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Communication

Molecules and Cells

Soluble Epithin/PRSS14 Secreted from Cancer Cells Contains Active Angiogenic Potential

Sang Bum Kim[1,4], Deokjae Lee, Joo-Won Jeong[2], Chungho Kim[3], Dongeun Park[1], and Moon Gyo Kim*

Epithin (PRSS14/matriptase/ST14), a type II membrane protein, is involved in progression of epithelial cancers and metastasis as well as in the normal epidermal barrier function. When activated, it translocates into the cell-cell contacts and sheds into media. In order to understand the specific mechanism during tumor progression, we tested the angiogenic potential of secreted form of epithin. Epithin produced from the cancer cells shed more in hypoxia and induced motility of endothelial cells. Epithin enhanced the migration and invasion of mouse and bovine endothelial cells without cell proliferation. Furthermore, soluble epithin induced endothelial differentiation in the assay of the human endothelial microvessel-like tube formation and in that of the chicken chorioallantoic membrane. The knock-down of epithin in the 427 thymoma cell line abolished the protease activity of secreted epithin fraction, reduced the invasion of endothelial cells through matrigel, and tube formation activity. Only specific antibodies abolished the migration of endothelial cell and the vessel morphogenesis, suggesting that epithin specifically functions in these systems. Therefore, we propose that the secreted epithin in the hypoxic cancer microenvironment plays a role as a proangiogenic factor, and can be modulated with specific antibodies.

INTRODUCTION

Among the molecules up-regulated during tumor progression, proteases are especially important. Many proteases are involved in the various stages of cancer, such as initiation, progression, and metastasis. They play roles in cell migration by degrading the extracellular matrix, invasion into basement membrane, and angiogenesis (Affara et al., 2009; Mohamed and Sloane, 2006; Park et al., 1999). Angiogenesis is critical for fast growing tumor cells to overcome lack of oxygen and can be induced by angiogenic factors, such as VEGF, HGF and FGF(Ferrara and Kerbel, 2005; Ha and Jin, 2009). In addition, many proteases including matrix metalloproteases (MMPs), ADAM and ADAMTS families, as well as cysteine and serine proteases, have been implicated in extracellular matrix (ECM) remodeling and signal transduction for endothelial cells to migrate, invade, proliferate, and differentiate in order to form new vessels (Roy et al., 2006; Seiki and Yana, 2003).

The TTSPs perform complex functions at the plasma membrane and within the extracellular matrixes (Netzel-Arnett et al., 2003; Wu, 2003). Epithin (Kim et al., 1999), also known as matriptase (Lin et al., 1999), MT-SP1 (Takeuchi et al., 1999), and ST14 (Basel-Vanagaite et al., 2007) is a serine protease (*Prss14*) (Kim et al., 1999) that belongs to a subfamily of the TTSP. It has a short intracellular and a long extracellular region containing serine protease domain in addition to SEA, CUB, and LDLR domains. The extracellular portion is first cleaved in the intracellular compartment and then stays associated on the membrane until it is shed (Cho et al., 2005; Lin et al., 2008; List et al., 2006). Upon activation with a tumor promoting agent like PMA, it is shed from the membrane in a filamin dependent manner (Kim et al., 2005). Its dysregulated expression has been widely documented during the progression of various epithelial cancers (Jin et al., 2006; Saleem et al., 2006; Uhland, 2006). Furthermore, epithin interacts and/or activates many well known tumor associated proteins such as protease activated receptor-2 (PAR-2), hepatocyte growth factor (HGF) and pro-urokinase plasminogen activator (pro-uPA) (Lee et al., 2000; Takeuchi et al., 2000) while it remains inactivated by endogenous inhibitor HAI (Oberst et al., 2002; Szabo et al., 2009). When epithin is ectopically overexpressed using Keratin 5 promoter, mice developed spontaneous skin carcinoma (List et al., 2005). Furthermore, epithin overexpressing cancer cells grew faster, produced much larger solid tumors with more vascularization (Jin et al., 2006). These results suggest that epithin can affect vascular morphogenesis in the tumor microenvironment.

To elucidate the role of epithin in the tumor angiogenesis, we applied the soluble epithin protein recovered from various cell types into angiogenic assays *in vitro* and *in vivo*. Our results show that epithin significantly increases the migration, invasion, and tube formation capacities of endothelial cells as well as the change of vessel morphogenesis.

