Jpn. J. Cancer Res. 85, 53–62, January 1994

Cooperative Roles of Hepatocyte Growth Factor and Plasminogen Activator in Tubular Morphogenesis by Human Microvascular Endothelial Cells

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Epidermal growth factor (EGF) or transforming growth factor-α (TGF-α) stimulated cell migration, chemotaxis, and the expression of tissue-type plasminogen activator (t-PA) in human omental microvascular endothelial (HOME) cells. Hepatocyte growth factor (HGF) stimulated cell proliferation, but had a negligible stimulatory effect on cell migration, the expression of t-PA and tube-like formation into collagen gel in HOME cells. Basic fibroblast growth factor stimulated cell proliferation, cell migration, tubulogenesis and the expression of urokinase-type plasminogen activator (u-PA) in bovine aortic endothelial (BAE) cells. HOME and BAE cells had both high- and low-affinity receptors for HGF. In BAE cells, u-PA activity and tube-like structures in collagen gel were induced in the presence of HGF alone. In contrast, in HOME cells, t-PA activity and tube-like structures were induced in the presence of TGF-α alone, but not in the presence of HGF alone. However, we observed a marked induction of tube formation by HOME cells when both t-PA and HGF were added simultaneously. In the model system for tumor angiogenesis, when HOME cells were co-cultured with a renal cancer cell line, KPK13, tube-like structures were induced in the presence of HGF: KPK13 cells expressed large amounts of t-PA mRNA. Our present study suggested that HGF in concert with active t-PA could be angiogenic in HOME cells.

Key words: Hepatocyte growth factor — Plasminogen activator — Angiogenesis

aFGF,[5] bFGF, EGF and TGF-α are angiogenic growth factors,[1-5] but their underlying mechanisms of action remain unknown. In our laboratory, we have established a model system of *in vitro* angiogenesis with HOME cells. In this system, EGF or TGF-α is the most potent growth factor in terms of stimulating chemotaxis, cell migration, synthesis of t-PA, and the formation of tube-like structures in type I collagen gel.[6-11] The expression of the t-PA gene appears to be indispensably coupled with the EGF-induced angiogenesis of HOME cells in culture. On the other hand, bFGF stimulated cell proliferation, cell migration and the expression of u-PA and induced the formation of tube-like structures by BAE cells.[12-14]

HGF stimulates the proliferation of hepatocytes, melanocytes, and other epithelial cell lines.[15-21] The *met* proto-oncogene has recently been identified as the receptor for HGF/scatter factor.[22-24] HGF is thought to be involved in cell differentiation and malignant transformation[25,26] and it has been shown to induce the expression of u-PA and its receptor and the formation of epithelial tubules by Madin-Darby canine kidney (MDCK) cells in collagen gel.[27-29] In contrast, the biological effects of HGF on vascular endothlial cells are controversial.[30,31] Rosen *et al.*[32] have reported that HGF stimulates both the migration and the formation of tube-like structures by bovine brain capillary and bovine pulmonary artery endothelial cells. Bussolino *et al.*[33] have reported that HGF stimulates the proliferation and the migration of human umbilical vein endothelial cells and induces angiogenesis in rabbit cornea.

On the other hand, we have reported that HGF stimulates cell proliferation and chemotaxis, but does not significantly stimulate t-PA gene expression and the migration of HOME cells.[11,34] One can ask why HGF itself cannot induce cell migration in HOME cells, an initial step for angiogenesis. If the expression of the t-PA gene is a prerequisite for angiogenesis in HOME cells, it is possible that the exogenous addition of t-PA to the culture medium might support migration and tubulogenesis in the presence of HGF. We investigated whether tube formation by HOME cells was induced in a model system for tumor angiogenesis[9] when they were co-

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[5] Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; TGF-α, transforming growth factor-α; HGF, hepatocyte growth factor; HOME, human omental microvascular endothelial; BAE, bovine aortic endothelial; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; M-199, medium 199; DMEM, Dulbecco's modified essential medium; FBS, fetal bovine serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

cultured with t-PA-producing human tumor cells in the presence of HGF. We also investigated whether HGF could induce expression of u-PA as well as tube formation in BAE cells.

MATERIALS AND METHODS

HOME cells and other cell lines HOME cells were isolated from human omental tissues and cultured in M-199 supplemented with 10% FBS, 100 units/ml penicillin, and 60 μg/ml kanamycin as previously reported.[6-10, 34, 35] Since HOME cells decreased their responsiveness to growth factors such as EGF during serial cultivation,[8] we used cells at 3 to 5 passages. BAE cells were isolated from bovine aorta and cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 60 μg/ml kanamycin.[36] Two human renal cancer cell lines, KPK1 and KPK13, which were independently isolated from renal cancer patients[37, 38] were used in this study.

