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Angiogenin promotes invasiveness of cultured endothelial cells by stimulation of cell-associated proteolytic activities

(cell invasion/plasminogen activator/extracellular matrix/actin/matrix metalloproteinase)

GUO-FU HU, JAMES F. RIORDAN, AND BERT L. VALLEE

Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, 250 Longwood Avenue, Boston, MA 02115

Contributed by Bert L. Vallee, August 18, 1994

ABSTRACT **Angiogenin, a potent inducer of neovascularization in the chicken chorioallantoic membrane and rabbit cornea, promotes endothelial cell invasion of Matrigel basement membrane. A transformed bovine aortic endothelial cell line, GM 7373, is 5 times more invasive when cultured in the presence of 1 μg of bovine angiogenin per ml than in its absence. A polyclonal anti-angiogenin antibody and α_2-antiplasmin neutralize the effect of angiogenin, but an angiogenin-binding protein (actin) does not. Further, this concentration of angiogenin induces a 14-fold increase in the cell-associated proteolytic activity of cultured endothelial cells, determined with a tissue-type plasminogen activator-specific peptide as the substrate. In addition, cells cultured on a three-dimensional fibrin gel in the presence of angiogenin are 3 times more capable of dissolving the gel and forming focal defects in the underlying matrix. The results indicate that angiogenin can enhance the ability of endothelial cells to digest extracellular matrix components and degrade basement membrane, thereby facilitating cell invasion and migration. Binding of angiogenin to its cell-surface binding protein (actin) followed by dissociation of the angiogenin-actin complex from the cell surface and subsequent activation of tissue-type plasminogen activator/plasmin are likely steps involved in the processes of endothelial cell invasion and angiogenesis.**

Angiogenin is a potent inducer of blood vessel formation on the chicken embryo chorioallantoic membrane and in the rabbit cornea and meniscus (1, 2). It is a 14-kDa protein with 33% sequence identity to bovine pancreatic ribonuclease A and an overall sequence similarity of 53% (3). It was first isolated from the conditioned medium of the human colon adenocarcinoma cell line HT-29 based on its angiogenic activity *in vivo* (4). Subsequently, angiogenin was isolated from human plasma (5) and bovine milk (6), and its mRNA has been detected in both normal and tumor cells (7, 8). The widespread existence of angiogenin *in vivo* and its potent blood vessel-inducing activity suggest that it may play a role in embryologic, neoplastic, inflammatory, and immunogenic angiogenesis. The protein binds to endothelial cells (9), stimulates second messenger responses in both endothelial and smooth muscle cells (10, 11), and modulates mitogenic stimuli of certain cell types (12). In addition, angiogenin acts as an adhesion molecule for endothelial cells (13). It is also internalized by endothelial cells, where it is translocated to the nucleus and accumulates in the nucleolus (14).

An angiogenin-binding protein has been isolated from the endothelial cell surface and characterized (15, 16). Tryptic peptide mapping and amino acid sequence analysis reveal that it is a member of the muscle actin family (16). Indeed, angiogenin binds to bovine skeletal muscle actin with about the same affinity as to angiogenin binding protein. An α-smooth muscle type actin has been detected on the surface of endothelial cells by immunofluorescent staining (17). Both exogenous actin and an anti-actin antibody inhibit the angiogenin-induced neovascularization of the chorioallantoic membrane (16) as well as the nuclear translocation of angiogenin in cultured endothelial cells (14). We have reported previously that upon binding of angiogenin to cell surface actin, some of the angiogenin-actin complex dissociates from the cell surface (15). Furthermore, this complex accelerates tissue-type plasminogen activator (tPA)-catalyzed generation of plasmin from plasminogen (18). Since the angiogenin–actin complex does not inhibit the resultant plasmin activity, whereas actin alone does, the binding of angiogenin to cell-surface actin could lead to the activation of several protease cascades, including the plasminogen activator/plasmin serine protease system and the matrix metalloproteinase system. Therefore, through the formation of its actin complex, angiogenin may promote the degradation of basement membrane and extracellular matrix (ECM) and thus allow endothelial cells to penetrate and migrate into the perivascular tissue, an essential feature of the angiogenesis process.

To test this hypothesis, we have investigated the effect of angiogenin on endothelial cell invasion. Angiogenin indeed stimulates the cell-associated proteolytic activity of endothelial cells and promotes their invasion of fibrin gel and Matrigel basement membrane. The results suggest that angiogenin plays an important role in the regulation of these protease systems and therefore may modulate the ECM-degrading abilities of endothelial cells and their subsequent invasion and migration.

MATERIALS AND METHODS

Reagents. Bovine angiogenin was purified from bovine milk as described (19). Fibrinogen, tPA, α_2-antiplasmin and thrombin were purchased from Calbiochem; chromozym tPA and Tween-80 were from Boehringer Mannheim; biocoated Matrigel invasion chambers and Nuserum were from Becton Dickinson; bovine muscle actin was from Sigma; and rabbit polyclonal anti-angiogenin antibody was provided by K. Olson (Harvard Medical School).

Cell Culture. Calf pulmonary artery endothelial cells and GM 7373 cells, the fetal bovine aortic endothelial cells transformed by benzo[*a*]pyrene, were routinely maintained as described (15). Cells at confluence were trypsinized, washed twice with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) Nuserum, 2 mM glutamine, and 1 μg of fungizone, 100 μg of streptomycin, and 100 units of penicillin per ml and were resuspended in the same medium for experiments. Angiogenin, when present,

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Abbreviations: ECM, extracellular matrix; PAI, plasminogen activator inhibitor; TGF-β, transforming growth factor β; tPA, tissue-type plasminogen activator.

was added at the concentrations indicated immediately after the cells were seeded.

