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Effect of Fibroblast Growth Factor-2 on Migration and Proteinases Secretion of Human Umbilical Vein Endothelial Cells

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Abstract Fibroblast growth factor-2 (FGF-2) is known to modulate numerous cellular functions in various cell types, including cell proliferation, differentiation, survival, adhesion, migration, and motility, and also in processes such as wound healing, angiogenesis, and vasculogenesis. FGF-2 regulates the expression of several molecules thought to mediate critical steps during angiogenesis. This study examines the mechanisms underlying FGF-2-induced cell migration, using human umbilical vein endothelial cells (HUVECs). FGF-2 induced the nondirectional and directional migration of endothelial cells, which are inhibited by MMPs and plasmin inhibitors, and induced the secretion of matrix metalloproteinase-3 (MMP-3) and MMP-9, but not MMP-1 and MMP-2. FGF-2 also induced the secretion of the tissue inhibitor of metalloproteinase-1 (TIMP-1), but not of TIMP-2. Also, the pan-PKC inhibitor inhibited FGF-2-induced MMP-9 secretion. It is, therefore, suggested that FGF-2 induces the migration of cultured endothelial cells by means of increased MMPs and plasmin secretion. Furthermore, FGF-2 may increase MMP-9 secretion by activating the PKC pathway.

Key words: FGF-2, migration, HUVECs, MMPs, plasmin

FGF-2, one of the nine members of the FGF family [2], is known to modulate numerous cellular functions in various cell types, including cell proliferation, differentiation, survival, adhesion, migration and motility, and in processes such as wound healing, angiogenesis and vasculogenesis [38]. FGF-2 regulates the expression of several molecules thought to mediate critical steps during angiogenesis. These include interstitial collagenase, urokinase-type plasminogen activator (uPA), plasminogen activator inhibitor, uPA receptor (uPAR), and β1 integrins [16, 23]. These molecules may be involved in the invasive phenotype displayed by endothelial cells during angiogenesis [4]. Angiogenesis induced by FGF-2 also involves αvβ3 integrin and extracellular matrix (ECM) [5].

Angiogenesis is a process involving cell attachment, basement membrane degradation, migration, proliferation, and cell differentiation, as well as the formation of a new capillary structure [11, 33]. Emerging evidence suggests that proteinases play an important role in angiogenesis. Vascular endothelial cells release proteinases that degrade the ECM. Degradation of the ECM *in vivo* paves the way for the migration of vascular endothelial cells during angiogenesis and vasculogenesis [12, 32]. The ECM is a substance of tightly interwoven components, and requires several enzymes, particularly MMPs and plasmin. MMPs are secreted in proenzyme form and require proteolytic cleavage for activation. MMPs are inhibited by endogenous inhibitors, TIMPs, which form 1:1 complexes with MMPs [26]. The TIMPs act by inhibiting both the proteolytic activation of the proMMPs and the enzymatic activity of active MMPs, and the balance between the levels of MMPs and TIMPs is believed to be critical in regulating the breakdown of connective tissues by migrating cells [27]. Plasmin degrades a variety of ECM components and activates several MMPs with different substrate specificities, including MMP-1 (interstitial collagenase), MMP-3 (stromelysin 1), and MMP-9 [14, 30, 40].

In the present study, the possible mechanisms underlying FGF-2-induced cell migration were examined using human umbilical vein endothelial cells (HUVECs). FGF-2 induced the migration of HUVECs, which were inhibited by MMPs and plasmin inhibitors. FGF-2 also induced the secretion of MMPs and plasmin. It is suggested, therefore, that FGF-2-induced migrating activity in endothelial cell may be accomplished by increased secretion of proteinases.

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MATERIALS AND METHODS

Materials

Recombinant human FGF-2 was purchased from R & D systems (Minneapolis, MN); Recombinant human TIMP1, TIMP2, MMP-1, MMP-2, MMP-3, and MMP-9 enzyme immunoassay kits from Fuji Chemical Industries (Toyama, Japan); Recombinant human plasmin enzyme immunoassay kit from Progen (Heidelberg, Germany); Media and sera from Life Technology, Inc. (Gaithersburg, MD); BB-94 and α_2-antiplasmin from British Biotechnology (Oxford, U.K.). Plasmin standard was purchased from Boehringer Mannheim (Mannheim, Germany), and MMP-3 inhibitor was from Calbiochem (La Jolla, CA, U.S.A.). Unless otherwise specified, other biochemical reagents, including GF109203X, Giemsa staining solution, antimycotics, and trypsin-EDTA, were purchased from Sigma (St. Louis, MO, U.S.A.).