Department of Biological Sciences, Inha University, Incheon 402-751, Korea, [1]School of Biological Sciences, Seoul National University, Seoul 151-747, Korea, [2]Department of Anatomy and Neurobiology, Biomedical Science Institute, School of Medicine, Kyung Hee University, Seoul 130-701, Korea, [3]Department of Medicine, University of California, San Diego, La Jolla, California 92093, USA, [4]Present address: Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA
*Correspondence: mgkim@inha.ac.kr

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MATERIALS AND METHODS

Cell culture

All the reagents were purchased from Invitrogen. Cancer cell lines (427.1.86, 4T1, B16, TC-1) and Bovine aortic endothelial cell (BAEC) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal Bovine Serum (FBS). Mouse endothelial cell MS1 was maintained in DMEM with 5% FBS and Human umbilical vein endothelial cell (HUVEC) in M199 with 20% FBS in the presence of 3 ng/ml bFGF and 5 unit/ml heparin, and NIH3T3 cells in DMEM with 10% calf serum. To obtain the epithin protein, culture medium of cancer cells (427.1.86, 4T1, B16, and TC-1) or NIH3T3 cells transfected with pcDNA3/epithin were grown until confluent. Collected media were clarified by centrifugation and kept at 4°C until used in wound healing migration assays. In other experiments, media of confluent cultures were changed with 5 ml of Tris Buffered Saline (TBS, 10 mM Tris-HCl pH7.5, 150 mM NaCl). After 1 h incubation, TBS was collected and filtered. Conditioned media were concentrated (10X) using Centricon (YM-30, MILLIPORE). For the hypoxic culture, cells were incubated with 1% O_2 using hypoxic chamber (Forma). Epithin knock-down (KD) cells were generated using the pSUPER-st14_2 construct containing two synthetic oligonucleotides (5′-GATCCCC**GGTGCGCTTCAAACTCTTC**TTCAAGAGA**GAAGAGTTTGAAGCGCACC**TTTTTA-3′, reverse strand: 5′-TCGATAAAAA**GGTGCGCTTCAAACTCTTC**TCTCTTGAA**GAAGAGTTTGAAGCGCACC**GGG-3′, bold: epithin targeting sequence). One line (EpiKD17) consistently showed lower expression of protein on Western blotting.

Antibodies

Anti-epithin (Matriptase) antibodies (H-270 and C-16) were purchased from Santa Cruz Biotechnology, INC. Polyclonal anti-epithin antibody and monoclonal antibody mAb5 has been described previously (Cho et al., 2001).

Western blot analysis

For soluble protein preparations, conditioned media were collected and all proteins in the media were precipitated with 10% TCA solution. For cell lysate preparation, Laemmli SDS reducing sample buffer was directly added into cells. Protein samples were separated on 10% acrylamide gel under reducing condition. To visualize, horseradish peroxidase-conjugated secondary antibody was used and treated with the enhanced chemiluminescence reagents.

Protease activity assays

For Gelatin-based zymography, the CM of 427.1.86 cells was collected and concentrated using centricon (YM-30). Epithin depletion was accomplished with anti-epithin antibody (Cho et al., 2005) and Protein A Sepharose bead and concentrated. 32μl of concentrated CM were separated by SDS-PAGE gel containing 0.1% gelatin. After washing with 2.5% Triton X-100 for 2 h, the gel was incubated for 18 h in TBS with 0.01 M $CaCl_2$, 1 M $ZnCl_2$, 0.02% NaN_3. The gelatinase activity was visualized by the staining with 0.5% Coomassie brilliant blue R250 in 45% methanol and 10% acetic acid. To measure the protease activity, 1 μM QAR-AMC was added into 100 μl of soluble epithin fraction or control fraction, and fluorescence intensity was measured for 2 h.

Wound healing migration assay

For wound healing migration assay, MS1 cells grown confluently were scratched using a blue tip. Same volume of epithin-containing conditioned media was added to MS1 media. After culturing 40 h with epithin containing media, cells were fixed with 4% paraformaldehyde. Epithin-depleted media were prepared using anti-epithin antibodies and Protein A Sepharose bead.

For quantitative wound healing migration assays, BAECs at 90% confluence were wounded with a razor blade and supplied with fresh growth media containing 1 mM thymidine and CM (20%) for 16 h. The number of cells that moved beyond the reference line was counted as the migrated cells. 6-8 fields were counted and the total cell number was divided by the numbers of counted field in each assay. Cells were then stained with Giemsa.

