGF-receptor. (A) Endothelial cells were incubated with 10 ng/ml EGF or HB-EGF for the indicated periods. (B) Endothelial cells were incubated with or without 200 ng/ml of EGF at 37°C for 12 h and then with 10 ng/ml EGF (E) or HB-EGF (H), or without these factors (N) for 30 min. Total RNAs were extracted, then Northern blotted with a c-*fos* ^{32}P-labeled cDNA probe. Ribosomal 18S and 28S RNAs loaded on the gels are also presented.

fate of EGF receptor under down-regulation was followed by measuring the amount of cell surface-bound anti-EGF receptor antibodies using ^{125}I-protein A and by a confocal study with immunofluorescence analysis.

We first compared the amount of cell surface EGF receptors in endothelial cells by ^{125}I-protein A immunoprecipitation.[47] As seen in Fig. 3, EGF binding induced the loss of 70–80% of the receptors from the cell surface within 15–30 min after ligand binding, consistent with a previous study.[28] Down-regulation of EGF receptors appeared to proceed at similar rates in the presence of EGF and HB-EGF.

Induction of c-*fos* proto-oncogene and activation of p91 by HB-EGF EGF receptor was autophosphorylated and down-regulated in response to HB-EGF in microvascular endothelial cells. We examined whether HB-EGF could trigger intracellular signaling via the EGF receptor in the microvascular endothelial cells. We reported that the

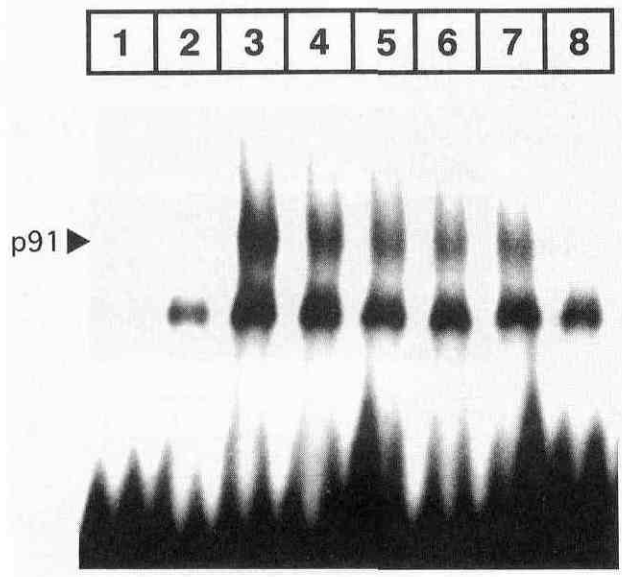

Fig. 5. Effect of EGF or HB-EGF on nuclear factor to the p91 consensus fragment. Endothelial cells were first incubated with or without 10 ng/ml EGF and HB-EGF and a nuclear extract of the cells was incubated with ^{32}P-labeled p91 consensus fragment and resolved by gel electrophoresis. Lane 1, complexes formed in the absence of nuclear extract; lane 2, complexes formed by nuclear extract from cells in the absence of factors; complexes formed by nuclear extract from cells in the presence of 10 ng/ml EGF for 30 (lane 3) and 60 min (lane 4); complexes formed by nuclear extract from cells in the presence of 10 ng/ml HB-EGF for 30 (lane 5) and 60 min (lane 6); complexes formed by nuclear extract from cells in the presence of 10 ng/ml HB-EGF for 30 min and a 50-fold excess of unlabeled SP-1 DNA fragments (lane 7) or unlabeled SIE DNA fragments (lane 8) for competition. The arrow indicates the specific, retarded DNA-protein complex of p91.