Materials Wild-type t-PA and recombinant human HGF were donated by K. Higashio and N. Shima (Snow Brand Milk Products Company, Tochigi).[39] HGF employed in this study has a deletion of five amino acids in the first kringle domain.[39] Human EGF receptor cDNA was also used in this assay.[40, 41] TGF-α was purchased from Collaborative Research Inc., Bedford, MA; aprotinin and FOY were from Sigma, St. Louis, MO. The two deletion mutants of t-PA, 820 (t-PA^{E}) and 2600 (t-PA^{FE}), were kindly donated by Tsukuba Research Laboratories, Eisai Co., Tsukuba; 820 (t-PA^{E}) contains a deletion mutation of the EGF homologous domain and 2600 (t-PA^{FE}) contains a deletion mutation of both finger and EGF homologous domains.[42] Human t-PA cDNA was obtained from W-D. Schleuning (Schering Aktiengesellshaft Pharma Forschung, Berlin, Germany)[43]; plasminogen activator inhibitor-1 (PAI-1) cDNA was from D. J. Loskutoff (Research Institute of Scripps Clinic, La Jolla, CA)[44]; and proto-oncogene c-*met* cDNA was from G. Vande Woude (NCI-FCRDC, Maryland).[45] Anti-t-PA antibody was purchased from Oncogene Science, New York.

Iodination of HGF Iodination of HGF was performed by the chloramine T method as described previously.[40] K-phosphate buffer, pH 7.5 (25 μl) and Na-^{125}I (1 mCi) were added to 5 μg of recombinant HGF. The reaction was started by adding 5 μl of chloramine-T solution (100 μg/ml), four times at 30-s intervals. The reaction was halted by adding 100 μl of L-tyrosine (1 mg/ml) and 15 μl of $Na_2S_2O_5$ (10 mg/ml) solution, then ^{125}I-HGF was separated from free iodine on a heparin-Sepharose column. The column was washed with 10 ml of PBS and ^{125}I-HGF was eluted with 5 ml of 2 *M* NaCl. The specific activity of ^{125}I-HGF was 208 cpm/fmol.

^{125}I-HGF binding assay Confluent monolayers of HOME cells or BAE cells were washed twice with PBS, and a binding buffer, containing 20 m*M* Hepes, 0.2% BSA, and various concentrations of ^{125}I-HGF, with or without an excess of unlabeled HGF, was added. After incubation for 2 h at 4°C, the binding buffer was aspirated. The cells in monolayers were washed three times with PBS and ^{125}I-HGF binding to heparin-like molecules of cells surface was excluded by washing with PBS containing 0.1 mg/ml of heparin. After detachment of the monolayer with 1 *N* NaOH, the radioactivity of bound ^{125}I-HGF was measured in a γ-counter.

Tyrosine-specific phosphorylation of EGF receptor Confluent HOME cell cultures were treated with or without 100 ng/ml TGF-α or 1 μg/ml HGF for 10 min, washed three times with ice-cold PBS, and solubilized with a lysis buffer containing 0.5% Triton X-100. The lysate was immunoprecipitated with anti-EGF receptor antibody (Oncogene Science, Inc.), subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and transferred electrophoretically to a nitrocellulose filter. The filter was incubated with anti-phosphotyrosine antibody (PY20, ICN) and detection was performed with the enhanced chemiluminescence (ECL) Western blotting method using ECL Western blotting detecting reagents by autoradiography on X-ray film for about 30 min at room temperature, as described previously.[10, 41]

Northern blot analysis Northern blot analysis of HOME cells was performed as described previously.[7, 46] Briefly, HOME cells were incubated with HGF or TGF-α in M-199 containing 1% FBS. Total RNA was isolated, and fractionated on 1% agarose containing 2.2% formaldehyde. The filter-bound RNA was hybridized to ^{32}P-labeled probes and autoradiographed.

Zymographic assay Cellular PA activity was assayed with membrane fractions. The membrane fraction was prepared as previously described.[47] Briefly, HOME cells and BAE cells growing in 100-mm dishes were harvested with a rubber scraper and pelleted by centrifugation. Cell pellets were then homogenized in 2 ml of 50 m*M* NaCl, 10 m*M* Tris-HCl (pH 7.4), and the homogenate was centrifuged at 1500*g* for 10 min. After centrifugation, the supernatant was recentrifuged at 100,000*g* for 45 min and the pellet corresponding to the membrane fraction was resuspended in buffer containing 2% SDS, 10% glycerol and 0.125 m*M* Tris-HCl (pH 6.8). After the samples were heated at 60°C for 20 min, SDS-PAGE was performed and the gel was soaked in 2.5% Triton X-100 to remove SDS for 2 h. To test the PA activity of both t-PA and u-PA, the polyacrylamide gel was placed in contact with fibrin detector gel containing fibrinogen, plasminogen and thrombin, and incubated at 37°C under humid conditions.