Invasion Migration assay

Invasion assay was performed in Transwell (Costar) with 8 μm filters coated with 0.5 mg/ml gelatin and 0.5 mg/ml Matrigel (BD Biosciences). The lower compartment contained 600 μl DMEM complemented with 0.1 mg/ml bovine serum albumin, the upper compartment contained 2×10^4 cells in 100 μl DMEM. After incubation for 18 h, cells were fixed and stained using hematoxylin. Cells on the upper surface of the filter were removed, and the cells at the lower compartment were counted. Four fields were counted and the total cell number was divided by the numbers of counted field in each assay.

Tube formation assay

5×10^4 HUVECs or 1×10^5 BAECs were grown on the surface of Matrigel in 48-multiwell plates. After 30 min, epithin-containing media were added in culture media for indicated periods.

CAM assay

At the stage of 4-days-old, the shell and the membrane of the air sac was removed. A sample (30 μl of concentrated conditioned media)-loaded thermanox-coverslips were applied at 9-day-old embryos. After 3 days, 10% Intralipose was injected into chorioallantois, and observed.

Cell proliferation assay

BAECs were seeded at a density of 5×10^3 cells per well into 48-well culture plates. After 21 and 45 h cultures in the presence of epithin, the cells were incubated with 50 μg/ml MTT (Mosmann, 1983) for 3 h. After the medium containing MTT was removed, the cells were dissolved in 200 μl DMSO. bFGF (10 ng/ml) was used as a positive control. Absorbance at 540 nm was measured.

RESULTS

Epithin shedding is increased in the hypoxic condition

The 427.1.86 thymic epithelial cancer line produces the epithin constitutively and secretes it into culture media under various conditions (Cho et al., 2001; Kim et al., 1999; 2005). We found that soluble epithin can be easily obtained with TBS treatment without contamination of serum proteins. The soluble epithin recovered in this condition showed the proteolytic activity in zymography as shown in Fig. 1A. The gelatin digesting activity by the epithin protein was specifically abolished by anti-epithin antibody, but not the activity of MMP2. There was no other gelatinase activity besides of MMP2 that showed the identical migration in zymography to the MMP2 activity of well known HT1080 fibrosarcoma cells (data not shown).

In order to investigate the significance of soluble epithin in tumor progression process, we tested the effect of hypoxia for its shedding from 427.1.86 cell. The amount of epithin shedded from cell in 1% O_2 hypoxia was more increased than that in