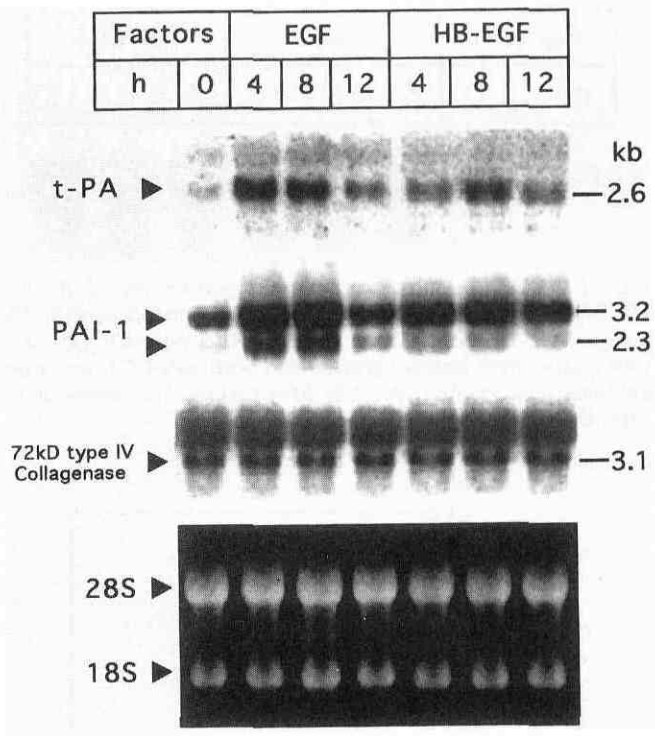

Fig. 6. Effect of EGF or HB-EGF on the expression of t-PA, PAI-1 and 72 kD type IV collagenase genes. Endothelial cells were first incubated with 10 ng/ml of EGF or HB-EGF for the indicated periods. Total RNAs were Northern blotted with t-PA and PAI-1 ^{32}P-labeled cDNA probes. Ribosomal 18S and 28S RNAs loaded on the gels are also presented.

expression of the c-*fos* proto-oncogene is enhanced by EGF within 30 min in microvascular endothelial cells.[20] We examined whether the expression of c-*fos* gene was also enhanced by HB-EGF. The expression of c-*fos* mRNA was increased to similar levels after 30 min in the presence of EGF and HB-EGF (Fig. 4A). Both EGF- and HB-EGF-dependent inductions of c-*fos* mRNA were almost completely abrogated when cell surface EGF receptors were down-regulated after a 12 h exposure to 200 ng/ml EGF (Fig. 4B). These results demonstrated that HB-EGF induced c-*fos* gene expression through the EGF receptor.

Fu and Zhang[52] have reported that the transcription factor p91 directly interacts with the EGF receptor, and that p91 mediates activation of the c-*fos* gene promoter through its translocation into the nucleus in response to EGF: p91 contains an SH2 domain which is activated by EGF receptor tyrosine phosphorylation. We examined whether HB-EGF or EGF modulated the activation of p91 by means of a gel mobility shift assay. As shown in Fig. 5, exposure to EGF and HB-EGF for 30 and 60 min induced the activation of p91. The EGF receptor pathway appeared to activate p91 in response to HB-EGF as well as to EGF in microvascular endothelial cells.

Expression of t-PA and PAI-1 genes induced by HB-EGF We demonstrated that t-PA gene expression was closely correlated with EGF/TGF-α-induced cell migration and tubulogenesis in our microvascular endothelial cell system, suggesting a key role of t-PA gene expression in the initial step of angiogenesis.[19, 23, 24, 26] Consistent with those results, exogenous 10 ng/ml EGF enhanced the expression of t-PA mRNA as well as PAI-1 mRNA more than 6-fold at time 4 h in comparison with that at time 0 h (Fig. 6). Expression of both PAI-1 transcripts, 3.2 and 2.3 kb, possible products of alternative splicing, was enhanced to similar levels by EGF. Cellular t-PA mRNA levels were increased 2- and 4-fold at 4 and 8 h, respectively, after addition of 10 ng/ml HB-EGF (Fig.