Tube formation by HOME cells or BAE cells in type I collagen gel and quantitative analysis Eight volumes of type I collagen solution (Nitta Gelatin, Osaka), one volume of 10×M-199 or 10×DMEM, and one volume of 0.05 *N* NaOH, 200 m*M* Hepes, and 260 m*M* $NaHCO_3$ were mixed on ice, poured into 35 mm plastic dishes (Corning, NY), and allowed to gel at 37°C. HOME cells or BAE cells were plated onto the surface of type I collagen gel in M-199 or DMEM containing 10% FBS. When HOME cells or BAE cells became confluent on type I collagen gel, the medium was replaced with M-199 or DMEM containing 1% FBS, various factors were added and the plate was incubated for 3 days.[6, 9, 10, 36] On the third day, phase contrast microscopic pictures of each dish were recorded on a still video camera recorder (R5000H; Fuji, Tokyo), and the total length of tube-like structures per field was measured by using a Cosmozone IS image analyzer (Nikon, Tokyo) as described previously.[9, 10]

Tube formation assay with co-culture system We constructed a tube formation assay system for endothelial cells in type I collagen gel of HOME cells co-cultured with tumor cells or keratinocytes.[9, 10, 36] Human tumor cells were cultured on outer chamber 6-well plates [each well being 38×7 mm (Corning)] in 2 ml of M-199 containing 10% FBS. At confluence, the medium was changed for 2 ml M-199 containing 1% FBS. Separately, HOME cells were seeded in the inner chamber (30×7 mm) in 2 ml of M-199 containing 10% FBS on 1 ml of type I collagen gel in culture plates equipped with 0.4 μm Millipore filters (Millicell-CM, Millipore Products Division, Bedford, MA). When endothelial cells were at confluence, the serum concentration was changed from 10% FBS to 1% FBS and the culture in the inner chamber was transferred to the outer chamber.[9, 10] On the third day, quantitative analysis of the total length of tube-like structures was performed with an image analyzer.[9, 10, 36]

RESULTS

We have previously reported that the proliferatoin of HOME cells was stimulated in response to HGF.[34] Scatchard analysis from the saturation kinetics of ^{125}I-HGF binding to the HOME cells was performed (Fig. 1A). HOME cells showed high-affinity ($K_d = 3.1 \times 10^{-12}$ *M*) and low-affinity ($K_d = 1.5 \times 10^{-9}$ *M*) binding sites for HGF. The receptor numbers were estimated to be 500 sites/cell and 4000 sites/cell, respectively. HGF/scatter factor receptor is the proto-oncogene c-met encoded receptor.[21, 22] One subunit (β-subunit) of the HGF receptor contains the tyrosine kinase domain.[48, 49] On the other hand, a recent study has demonstrated that HGF itself stimulates cell migration, proliferation and tubulogenesis

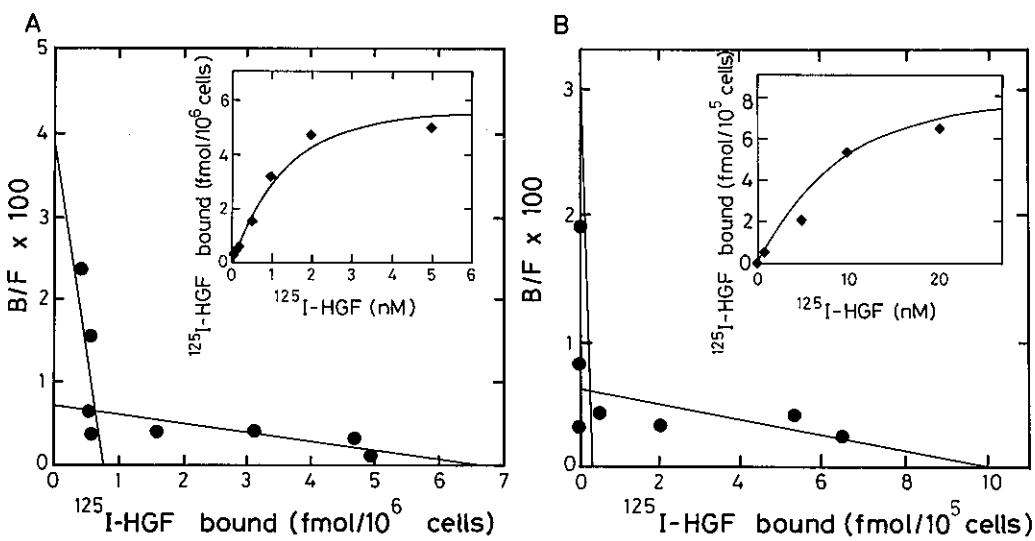

Fig. 1. Saturation kinetics and Scatchard analysis of ^{125}I-HGF binding to HOME cells and BAE cells. ^{125}I-HGF binding at 4°C on the cell surface was measured; the results are expressed as specific binding after subtraction of nonspecific binding. (A) In HOME cells, both high- and low-affinity binding sites are observed, at 500 sites/cell ($K_d = 30.5 \times 10^{-12}$ *M*) and 4000 sites/cell ($K_d = 1.5 \times 10^{-9}$ *M*), respectively. (B) In BAE cells, both high- and low-affinity binding sites are observed, at 2200 sites/cell ($K_d = 95 \times 10^{-12}$ *M*) and 6×10^4 sites/cell ($K_d = 12 \times 10^{-9}$ *M*), respectively. The inset shows the saturation curve of ^{125}I-HGF binding to its receptor on HOME cells or BAE cells. Each point shows the amount of ^{125}I-HGF specifically bound to the cells at the indicated concentration of added ^{125}I-HGF.