Cell Culture

All cells used in this study were HUVECs. HUVECs were prepared from human umbilical cords by collagenase digestion, as previously described [19, 34]. The endothelial origin of the cultures was confirmed by immunofluorescent staining with an anti-von Willebrand factor antibody; acceptable cultures had >95% fluorescent cells. These endothelial cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 20% (v/v) heat-inactivated fetal bovine serum, at 37°C in 5% CO_2. The primary cultured cells used in this study were between passages 2 and 4.

Migration Assays

For the nondirectional assay, the microcarrier bead migration method was used [35]. HUVECs were grown to confluence on microcarrier beads (diameter 175 μm; Sigma) and placed in a gelatinized 24-well plate (30–40 beads/well) in serum-free DMEM with various reagents and incubated for 20 h. The wells were washed with PBS, fixed with methanol, dried, and stained with Giemsa staining solution for 5 min. The cells that had migrated from the beads and attached to wells were counted at 100× magnification using an inverted phase-contrast microscope. For the directional migration assay, the method using a modified 48-well Boyden chamber (Neuroprobe, Cabin John, MD, U.S.A.) was used [18]. Indicated reagents in DMEM containing 1% bovine serum albumin were placed in the bottom or upper wells of the chamber. Polycarbonate filters with 8 μm pores (Poretics, Livermore, CA, U.S.A.) were coated with 50 μg/ml fibronectin and 0.2% gelatin, and placed between the test substances and the upper chambers. Cells were trypsinized, washed twice in DMEM, and resuspended in 1% bovine serum albumin. 5×10^4 cells were placed into each well in the upper chamber and then incubated for 12 h at 37°C in a humidified chamber with 5% CO_2. After incubation, the nonmigrated cells were removed from the upper side of the filters with a cotton ball. The filters were fixed with methanol, mounted onto microscope slides, and stained with Diff-Quik solution (HEMA-3 stain set; Biochemical Sciences, Swedesbord, NJ, U.S.A.). The migrated cells were counted at 100× magnification using a microscope.

Enzyme Immunoassay

HUVECs were seeded into 24-well plates at a density of 5×10^4 cells/cm^2 and were grown for 24 h in the DMEM supplemented with 20% serum. Then, confluent HUVECs were incubated in serum- and phenol red-free DMEM for 12 h. After the cells were washed with fresh medium, a control buffer or FGF-2 was applied for 12 h. The actual amounts of the MMPs, TIMPs, and plasmin were assayed by enzyme immunoassay according to the manufacturer's protocol (Fuji Chemical Industries, Toyama, Japan; Progen, Heidelberg, Germany).

Zymography

HUVECs were seeded into 24-well plates at a density of 5×10^4 cells/cm^2 and were grown for 24 h in the DMEM supplemented with 20% serum. Then, confluent HUVECs were incubated in serum- and phenol red-free DMEM for 12 h. After the cells were washed with fresh medium, a control buffer or the indicated reagents were applied for 12 h. The hydrolytic activities of MMPs were measured by gelatin zymography [17]. Thus, samples were mixed with 5× sample buffer (4 M Tris-HCl, pH 6.8, 5% SDS, 20% glycerol, 0.1% bromophenol blue) and were applied to 10% SDS-PAGE containing 0.1% gelatin. Reference standards were MMP-2 and MMP-9 (Chemicon International, Inc., Temecula, CA, U.S.A.). After activity ceased , the gel was incubated in 2.5% Triton X-100 for 1 h and then incubated in enzyme buffer (0.05 M Tris-HCl, pH 7.5, 0.02 M NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35) for 24 h at 37°C. The gel was stained with 0.5% Coomassie brilliant blue 250 solution and destained with several changes of solution consisting 30% methanol and 10% acetic acid. Next, the hydrolytic activity of the plasmin was measured by fibrin zymography. Thus, equal amounts of proteins (10 μg/lane) from supernatants were mixed with SDS sample buffer and electrophoresed in 10% acrylamide gel containing 0.1% gelatin and 0.2% plasminogen (Athens Research and Technology, Athens, GA, U.S.A.). The gel was washed twice in 10 mM Tris-HCl (pH 8.0) containing 2.5% Triton X-100 for 30 min while agitating at room temperature. The gel was further rinsed in 10 mM Tris-HCl for 30 min, and then incubated in a buffer containing 0.1 M glycine-NaOH (pH 8.3) at 37°C for 16 h. The gel was stained with 1% Coomassie brilliant blue 250 in 5% acetic acid and 10% methanol, and then destained in the same mixture without dye.