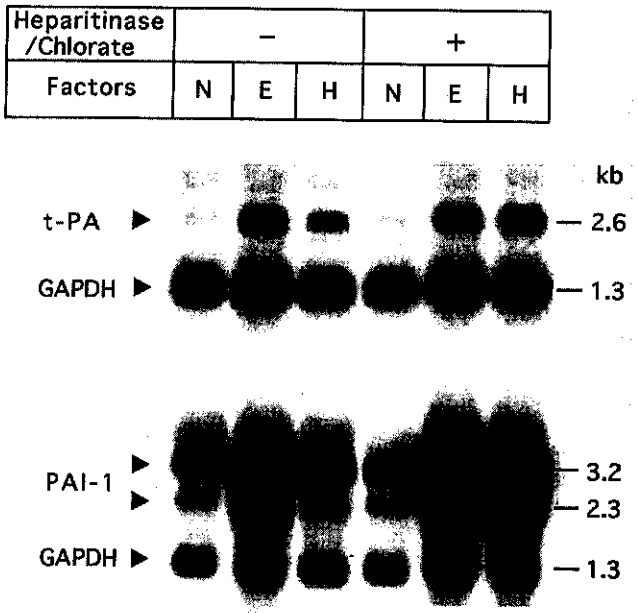

Fig. 7. Role of cell surface heparan sulfate proteoglycans in expression of the t-PA/PAI-1 gene induced by EGF or HB-EGF in microvascular endothelial cells. Endothelial cells with or without exposure to heparitinase and chlorate were incubated with 10 ng/ml of EGF and HB-EGF for 4 h. Total RNAs were Northern blotted with t-PA and PAI-1 ^{32}P-labeled cDNA probes.

6). Maximal increase of t-PA mRNA levels was observed at an earlier period (4 h) in EGF-treated cells than in HB-EGF-treated cells. By contrast, HB-EGF only 1.5-fold enhanced PAI-1 mRNA, while PAI-1 mRNA was increased more than 4-fold in the presence of EGF. The stimulatory effect of HB-EGF on both t-PA and PAI-1 gene expression thus appeared to be less than that of EGF. The relative rate of t-PA per PAI-1 mRNA level in HB-EGF-treated cells was found to be comparable to that in EGF-treated cells. There was no change in the 72 kD type IV collagenase mRNA levels in the absence or presence of EGF or HB-EGF.

To examine whether the decreased response to HB-EGF in the expression of PAI-1 mRNA was due to the heparin-binding domain of HB-EGF, we incubated vascular endothelial cells in dishes with heparitinase/chlorate and analyzed the mRNA levels of both t-PA and PAI-1 genes in the presence of HB-EGF. As shown in Fig. 7, the expression of PAI-1 mRNA in response to HB-EGF was 2.2 times more enhanced after treatment with heparitinase/chlorate, while t-PA mRNA showed only a slight, 1.1 times, increase after treatment with heparitinase/chlorate by densitometric analysis. The levels of both t-PA and PAI-1 mRNA in heparitinase/chlorate-treated cells in the presence of HB-EGF were similar to those of untreated cells in the presence of EGF (Fig. 7). The differential effects of HB-EGF on t-PA and PAI-1 gene expression appeared to be due to the heparin-binding domain of HB-EGF.

Formation of tube-like structures in type I collagen gel

Microvascular endothelial cells migrate into type I collagen gels and form numerous tube-like structures there in the presence of EGF/TGF-α.[21-23] We examined the effect of HB-EGF on tubular morphogenesis by microvascular endothelial cells in comparison with that of EGF. Microvascular endothelial cells showed a typical cobblestone-like appearance on type I collagen gel at 0 h (Fig. 8a) and a few tube-like structures in the gel (data not shown). This cobblestone-like appearance on the gel and tube-like structures in the gel showed little change for 72 h in the absence of any growth factor (Fig. 8d), but HB-EGF (Fig. 8c) as well as EGF (Fig. 8b) at 10 ng/ml induced numerous tube-like structures in the gel. HB-EGF at 1 ng/ml also significantly stimulated tubular morphogenesis (data not shown), suggesting that HB-EGF as well as EGF is tubulogenic in human microvascular endothelial cells *in vitro* in type I collagen gel. The tube-like structures were quantified, and total lengths were estimated to be 2207 ± 390 μm and 2311 ± 499 μm in the presence of EGF (Fig. 8b) and HB-EGF (Fig. 8c) at 10 ng/ml, respectively, when those of the control were 651 ± 123 μm in the absence of those factors (Fig. 8d). HB-EGF and EGF appeared to stimulate the formation of tube-like structures to a level about 3-fold higher than that in the absence of the factors.