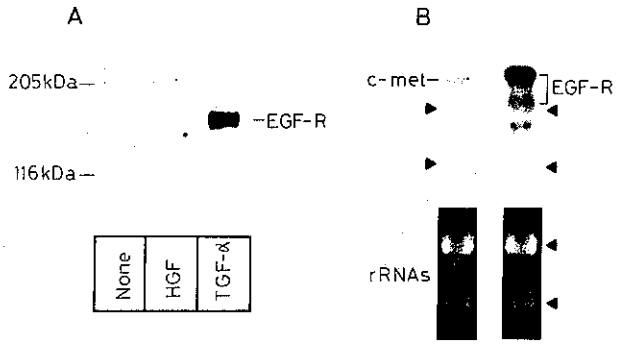

Fig. 2. Tyrosine-specific phosphorylation of EGF receptor (A) and expression of *met* and EGF receptor mRNA in HOME cells (B). In (A), HOME cells were treated with or without 100 ng/ml TGF-α and 1 μg/ml HGF. The immunoprecipitate of each lysate by anti-EGF receptor antibody was analyzed by immunoblotting with anti-phosphotyrosine antibody. An EGF receptor of 170 kDa molecular weight is shown. Molecular weight is shown by electrophoresis of standard marker protein. In (B), confluent HOME cells were harvested and total RNA was extracted. Fifteen μg of total RNA was fractionated on 1% agarose gel and transferred to a Nytran filter. Northern blot hybridization was performed with ^{32}P-labeled *met* cDNA and EGF receptor cDNA. Ribosomal RNAs (rRNAs) loaded on the gel are shown after staining with ethidium bromide: arrows indicate 28s and 18s rRNA.

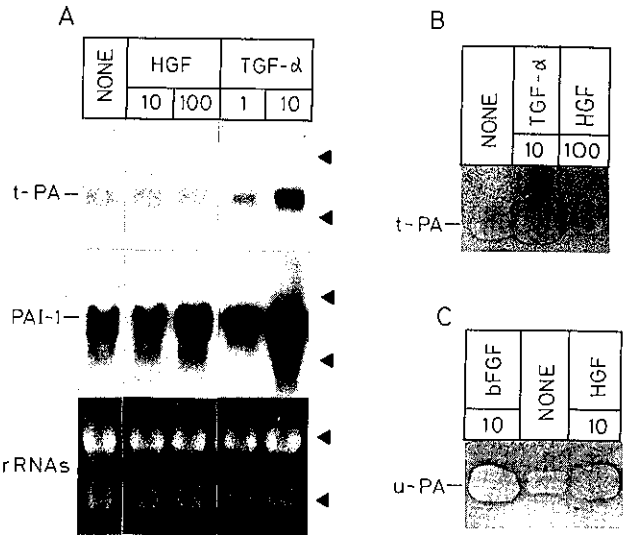

Fig. 3. Induction of t-PA and PAI-1 mRNA in HOME cells by HGF or TGF-α (A) and induction of PA activity in HOME cells by TGF-α (B) and in BAE cells by bFGF or HGF (C). In (A), confluent HOME cells were incubated with 10 or 100 ng/ml of HGF or 1 or 10 ng/ml of TGF-α for 12 h and harvested. After total RNA extraction, 15 μg of total RNA was fractionated on 1% agarose gel and transferred to a Nytran filter. Northern blot hybridization was performed with ^{32}P-labeled t-PA cDNA and PAI-1 cDNA. Ribosomal RNAs (rRNAs) loaded on the gel are shown after staining with ethidium bromide: arrows indicate 28s and 18s rRNA. In (B), confluent HOME cells were incubated with 10 ng/ml of TGF-α or 100 ng/ml of HGF for 24 h and harvested. After the membrane fractions were prepared and SDS-PAGE was performed, the gel was placed in contact with fibrin detector gel. In (C), confluent BAE cells were incubated with 10 ng/ml of bFGF or 10 ng/ml of HGF for 24 h and harvested. Detection of PA activity was the same as above.

of bovine endothelial cells[32] and human umbilical endothelial cells.[33] We performed Scatchard analysis of the saturation kinetics of ^{125}I-HGF binding to the BAE cells (Fig. 1B). BAE cells showed high-affinity ($K_d = 95 \times 10^{-12}$ *M*) and low-affinity ($K_d = 12 \times 10^{-9}$ *M*) binding sites for HGF. The receptor numbers were 2200 sites/cell and 6×10^4 sites/cell, respectively.