Data Analysis

Data are expressed as means±standard deviation (SD). Statistical significance was tested using one-way ANOVA followed by the Student-Newman-Keuls test. Statistical significance was set at $P<0.05$.

RESULTS AND DISCUSSION

Migration Assay of HUVECs

Placing microcarrier beads onto a confluent monolayer of HUVECs for 2 to 3 days produces beads covered by a confluent monolayer of cells with ≈25 to 30 cells per bead. When HUVEC-bearing microcarrier beads were placed onto gelatinized plastic dishes with a control buffer for 20 h, they yielded a basal level of nondirectional migration (≈50 to 55 cells per 10 beads, Fig. 1A). The number of migrating cells increased with FGF-2 stimulation in a dose-dependent manner. FGF-2 also induced directional (chemotactic) migration in a dose-dependent manner (Fig. 1B). This data indicate that FGF-2 is a potent factor in migration of HUVECs. This observation is consistent with studies of FGF-2 in other types of endothelial cells [6, 9].

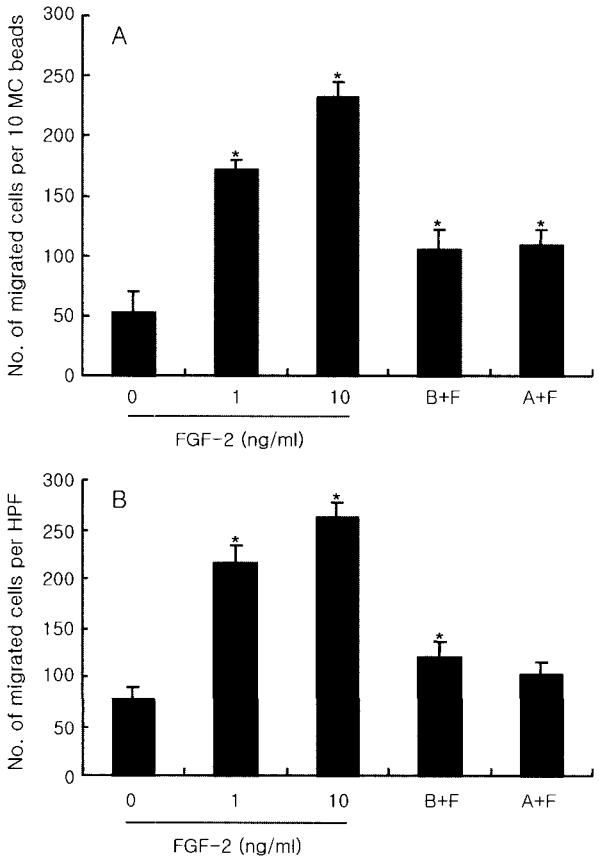

Fig. 1. Effects of FGF-2, MMPs and plasmin inhibitors on migratory activity of HUVECs.

HUVECs were grown to confluence on microcarrier beads, and the beads were placed into gelatinized 24-well plates in serum free DMEM and incubated for 20 h to examine nondirectional migratory activity (A). A modified Boyden chamber was used to examine directional migratory activity (B). Cells grown on microcarrier beads were treated with various amounts of FGF-2. BB-94 (20 ng/ml) plus FGF-2 (10 ng/ml) (B+F), and α_2-antiplasmin (100 mU) plus FGF-2 (10 ng/ml) (A+F). HPF indicates a high-power field (×100). This data represent means±SD from four independent experiments. Statistical significance was tested using one-way ANOVA followed by the Student's *t* test. *$P<0.05$ versus control buffer.