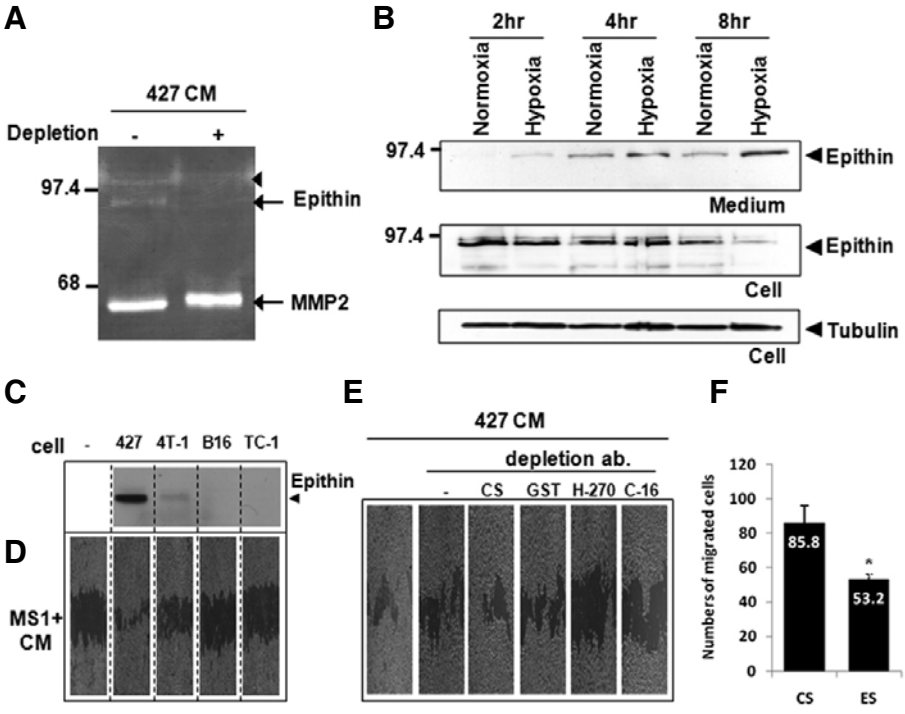

Fig. 1. Epithin shedding from 427.1.86 is increased in hypoxic condition and soluble epithin induces endothelial migration. (A) Protease activity of secreted epithin was measured in gelatin-based zymography. The protease activity of concentrated CM from 427.1.86 cells, (lane 1, arrow) and that of immunodepleted CM using anti-epithin polyclonal antibodies (lane 2) are shown. The upper band (arrowhead) above the indicated epithin was also depleted. (B) Western blot with anti-epithin mAb5. 427.1.86 cells were placed in the serum-free media and incubated in normoxia or hypoxia for indicated times. CMs were collected and precipitated with TCA solution. Epithin secreted to the media (top) and remaining in the cells (middle) and tubulin in the cell (bottom) were shown. (C) Expression of epithin in various cancer cell lines. Names of cancer cell lysates for western blotting are shown on top. (D) Epithin expressing cancer cell line-conditioned media (20%) induced the migration of MS1 in wound healing assay. (E) Anti-epithin antibodies blocked the MS1 migration induced by 427.1.86 CM (427CM). Immunodepletion with Protein-A sepharose only (–), controls rabbit serum (CS) and anti-GST antibody (GST) showed no apparent effects, while immunodepletion with anti-Matriptase antibodies (H-270 and C-16) reduced endothelial migration. (F) Quantitative wound healing migration assay with BAEC. Immunodepletion with anti-epithin serum (ES) diminished the endothelial migration about 38% over that of control serum (CS). The asterisk indicates $P < 0.05$.

normoxia condition (Fig. 1B). In contrast, the amount of epithin remaining in the cell was slightly decreased at 8 h. These results clearly suggested that hypoxia accelerate the shedding of epithin.

Soluble epithin induced migration of endothelial cells, but not proliferation

Endothelial cell migration is a critical and initiative event in the formation of new blood vessels. In this respect, the effect of epithin on the migration of MS1 was evaluated using the wound-healing assay. First, we examined the relationships between the expression level of epithin and the endothelial motility. As seen in Fig. 1C, epithin was detected in 427.1.86 and 4T-1 (breast carcinoma) but not in B16 (melanoma) and TC-1 (lung carcinoma). The CMs of these cells were used in MS1 endothelial migration assay. There was a clear correlation between the levels of epithin in the cell and the degrees of endothelial migration (Fig. 1D). Next, to verify if epithin is, in fact, the endothelial migration-enhancing factor among the medium components, epithin in 427.1.86 CM was depleted by anti-epithin antibodies (H-270 and C-16). Only epithin-specific antibodies inhibited the induced wound closure, while the control normal rabbit serum and anti-GST antibody did not (Fig. 1E). In the case of specific immunodepletion with anti-epithin antibody, the endothelial migration was decreased by 36% compared with that of control depletion with preimmune serum (Fig. 1F). These results strongly suggested that the soluble active epithin enhances the motility of endothelial cells.

Next, we introduced a plasmid encoding epithin into NIH3T3 fibroblast. We assumed that non-transformed, non-cancer cell line would secrete proteins different from cancer cells, therefore show less contaminating angiogenic activity. The epithin was detected in the cell as well as in the media (Fig. 2A, arrow). Unprocessed form of epithin was also detected in the cell (Fig. 2A, arrow head) (Cho et al., 2005). The soluble epithin fraction was collected from the CM and was used in BAEC wound healing assay. As shown in Figs. 2B and 2C, migration of the BAEC treated with CM from epithin transfected cells (Epithin CM) was increased by 33% compared to CM from control cells (Vector CM). These results indicate that epithin affects the stimulation of BAEC migration across the species (Figs. 1 and 2).

To address whether the soluble epithin fraction treatment causes proliferation of endothelial cells, one of the angiogenic phenotypes, we applied MTT assay. As shown in Fig. 2D, Epithin CM failed to induce the proliferation of BAEC at 24 or 48 h while bFGF was effective. The proliferation activity of Epithin CM that was depleted with either specific or control serum was similar. Therefore, we concluded that epithin induces the endothelial cell migration without affecting proliferation.