EGF or TGF-α stimulates cell migration, chemotaxis, and formation of tube-like structures in collagen gel.[6-10, 34] The EGF receptor with the tyrosine kinase domain of HOME cells is also phosphorylated in response to EGF or TGF-α.[10] The EGF receptor of 170 kDa was specifically phosphorylated in the presence of TGF-α, but not HGF (Fig. 2A). We were not able to detect HGF receptor by HGF receptor antibody or ^{125}I-HGF cross linking assay (unpublished data), possibly due to the very low receptor numbers in HOME cells. The expression of 9 kb c-*met* mRNA was observed in HOME cells (Fig. 2B). We also observed the expression of EGF receptor mRNA of two different sizes, 10.5 and 5.8 kb (Fig 2B). The band of 4.0 kb was also observed, but this band was not found reproducibly.

Our previous study demonstrated that the expression of the t-PA gene was closely correlated with EGF- or TGF-α-induced cell migration and with tubulogenesis in HOME cells, an initial step for angiogenesis.[7, 8, 34] HGF had a stimulatory effect on the proliferation of HOME cells,[34] but it did not enhance the expression of the t-PA gene or tubulogenesis in collagen gel. In contrast, EGF/TGF-α stimulated both expression of the t-PA gene and tubulogenesis of HOME cells.[7-10] In accordance with a previous study, exogenous addition of TGF-α enhanced the expression of t-PA mRNA in HOME cells, while HGF did not enhance the expression of t-PA gene (Fig. 3A). TGF-α induced 5-fold higher expression of PAI-1 mRNA in HOME cells, but HGF had only a slight effect (about 1.5-fold) (Fig. 3A). Zymographic assay showed that plasminogen-dependent fibrinolytic plaque (t-PA activity) was induced by TGF-α in HOME cells, but not by HGF (Fig. 3B). We also examined whether PA activity was enhanced in response to bFGF or HGF in BAE cells.

Bovine u-PA probe was not available, and we employed zymographic assay. As can be seen in Fig. 3C, BAE cells have u-PA activity as the main PA activity, and cellular u-PA activity was apparently increased by bFGF or HGF.

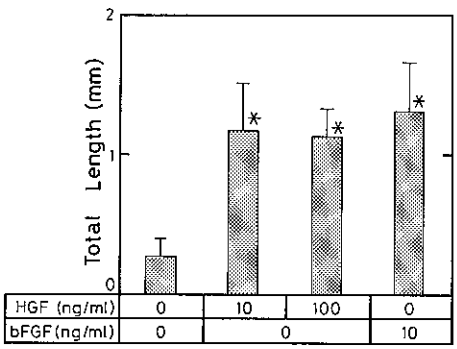

Fig. 4. Development of tube-like structures by BAE cells in type I collagen gel. BAE cells were plated onto collagen gel and incubated in medium containing 10% FBS. When BAE cells reached near-confluence and showed a cobblestone-like appearance, the medium was replaced by medium containing 1% FBS with or without the indicated factors. After three days of incubation, phase-contrast microscopic pictures were recorded with a still video camera recorder and the total length of the tube-like structures was determined with an image analyzer. Eight random fields per dish were measured and the total length per field was calculated. Columns represent means±SD (bars) of triplicate experiments. (*); significantly different from the value for control in the absence of bFGF and HGF ($P<0.01$).

We then examined the effect of HGF on tubulogenesis of BAE cells and also HOME cells in type I collagen gel. BAE cells efficiently form tube-like structures in type I collagen gel when bFGF is present. HOME cells also migrate into the type I collagen gel and form numerous tube-like structures there in the presence of EGF or TGF-α.[9,10] BAE cell proliferation and cell migration were stimulated by both bFGF and HGF (data not shown). We have recently reported that BAE cells form tube-like structures in the presence of bFGF or bFGF producing human glioma cells in type I collagen gel.[36] We examined the effect of HGF on the tubulogenesis by BAE cells in type I collagen gel. BAE cells formed a monolayer of cobblestone-like appearance on its surface in the absence of any growth factor, but formed tube-like structures in the type I collagen gel in the presence of bFGF. BAE cells migrated into the type I collagen gel and formed numerous tube-like structures there in the presence of 10 and 100 ng/ml HGF. HGF at 10 ng/ml induced tubu-like structures in type I collagen gel at similar levels to bFGF at 10 ng/ml (Fig. 4). Thus, HGF alone could induce tubulogenesis of BAE cells.