MMPs and TIMPs Secretion from HUVECs

To migrate *in vivo*, endothelial cells secrete proteinases to dissolve the adjacent ECM [36]. There are currently 24 known members of the MMPs. In addition, 4 members of the TIMPs have been identified to date [27, 31]. The stepwise activation processes of proMMPs suggest that MMP activities are controlled by the endogenous inhibitors such as α_2-macroglobulin or TIMPs before MMPs are fully activated. Indeed, the binding of activation intermediates of MMPs to a TIMP has been demonstrated for MMP-1, MMP-2, MMP-3, and MMP-9 [3, 8, 15, 39]. An enzyme immunoassay showed that the culture media from HUVECs contained clearly detectable amounts of MMP-3 and MMP-9, whereas MMP-1 and MMP-2 levels were low (Fig. 2A). The addition of FGF-2 (10 ng/ml) for 12 h produced ≈3.1-fold and ≈9.2-fold increase of MMP-3 and MMP-9 secretion, respectively, compared with the control buffer. The addition of FGF-2 produced ≈1.8-fold increase

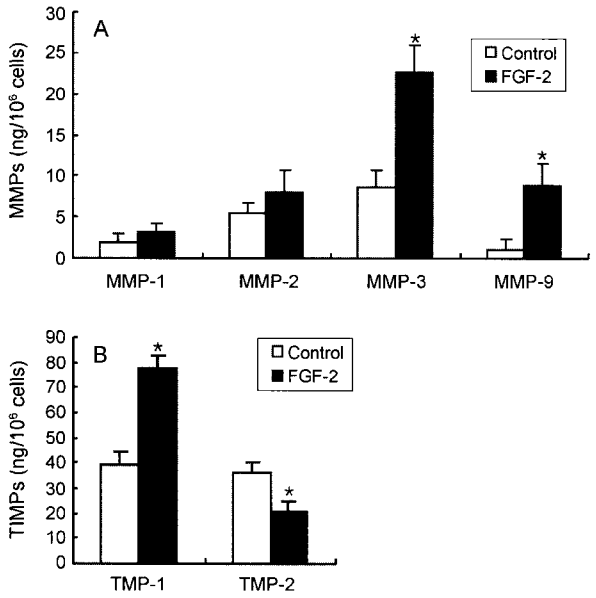

Fig. 2. Effects of FGF-2 on the secretion of MMPs (A) and TIMPs (B) of HUVECs.

Cells were incubated in serum- and phenol red-free DMEM for 12 h. Then, control buffer or FGF-2 (10 ng/ml) was added to 0.5 ml of culture medium, incubated for 12 h, and the medium was quantitatively assayed by enzyme immunoassay. Bars represent means±SD from four independent experiments. Statistical significance was tested using one-way ANOVA followed by the Student's *t* test. *$P<0.05$ versus control buffer.

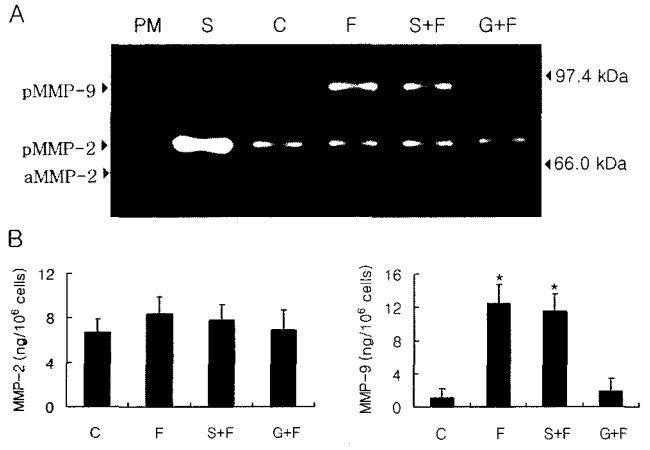

Fig. 3. Effects of NF-κB and PKC inhibitors on FGF-2-induced MMP-9 secretion.
(A) Cells were incubated in serum- and phenol red-free DMEM for 12 h. Sixty minutes prior to stimulation with FGF-2 (10 ng/ml), HUVECs were treated with the control buffer (C), SN50 (50 μg/ml) (S+F), and GF109203X (1 μM) (G+F). Conditioned media were collected 12 h later and analyzed by gelatin zymography. Equal amounts of proteins (10 μg/lane) from supernatants were loaded into each lane. Lanes PM and S contain standards of protein and MMPs, respectively. Results of three independent experiments were similar. (B) Conditioned media were quantitatively assayed by enzyme immunoassay. Bars represent means±SD from four independent experiments. Statistical significance was tested using one-way ANOVA followed by the Student's *t* test. $^*P<0.05$ versus control buffer.