Soluble epithin increased invasive migration of endothelial cells

We performed the transwell invasion migration assay. When treated with epithin CM, 29.5% more BAECs infiltrated the Matrigel and migrated into the lower compartment in comparison to the cells treated with vector CM (Fig. 3A).

Next, we used epithin knock-down cells generated with siRNA against epithin message (Figs. 3B-3D). After selection of transfected clones, Epi-KD-17 was chosen for the later experiment. In this clone, the lowest level of epithin was detected in the cell lysate and in the media (Fig. 3B). The epithin specific protease activity of the CM recovered from Epi-KD-17 was also significantly decreased in the assays with fluorescent peptide substrate (Fig. 3C). When the CM of 427.1.86 cells (427CM)

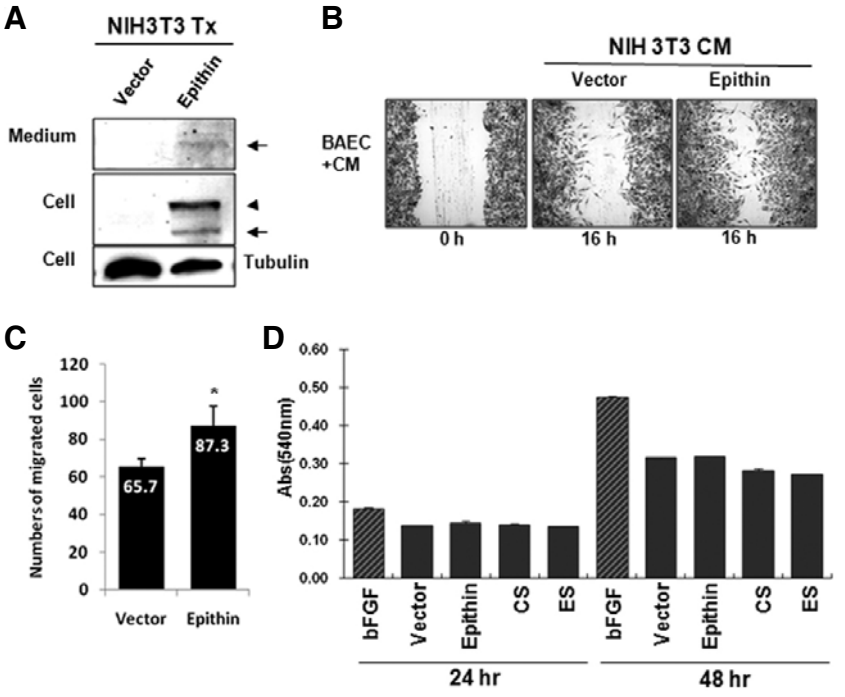

Fig. 2. Soluble epithin induces BAEC migration, not proliferation. (A) The epithin expression in the transfected NIH3T3 cells was analyzed by western blotting using mAb5. Epithin expression vector and empty vector were transfected into NIH3T3 cells. Epithin proteins in the media and cell lysates were analyzed. Unprocessed long-form of epithin (arrowhead) and processed epithin (arrow) are indicated. Tubulin was used as a loading control. (B) Wound healing migration assay. Representative fields show the wounded area at time zero and the cell migration into the wound after 16 h of treatment with Epithin CM or vector CM were observed using a light microscope (× 50). (C) Quantitative wound healing migration assay. The number of migrated cells was counted under light microscope. 6-8 fields were counted in each assay. The asterisk indicates $P < 0.05$. (D) The proliferation of BAECs was determined in the MTT assay after 24 and 48 h of stimulation with vector CM or Epithin CM. bFGF was used as a positive control. Immunodepletion with anti-epithin serum (ES) and control serum (CS) of epithin CM are also shown. The data are displayed with a 95% confidence interval of the means of three independent measurements ($n = 3$).