HOME cells showed a typical cobblestone-like appearance on the surface of type I collagen gel in the absence of any growth factor (Fig. 5A). TGF-α induced numerous tube-like structures in type I collagen gel (Fig. 5B), but HGF alone had no stimulatory effect on tubulogenesis of HOME cells. Coadministration of t-PA and HGF, however, stimulated formation of tubu-like structures (Fig. 5C). Quantitative analysis of the tube-like structures was performed (Fig. 6). As can be seen in Fig. 6, HGF alone did not induce tube-like structures,

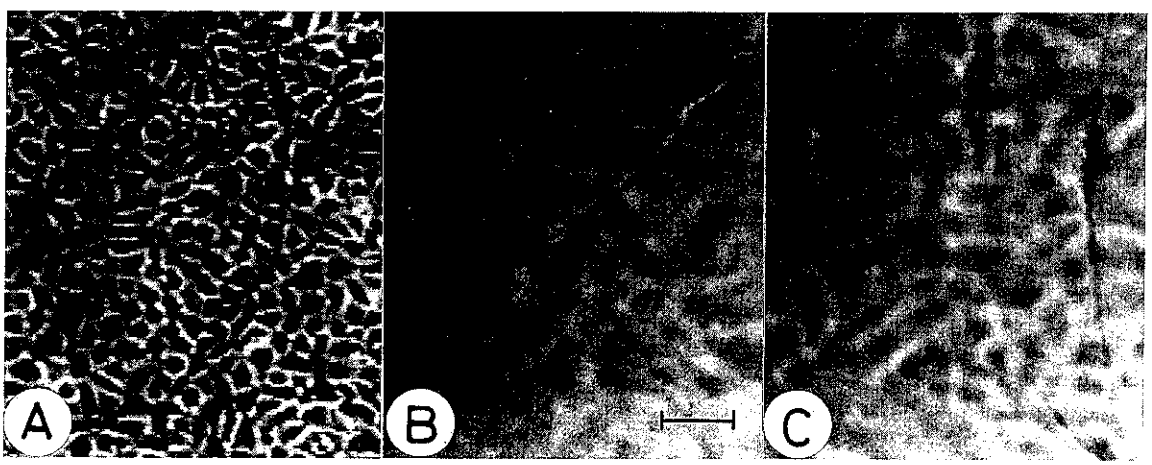

Fig. 5. Development of tube-like structures by HOME cells in type I collagen gel. HOME cells showed a monolayer of cobblestone-like appearance on type I collagen gel (A). TGF-α (10 ng/ml) induced numerous tube-like structures into gel (B). HGF (100 ng/ml) alone did not induce tube-like structures so much, but coadministration of HGF (100 ng/ml) and t-PA (100 U/ml) induced numerous tube-like structures comparable to those seen with TGF-α (C). Bar=100 μm.

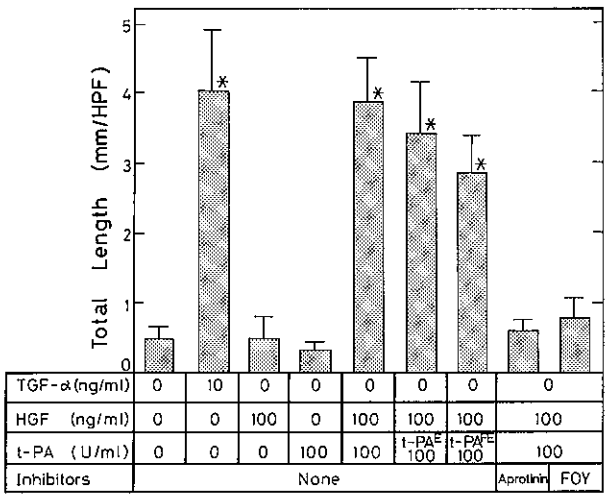

Fig. 6. Development of tube-like structures by HOME cells in type I collagen gel. HOME cells were plated onto collagen gel and incubated in medium containing 10 % FBS. When HOME cells reached near-confluence and showed a cobblestone-like appearance, the medium was replaced by medium containing 1% FBS with or without the indicated factors and protease inhibitors. After three days of incubation, phase-contrast microscopic pictures were recorded with a still video camera recorder and the total length of the tube-like structures was determined with an image analyzer. Eight random fields per dish were measured and the total length per field was calculated. Columns represent means±SD (bars) of triplicate experiments. (*); significantly different from the value for control in the absence of TGF-α, HGF, t-PA and inhibitors ($P<0.01$).

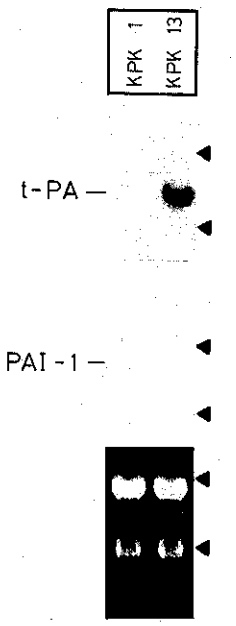

Fig. 7. Expression of t-PA and PAI-1 mRNA in kidney cancer KPK1 and KPK13 cells. Confluent renal cancer cells were harvested and total RNA was extracted. Fifteen μg of total RNA was fractionated on 1% agarose gel and transferred to a Nytran filter. Northern blot hybridization was performed with ^{32}P-labeled t-PA cDNA, and PAI-1 cDNA. Ribosomal RNAs (rRNAs) loaded on the gel are shown after staining with ethidium bromide: arrows indicate 28s and 18s rRNA.