of TIMP-1 secretion. On the other hand, the secretion of TIMP-2 was significantly suppressed (≈2.0-fold) (Fig. 2B). It has been reported that MMP-3 binds to TIMP-1 more readily than to TIMP-2, and that TIMP-2 may be required for the conversion of proMMP-2 to active MMP-2 [28]. A balance between the levels of MMPs and TIMPs is a critical factor in regulating the breakdown of ECM by MMPs [27]. Therefore, an increased ratio of MMP-3 over TIMP-1 caused by FGF-2 is favorable for the degradation of ECM in HUVECs.

The profiles of MMP-2 and MMP-9 in the media were quantitatively assayed by gelatin zymography and enzyme immunoassay. FGF-2 (10 ng/ml) was capable of inducing MMP-9 secretion for 12 h, compared to addition of a control buffer (Fig. 3). MMP-9 secretion was increased in a dose-dependent manner (data not shown). Consistent with the enzyme immunoassay, FGF-2 did not produce any changes in the level of MMP-2 secretion (Figs. 2A and 3). FGF-2 has been shown to be able to induce the expression of MMP-9 in many cell types [7, 24, 29], however, little is known about the signaling pathways involved in the FGF-2-induced MMP-9 secretion. In osteoclasts, FGF-2 acts on MMP-9 expression through activation of the $MEK_{1/2}/ERK_{1/2}$ pathway [25]. Liu *et al.* [22] showed FGF-2 induced MMP-9 secretion in MCF-7 cells, and suggested that FGF-2 upregulates MMP-9 production by activating the PKC pathway. Therefore, to examine the involvement of PKC, PKC inhibitor was applied to HUVECs. As shown in Fig. 3, pan-PKC inhibitor, GF109203X (1 μM), completely suppressed FGF-2-induced MMP-9 secretion (Fig. 3), indicating that FGF-2-induced MMP-9 secretion in HUVECs occurs through a PKC-dependent pathway. NF-κB has thus far been found in the MMP-9 promoter, and this is one characteristic determining the unique expression pattern of this gene [10]. Therefore, targeted inhibition of NF-κB might be a logical step in modulating the MMP-9 activity. To examine the involvement of NF-κB activation, cells were preincubated with NF-κB inhibitors, however, a specific NF-κB inhibitor peptide, NF-κB SN50 (50 μg/ml) had no effect on FGF-2-induced MMP-9 secretion. Although the actual amount of MMP-2 was lower than those of MMP-3 and MMP-9 (Figs. 2A and 3), the elevated MMP-2 activation level could not exclude the possibility that it was more activated in migration. The mechanisms of MMP-2 activation and its involvement in endothelial cell migration should be examined in future studies.

Plasmin Secretion in HUVECs

To migrate in response to FGF-2 stimulation in an *in vitro* fibrin gel, endothelial cells must secrete fibrinolytic enzymes. To date, the ability of endothelial cells to mediate fibrinolytic