DISCUSSION

HB-EGF was originally isolated from histiocytic lymphoma cell line U-937.[29,30] However, it is distributed in a wide range of tissues.[34,35] HB-EGF is mitogenic for fibroblasts and bovine vascular smooth muscle cells but not for bovine endothelial cells.[29,30] Temizer *et al.*[39] have reported that phorbol ester and angiotensin II increase HB-EGF mRNA levels in rat aortic smooth muscle cells. Yoshizumi *et al.*[38] have further reported that tumor necrosis factor-α enhances HB-EGF mRNA levels in human umbilical vein endothelial cells. These results suggest the involvement of HB-EGF in hyperplasia or hypertrophy of smooth muscle cells, resulting in an association with the pathogenesis of vascular diseases. In fact, eosinophils expressing HB-EGF are localized around lung microvessels in pulmonary hypertension.[36] Although HB-EGF is localized in vascular endothelial cells,[38] a stimulatory biological effect of HB-EGF on vascular endothelial cells has not yet been reported. In this study, we compared the effects of HB-EGF and EGF in human microvascular endothelial cells. HB-EGF, a novel heparin-binding member of the EGF family, bound

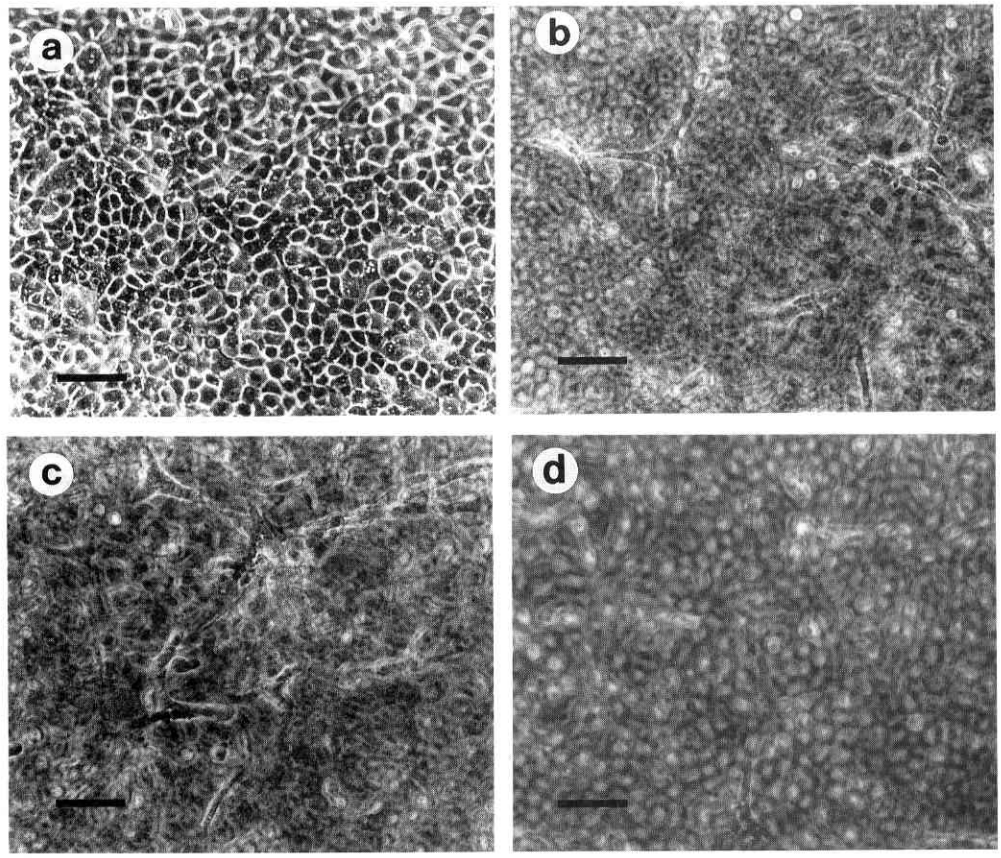

Fig. 8. Light microscopic morphology of human microvascular endothelial cells cultured on type I collagen gel for the indicated periods, (a), 0 h; (b), cultured with 10 ng/ml EGF for 72 h; (c), cultured with 10 ng/ml HB-EGF for 72 h; (d), cultured without any factor for 72 h. Photographed at the same magnification. Bar = 100 μm.

to the EGF receptor and autophosphorylated it in microvascular endothelial cells (Figs. 1 and 2). This is consistent with the results of another study of human cancer A431 or smooth muscle cells.[30] HB-EGF, as well as EGF, induced a loss of cell surface EGF receptors within 15 min in microvascular endothelial cells (Fig. 3). The HB-EGF-dependent induction of c-*fos* mRNA was almost completely abrogated under the EGF-induced EGF receptor down-regulation (Fig. 4). A transcriptional factor, p91, which directly interacts with the EGF receptor, mediates activation of the c-*fos* gene promoter through its translocation from the cytosol to the nucleus in response to EGF.[52] This transcriptional factor was activated in response to HB-EGF as well as EGF (Fig. 5), suggesting that HB-EGF induces its signaling through the EGF receptor in common with EGF in microvascular endothelial cells.