but co-administration of HGF and t-PA stimulated formation of tube-like structures at similar rates to that of TGF-α alone (Fig. 6), suggesting an indispensable role of t-PA in the tubulogenesis by HOME cells. To examine the role of t-PA in the formation of the tube-like structures by HOME cells, we used two deletion mutants of t-PA^{E} and t-PA^{FE}: t-PA^{E} with a deleted EGF homologous domain and t-PA^{FE} with both deleted finger and EGF homologous domains of PA after side mutagenesis.[42] Zymographic assay showed that these two deletion mutants had almost the same specific PA activity as wild-type t-PA, and also that their PA activity was almost completely inhibited in the presence of two serine protease inhibitors, aprotinin and FOY (data not shown). The exogenous addition of t-PA, t-PA^{E} and t-PA^{FE} at 100 U/ml alone had no effect on the formation of tube-like structures by HOME cells in type I collagen gel (Fig. 6 and unpublished data). Coadministration of 100 U/ml of t-PA and HGF at 100 ng/ml was found to induce formation of tube-like structures. When 100 U/ml of t-PA^{E} or t-PA^{FE} was added simultaneously with HGF at 100 ng/ml, we also found significant induction of the formation of tube-like structures in the gel, and the effect was comparable to that of wild-type t-PA (Fig. 6). The two serine protease inhibitors almost completely inhibited the induction of tubulogenesis in HOME cells brought about by t-PA and HGF. The t-PA activity appeared to be essential for the tubulogenesis of HOME cells in type I collagen gel.

We have recently established an *in vitro* co-culture system for angiogenesis.[9,10] In this assay system, HOME cells or other cells on the surface of the collagen gel in the inner chamber are expected to undergo tubulogenesis when tumor cells or other cells cultured in the outer chamber secrete any potent angiogenic factor. We examined whether human renal cancer cell lines, KPK1 and KPK13, could induce formation of tube-like structures of HOME cells in type I collagen gel. KPK13 cells expressed much higher levels of t-PA mRNA than KPK1 cells, whereas neither cell line showed significant expression of PAI-1 mRNA (Fig. 7) or TGF-α and HGF mRNA (data not shown). The zymographic assay with cell extracts of KPK13 also showed high t-PA activity,

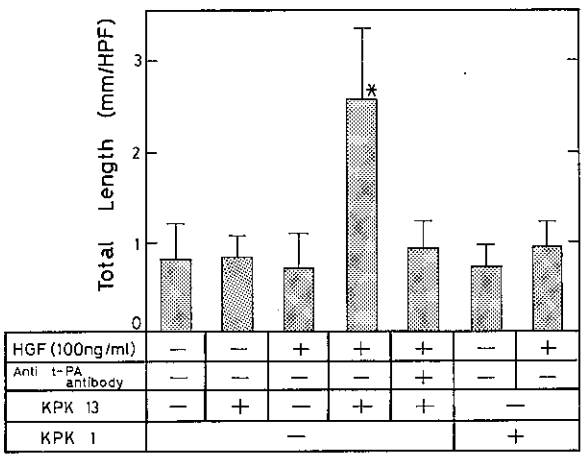

Fig. 8. Development of tube-like structures by HOME cells co-cultured with human renal cancer cells. Tubulogenesis by HOME cells was examined when they were co-cultured with or without KPK1 or KPK13 cells in the absence or presence of HGF. Antibody against t-PA was also incubated during the assay with co-cultured KPK13 cells in the presence of HGF. On the third day after the co-culture, quantitative analysis was performed with a still video camera recorder and image analyzer. Columns are means±SD (bars) of triplicate experiments. (*); significantly different from the value for control in the absence of HGF, anti-t-PA antibody and co-cultured cells ($P<0.01$).

consistent with the level of t-PA mRNA, but only negligible u-PA activity was observed (unpublished data). Tube formation by HOME cells was not significantly induced when they were co-cultured with KPK1 or KPK13 cells alone (Fig. 8). However, KPK13 cells induced tube formation by HOME cells at much higher rates than KPK1 cells when HGF was present. KPK13-induced tube formation was almost completely blocked by anti-t-PA antibody (Fig. 8), but not by anti-TGF-α antibody or anti-u-PA antibody (data not shown).

DISCUSSION

bFGF shows potent angiogenic activity,[5] and it stimulates cell migration and protease synthesis by bovine endothelial cells, as well as human umbilical endothelial cells.[4, 50] Bovine endothelial cells produce bFGF, and this endogenous bFGF stimulates u-PA synthesis, cell migration, and tubulogenesis in bovine endothelial cells in type I collagen gel,[13, 36] while the invasion activity of endothelial cells is blocked by serine protease inhibitors or anti-PA antibody.[51] The expression of PA in response to bFGF is a prerequisite for the *in vitro* angiogenesis of bovine endothelial cells and human umbilical endothelial cells.