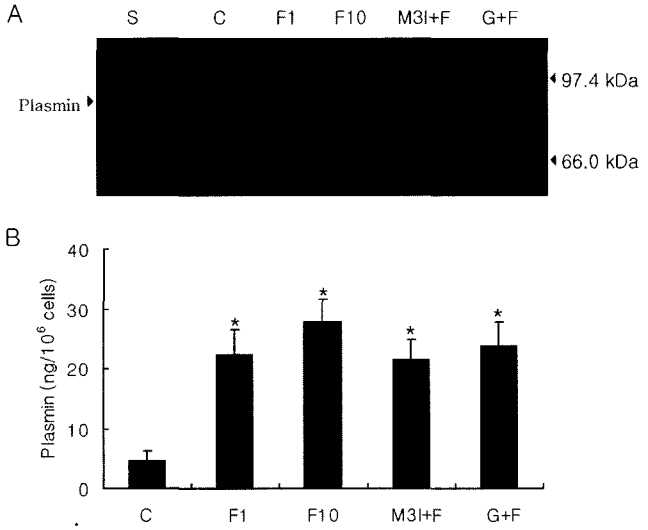

Fig. 4. Fibrin zymography and enzyme immunoassay of plasmin in culture medium of HUVECs treated with FGF-2.
(A) Cells were incubated in serum- and phenol red-free DMEM for 12 h. Then, the cells were incubated for 12 h after the addition of the control buffer (C) or FGF-2 (1, 10 ng/ml, respectively). Sixty minutes prior to stimulation with FGF-2 (10 ng/ml), HUVECs were treated with MMP-3 inhibitor (1 μM) (M3I+F) and GF109203X (1 μM) (G+F). Conditioned media were collected 12 h later and analyzed by fibrin zymography. Equal amounts of proteins (10 g/lane) from supernatants were loaded into each lane. Lane S contains standards of plasmin. (B) Conditioned media were quantitatively assayed by enzyme immunoassay. Bars represent means±SD from four independent experiments. Statistical significance was tested using one-way ANOVA followed by the Student's *t* test. $^*P<0.05$ versus control buffer.

activity has been largely attributed to the fibrinolysin plasmin [13, 20]. In the present study, the plasmin secretion was confirmed by fibrin zymography and enzyme immunoassay. The culture medium from FGF-2-treated cells clearly had increased ≈85 kDa fibrinolytic bands, compared with the cells treated with buffer alone. The addition of FGF-2 (10 ng/ml) for 12 h produced ≈4.1-fold increase in plasmin secretion, compared with the addition of a control buffer (Fig. 4). Plasmin secretion was increased in a dose-dependent manner. MMPs are generally secreted as zymogens that are extracellularly activated by several proteinases. MMPs, alone or in concert with the plasminogen/plasmin system, are involved in the degradation of ECM components, a requirement for cell migration [21]. *In vitro*, plasmin directly activates MMPs [14, 37], and activation of MMP-2 involves hydrolysis by MT1-MMP, yielding an intermediate that is activated by plasmin [1]. MMP-3 specifically interacts with the main inhibitors of the fibrinolytic system. Thus, MMP-3 specifically hrdrolyzes human α_2-antiplasmin, the major physiological plasmin inhibitor [1]. Given the fact that FGF-2 induced markedly increased amounts of MMP-3 (Fig. 2A), the effects of MMP-3 inhibitors on plasmin secretion were examined. As shown in Fig. 4, the addition of MMP-3 inhibitors (1 μM) produced ≈30% suppression of FGF-2-induced plasmin secretion (Fig. 4), indicating that FGF-2-induced MMP-3 secretion on endothelial cells was partially responsible for FGF-2-dependent plasmin secretion. On the other hand, the addition of pan-PKC inhibitor, GF109203X (1 μM), had no effect on FGF-2-induced plasmin secretion.

FGF-2 Enhances Migration of HUVECs through Increased MMPs and Plasmin Secretion

Endothelial cell migration is an initial step in angiogenesis and neovascularization [33]. This process requires cell migration and invasion into the ECM beneath the basement membrane. Since migration activities were measured in gelatinized plates, FGF-2-induced MMPs and plasmin secretion (Figs. 2 and 4) could be a major determinant for migration. To test directly whether the increased MMPs and plasmin were responsible for migration, the effect of MMPs and plasmin inhibitors on migration was examined. Consistent with this idea, the addition of BB-94 (20 ng/ml), a broad-spectrum MMP inhibitor, and α_2-antiplasmin (100 mU), a plasmin inhibitor, almost completely blocked the nondirectional and directional migratory effects of FGF-2 (Fig. 1). Therefore, it is highly likely that, FGF-2 may partially induce the migration of cultured endothelial cells through increased MMPs and plasmin secretion.

In summary, the present study provides deeper insight into FGF-2-induced endothelial cell migration. It is suggested that FGF-2 induces the migration of cultured endothelial cells through an increase in MMPs and plasmin secretion. This FGF-2-induced secretion of proteinases may enhance endothelial cell migration and cell invasion into the ECM, thereby enhancing sprouting that may initiate angiogenesis.

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