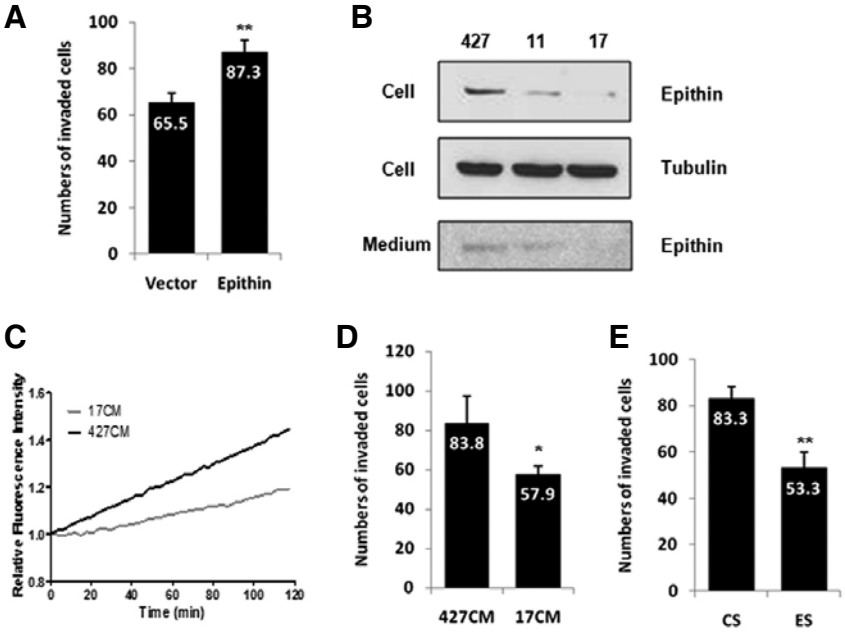

Fig. 3. Epithin induces the invasion of BAECs. (A) Invasion of BAECs into Matrigel after 16 h of treatment with Epithin CM (20%) or vector CM (20%). The double asterisk indicates $P < 0.05$. Each experiment was performed in triplicate. (B) Analysis of epithin expression in two epithin knock-down clones. Expression profiles of epithin in 427.1.86 cells (427), knock down Epi-KD-11 (11) and Epi-KD-17 (17) lines were analyzed with mAb5. Secreted epithin was also detected (Medium). Blot of tubulin was shown. (C) Epithin protease activity of 427 CM and EpiKD17 CM. CM of 427 and Epi-KD-17 cells were incubated with QAR-AMC and fluorescent intensity was measured for 2 h. (D) Epithin knock down reduced BAEC invasion. Cells were seeded on the Matrigel and incubated with 427.1.86 CM (427CM) or Epi-KD-17 CM (17CM). The asterisk indicates $P < 0.01$. (E) Immunodepletion of epithin reduced BAEC invasion. 427 CM was immunodepleted using control serum (CS) or anti-epithin serum (ES) and used in the invasion assay. Cell numbers found in the lower compartment of Transwell chamber is shown in the Y axis. The double asterisk indicates $P < 0.05$. Each experiment was performed in triplicate.

and Epi-KD-17 cells (17CM) were treated to BAEC invasion assay, 17CM-treated BAEC showed 31% less invasiveness than 427CM-treated cells (Fig. 3D).

We also investigated the invasion activity of BAEC with CM from 427.1.86 culture (427CM) which is immunodepleted with control serum (CS) or epithin specific serum (ES). In the case of specific immunodepletion with anti-epithin antibody (ES), the endothelial invasion through the Matrigel-layer was decreased by 36% compared with that of depletion with control serum (CS) (Fig. 3E). These results verified that the epithin containing CM promoted endothelial activation and invasiveness.

Epithin is involved in the angiogenic morphogenesis of the endothelial cell

Endothelial cells differentiate and form tubular networks when they grow on the surface of three-dimensional Matrigel. To

Table 1. Epithin in the media induces angiogenic morphogenesis in CAM assay. The induction of spoke-wheel-like pattern of blood vessels was considered as the indication of angiogenic effect of PMA, vector CM and epithin CM.

Compounds	Total eggs tested	Eggs showing angiogenesis	% of activation
PMA	15	14	93.3
Vector CM	12	3	25.0
Epithin CM	13	8	61.5

analyze the effect of epithin in tube formation, endothelial cells were cultured on the surface of Matrigel with CM recovered from 427.1.86 (Fig. 4A) and that from NIH3T3 overexpressing epithin (Fig. 4B). The morphology of BAECs treated with 427CM was changed into a tube-like structure at 2 h after seeding. However, the tube formation is retarded by Epi-KD-17 CM (Fig. 4A). The morphologies of HUVECs treated with conditioned media recovered from epithin-transfected NIH3T3 cells changed into a tube-like structure in 24 hours (Fig. 4B). These results clearly indicate that epithin stimulates angiogenic morphogenesis *in vitro*.