In HOME cells, EGF/TGF-α most efficiently stimulates cell migration, t-PA synthesis and tube formation.[6, 9, 10] Cell migration and tubular morphogenesis of HOME cells in the presence of EGF/TGF-α are almost completely abrogated when anti-t-PA antibody is present.[7, 9, 10] Coadministration of aprotinin, a serine protease inhibitor, inhibits the EGF/TGF-α-dependent tubulogenesis of HOME cells in collagen gel.[11] Addition of plasmin, instead of t-PA, could also induce formation of tube-like structures when HGF was present (unpublished data). The idea that the expression of PA is indispensably coupled with tube formation by HOME cells in type I collagen gel is also supported by our experiments in the co-culture assay system. We have previously reported that a human esophageal cancer cell line or human keratinocyte line induces tubulogenesis by HOME cells in the co-culture assay system: these esophageal cancer cells or keratinocytes express TGF-α.[9, 10] In this system, HOME cells are expected to show enhanced expression of t-PA in response to TGF-α.[9, 10] In a recent study, we also demonstrated that coadministration of insulin-like growth factor-1 (IGF-1) and t-PA induces tubulogenesis of HOME cells even though each factor alone does not[11]: IGF-1 cannot induce expression of t-PA gene in HOME cells. In our present study, we determined whether t-PA and t-PA mutants affected *in vitro* angiogenesis in HOME cells. Our present findings further support the idea of indispensability of PA activity in the angiogenesis in HOME cells.

Tubulogenesis of Madin-Darby canine kidney (MDCK) epithelial cells appears in collagen or fibrin gel when HGF/scatter factor is present.[28] HGF increases both u-PA and its receptor in MDCK cells.[29] This study by Montesano and his colleagues suggests a close coupling of expression of PA and tubulogenesis of MDCK cells. HGF induces tubulogenesis of endothelial cells derived from bovine brain capillary and bovine pulmonary artery.[32] Bussolino *et al.*[33] have reported that HGF induces tube-like structures of human umbilical endothelial cells and also neovascularization in rabbit cornea. Our present study demonstrates that HGF induces tubulogenesis of BAE cells with a concomitant increase of u-PA activity (Figs. 3 and 4). These results suggest that HGF might be a potent angiogenic factor against bovine endothelial cells, human umbilical endothelial cells and rabbit cornea. EGF or TGF-α lacked potent angiogenic activity against human umbilical endothelial cells which were highly susceptible to bFGF (unpublished data). In contrast, HGF alone cannot induce cell migration and tube formation of HOME cells.[11, 34] In our present study, PAI-1 mRNA expression was only slightly enhanced by HGF, but t-PA mRNA expression was not (Fig. 3A). Our previous study and the present one demonstrated that EGF/TGF-α stimulated expres-

sion of both t-PA and its inhibitor, PAI-1 (Fig.3A).[7, 33] Zymographic assay showed apparent enhancement of the t-PA activity in TGF-α-treated HOME cells (Fig. 3B). The inhibitory activity of PAI-1 might not be strong enough to inhibit the PA activity when HOME cells are treated with EGF/THG-α. However, further study is required to understand the precise role of PAI-1 in the tubulogenesis of HOME cells. The failure of HGF alone to induce tubular morphogenesis of HOME cells might be due to the loss of cellular induction of t-PA in response to HGF. Our present study indicates that coadministration of t-PA with HGF can induce tubular morphogenesis of HOME cells. We also observed that co-cultured KPK13 cells induce tubulogenesis by HOME cells at a higher rate than KPK-1 cells when HGF is present. This KPK13 cell-induced tube formation is almost completely blocked by co-administration of anti-t-PA antibody. This co-culture assay with KPK13 cells demonstrates that HGF is angiogenic only when HGF is in close contact with PA-producing cells. Our present study further supports the hypothesis that t-PA activity is required for tubular morphogenesis of human microvascular endothelial cells.

Two t-PA mutants, with a deleted finger domain and deleted EGF homologous domain, support tubulogenesis by HOME cells in the presence of HGF. These two t-PA mutants have levels of PA activity similar to that of wild type t-PA. While EGF/TGF-α is a potent tubulogenic factor for HOME cells,[6, 9, 10] the indispensable role of t-PA in tube formation by these cells does not appear to be due to the EGF homologous domain of t-PA. In contrast, two protease inhibitors, aprotinin and FOY, blocked t-PA-dependent action in HOME cells. Although HGF itself cannot induce t-PA synthesis, HGF can induce tubulogenesis in the presence of PA activity. The PA activity, which alone has no tubulogenic effect, appears to be a prerequisite for cell migration and tubular morphogenesis of HOME cells. The enhancement of cell motility by PA might be required as an initial key step for tubulogenesis. Further study is needed to determine how PA activity modulates cell migration and tubulogenesis in HOME cells in relation to the extracellular matrix and cell-cell contact.

ACKNOWLEDGMENTS

We thank Yasufumi Sato, Kazuki Okamura, Tatsuya Abe and Hiroto Izumi in our laboratory for fruitful discussions, and Ikuko Takemitsu for editorial help.

(Received July 22, 1993/Accepted September 29, 1993)

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