HB-EGF induced the formation of tube-like structures of microvascular endothelial cells in type I collagen gel (Fig. 8). HB-EGF was also mitogenic and stimulated cell migration in endothelial cells (S. Ushiro, unpublished data). These microvascular endothelial cells derived from human omental adipose tissue also develop capillary-like networks on Matrigel within 6 h.[26] In this *in vitro* model system, EGF/TGF-α stimulates cell proliferation, t-PA synthesis and the formation of tube-like structures.[19, 21, 23–25] Cell migration and tubular morphogenesis in the presence of EGF/TGF-α are almost completely abrogated when anti-t-PA antibody or aprotinin, a serine inhibitor, is present, suggesting that t-PA is essential for angiogenesis *in vitro*.[21, 23, 24] The notion that the expression of PA is indispensably coupled with the formation of tube-like structures by endothelial cells in type I collagen gels is also supported by the results of our experiments in the co-culture angiogenesis model assay system. We reported that human esophageal cancer cell lines or keratinocytes induce the formation of tube-like structures by human microvascular endothelial cells in a co-culture system.[21, 22] These esophageal cancer cells or keratinocytes express TGF-α, resulting in the promotion

of tube-like structures by endothelial cells in type I collagen gels.[21, 22] We also demonstrated that the co-administration of insulin-like growth factor-1 or hepatocyte growth factor and t-PA induces the formation of tube-like structures of human microvascular endothelial cells, whereas each factor alone does not.[23, 24, 53] HB-EGF also enhanced expression of the t-PA gene in endothelial cells (Fig. 6). The enhanced expression of t-PA mRNA by HB-EGF might again support the close coupling of tubulogenesis and t-PA in the model system. HB-EGF, like EGF, is angiogenic in the model system with human microvascular endothelial cells. Furthermore, human betacellulin, another EGF family member, which also interacts with the EGF receptor,[54, 55] enhances the expression of t-PA mRNA and the formation of tube-like structures in human microvascular endothelial cells (S. Ushiro *et al.*, unpublished data).

HB-EGF, containing up to 86 amino acids, is longer than EGF/TGF-α, which consists of 47–53 amino acids.[30] HB-EGF was also potent in the formation of tube-like structures (Fig. 8). In the microvascular endothelial cell system, HB-EGF interacted with the EGF receptor, autophosphorylated it and induced c-*fos* expression at similar levels to EGF. HB-EGF increased both t-PA and PAI-1 mRNA levels, but their mRNA levels were lower than in EGF-treated microvascular endothelial cells. This activity is a key factor for angiogenesis in the model system, as discussed above. Consistent with our previous study, EGF increased the cellular mRNA levels of both the t-PA and PAI-1 genes. Since the major structural difference between HB-EGF and EGF is the domain binding to heparan sulfate proteoglycan,[29, 30] depletion of the proteoglycan structure associated with endothelial cells should abrogate the differential biological effects of HB-EGF and EGF. HB-EGF increased both t-PA and PAI-1 mRNA to levels similar to those of EGF when vascular endothelial cells were first exposed to heparitinase/chlorate (Fig. 7). These results suggest that the heparan sulfate-binding domain of HB-EGF is closely involved in the poor induction of t-PA and PAI-1 mRNA. HB-EGF at 10 ng/ml induced tubulogenesis of microvascular endothelial cells at a rate comparable to that by EGF at 10 ng/ml (Fig. 8). One could ask why HB-EGF and EGF are equipotent in their tubular morphogenesis activities in the presence of heparitinase/chlorate. The relative rates of t-PA/PAI-1 mRNA levels were similar between EGF- and HB-EGF-treated cells (see Figs. 6 and 7), suggesting similar levels of PA activities. In fact, PA activity of HB-EGF-treated cells was found to be similar to that of EGF-treated cells when determined by fibrin-zymography (S. Ushiro, unpublished data).

In conclusion, HB-EGF, a novel EGF family growth factor, shows potent angiogenic activity in a model system with human microvascular endothelial cells, possibly through the modulation of t-PA/PAI-1 gene expression. HB-EGF appears to be active not only against vascular smooth muscle cells, but also against microvascular endothelial cells.

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