To test the effect of epithin on angiogenesis *in vivo*, we performed CAM assay. After 3 days of incubation with epithin-coated coverslip, it was clear that epithin strongly elicited an angiogenic response as a spoke-wheel-like pattern of blood vessels (Fig. 4C). Without epithin, this pattern was not observed. The degree of angiogenic pattern is comparable to that of PMA treatment. Quantitative analysis of CAM assays (Table 1) showed that the application of epithin CM exhibits 61.5% of PMA induced angiogenic responses, while vector CM showed just 25% of it. All together, we conclude that epithin behaves as a proangiogenic factor *in vivo*.

DISCUSSION

In this study, we utilized the soluble epithin fraction in various angiogenesis assays using chicken, mouse, bovine, and human endothelial cells. We showed that the soluble epithin fraction showed the gelatinase activity (Fig. 1) and specific peptide cleavage activity (Fig. 3). The secretion of epithin as a soluble

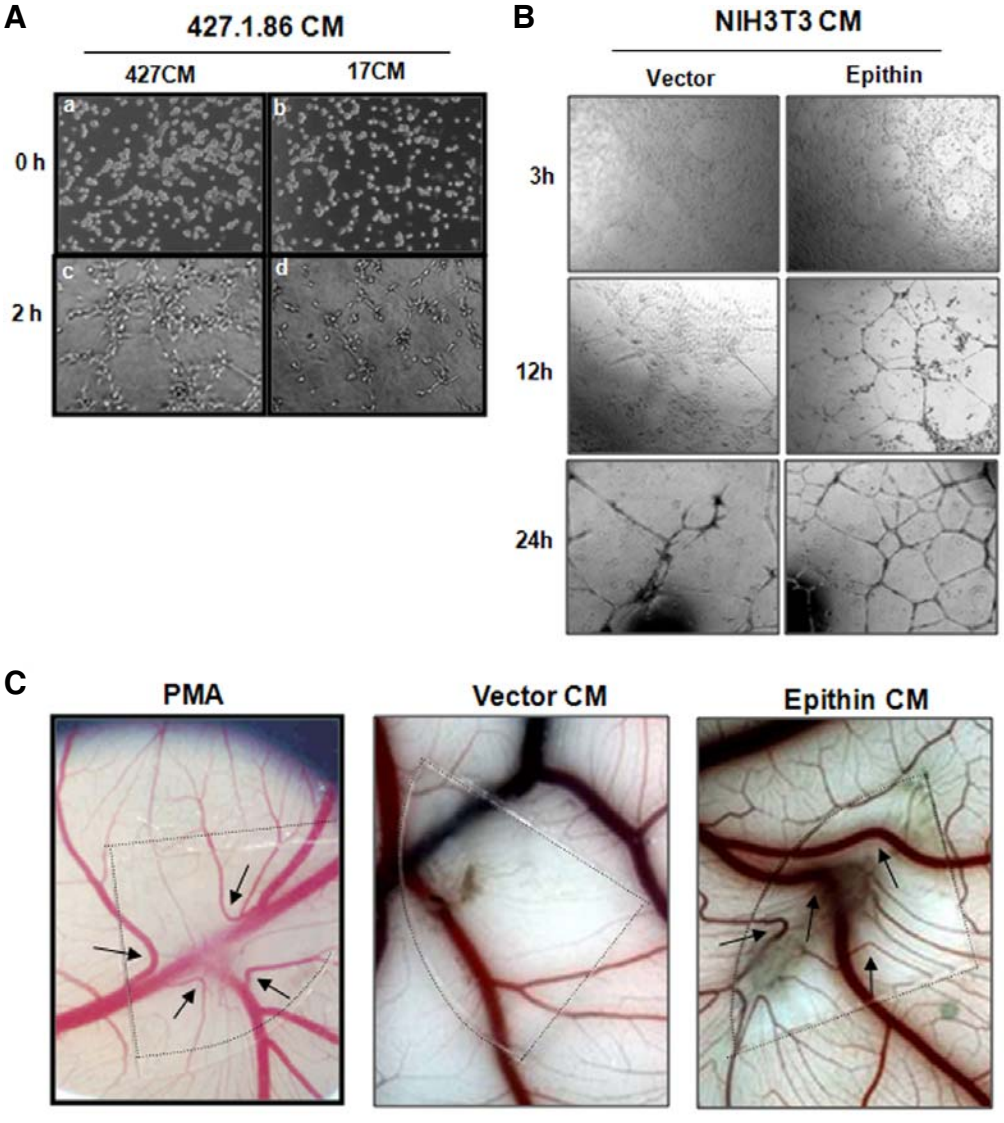

Fig. 4. Soluble epithin induces endothelial tube formation and branches. (A) The tubular structures of BAEC were more actively formed with 427CM than with 17CM. (B) The tubular structures of HUVEC were formed more with Epithin CM from NIH3T3 than with vector CM. Patterns of capillary tube formation at 3, 12, and 24 h are shown (50 ×). (C) CAM assay was performed as described in "Materials and Methods". The vessel structures were photographed and angiogenic responses (spoke wheel-like structures) were indicated by arrows. 120 ng PMA was used as a positive control.

form was upregulated by hypoxia, and revealed angiogenic activities *in vitro* and *in vivo*. Soluble epithin fraction increased endothelial cell migration and invasion (Figs. 2 and 3) as well as angiogenic differentiation and morphogenesis (Fig. 4). These results are the first direct demonstration of epithin's angiogenic potential.

So far, it is well established that epithin is associated with many stages of epithelial tumors (List, 2009). Therefore, epithin is currently considered as a marker and target for cancer diagnosis and therapy (Kang et al., 2003; Oberst et al., 2002). However, detailed mechanism on how epithin functions in the specific processes still remains to be studied.

Although epithin is TTSP that is associated with membranes of the cells, it is also present as a secreted form (Cho et al., 2001; 2005; Lin et al., 2008). The shedding of the protein is induced by PMA (Kim et al., 2005) and hypoxia (Fig. 1B), which are associated with both tumor progression and angiogenesis. Interestingly, increased epithin expression was described in the microvascular endothelial cells on the surface of Matrigel during vascular morphogenesis and angiogenesis (Aimes et al., 2003). However, we did not observe the epithin expression in normal 2D culture of BAEC (data not shown).

Epithin function in angiogenesis was initially suggested from a study with the overexpression of human ortholog matriptase in AZ521 cancer cell line. When transplanted into nude mice, cells with overexpression resulted in larger tumors and more angiogenesis within tumor mass (Jin et al., 2006). Authors proposed that matriptase from cancer cells can promote cancer angiogenesis either directly or indirectly through interaction with tumor stromal cells involving MMP3. It is well known that the activation of MMPs during tumorigenesis provides the conditions for angiogenesis (Overall and Kleifeld, 2006). Thus, if epithin activates MMPs in the tumor environment, it can affect angiogenic processes as a secondary effect.

However, we argue that epithin secreted from cancer cells directly affects endothelial cells rather than indirectly through other components. Supporting evidence comes from the facts that the immunodepletion of epithin abolished the induction of endothelial cell migration (Figs. 1E and 1F) and invasion assay (Fig. 3E). We did not find any active MMPs other than MMP2 in the CM (Fig. 1A) and the presence of MMP2 in epithin depleted CM failed to show efficient proangiogenic effects. This result suggested that at least MMP2 is not the factor involved, but epithin itself plays a major role. If there was any putative proangiogenic epithin substrate that could be activated, it should have been activated by active epithin present in CM when it was collected. Therefore, our results are not consistent to the presence of a secondary activatable molecule in the cancer CM. Although it is highly unlikely, however, we did not formally exclude the possibility of the putative activatable latent molecule present and/or induced in the endothelial cultures and activated in the presence of blocking antibody.

Second, the effects on the morphologic changes are fast (Figs. 4A and 4B). Endothelial cells formed tubes within 2 h that may not be sufficient for the production of secondary molecules. Third, overexpression of epithin in NIH3T3 resulted in the angiogenic soluble factor that can be depleted by epithin specific antibody in all the assays tested. Therefore, it is highly unlikely that there is a common secondary inducible angiogenic molecule present in both epithin expressing NIH3T3 and 427.1.86 CM. The direct approach using the purified active protein without contamination of any other protease activity will resolve this issue. However, we found such an experiment is technically challenging.

In conclusion, we demonstrated that epithin is a proangiogenic factor that can be controlled using specific antibodies. Our results also provide the further ground that epithin inhibitors may prevent angiogenesis as well as tumor growth. Consequently, epithin can be a key target for cancer prevention and therapy.

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