Enzymatic Assay. After 24 hr of incubation with or without exogenous angiogenin, cells were washed twice with phosphate-buffered saline and solubilized with 1% Tween-80 in 50 mM Tris·HCl (pH 8.5). The proteolytic activity of this lysate was determined chromogenically with the use of chromozym tPA as the substrate. The tPA-specific substrate was used because previous experiments (18) had shown that angiogenin and the angiogenin–actin complex activate tPA, a key protease believed to be involved in angiogenesis.

Culture of Endothelial Cells on Fibrin Gel. Three-dimensional fibrin gels were prepared essentially as described (20). A suspension of 10^4 cells in 0.5 ml of DMEM containing 10% Nuserum was placed in each well. The culture medium and angiogenin were renewed every 48 hr. The cells and the fibrin gel were observed and photographed under a phase-contrast microscope.

Cell Invasion Assay. Invasiveness of endothelial cells was determined with a biocoated Matrigel invasion chamber. DMEM containing 10% Nuserum (0.5 ml) was added to the culture well that serves as the chamber holder, and 0.2 ml of a cell suspension containing 10^4 cells in DMEM containing 10% NuSerum was added to the chamber itself. Two methods were used to assess cell invasion. In the first, cells were incubated for 28 hr at 37°C, after which time the culture medium was removed and the cells were stained with Wright–Giemsa stain. Invasive cells that accumulate at the bottom side of the membrane were counted over the entire membrane area. In the second method, 5×10^5 cells were plated on a T-25 flask in 5 ml of DMEM containing 10% (vol/vol) fetal calf serum and 10 μCi (370 kBq) of [^{3}H]thymidine (NEN) and were incubated at 37°C for 48 hr. Such [^{3}H]-labeled cells (5 cpm per cell) were used to test the effect of angiogenin under the same conditions as those used with unlabeled cells. After 8 hr of incubation at 37°C, the cells that had invaded the membrane were eluted with trypsin and lysed with 0.5 M NaOH, and the radioactivity was measured by liquid scintillation counting (21).

RESULTS

Angiogenin Stimulates Proteolytic Activity in Endothelial Cells. Calf pulmonary artery endothelial and GM 7373 cells have been shown to express a cell-surface actin that serves as an angiogenin-binding protein (15–17). Both exogenous actin and an anti-actin antibody inhibit angiogenin-induced neovascularization on the chorioallantoic membrane (17) and abolish nuclear translocation of angiogenin by endothelial cells (14), presumably by interfering with the binding of angiogenin to cell-surface actin. How actin is linked to the cell surface is unknown, but when it binds angiogenin, some of the resultant angiogenin–actin complex dissociates from the cell surface and can be detected in the culture medium (15). Moreover, actin, angiogenin, and the complex all modulate the activities of both plasmin and tPA and thus could have a pronounced activation effect on this serine protease system. In the presence of the angiogenin–actin complex, for example, the overall proteolytic activity of a mixture of plasminogen and tPA is 11-fold higher than in its absence and is 6-fold higher than in the presence of actin alone (18). Since this observation was obtained through the use of an *in vitro* assay, we examined the effect of angiogenin on the proteolytic activity of endothelial cells in culture.

Angiogenin stimulated the cell-associated proteolytic activity of GM 7373 cells in a concentration-dependent manner (Fig. 1). When the tPA-specific peptide substrate chromozym tPA was used to determine protease activity in the cell lysates, the basal proteolytic activity was 0.75 milliunit/μg of protein, with human two-chain tPA as the standard. Stimulation was readily detected at an angiogenin concentration as low as 1 ng/ml (2.5 milliunits/μg of protein). In the presence of 10 ng of angiogenin per ml, the cell-associated proteolytic activity was increased 9-fold to 7 milliunits/μg of protein. Stimulation was maximal (10.5–12.5 milliunits/μg of protein) at an angiogenin concentration of 0.5 μg/ml and remained at this level to at least 50 μg/ml. Similar effects of angiogenin on the stimulation of cell-associated proteolytic activity were also observed with calf pulmonary artery endothelial cells (data not shown).

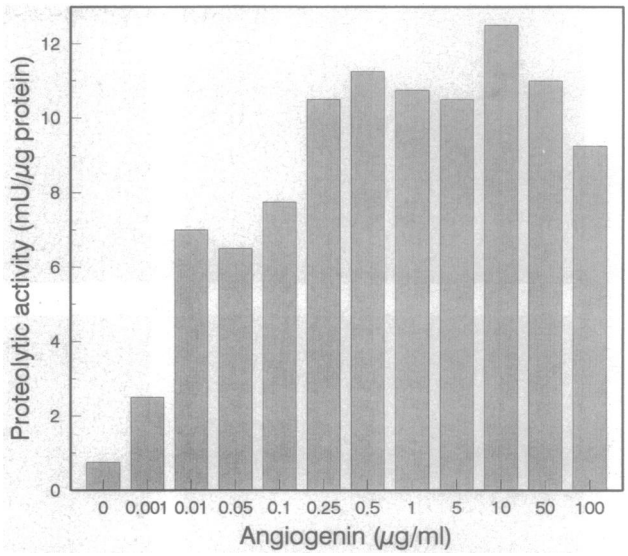

FIG. 1. Stimulation of cell-associated proteolytic activity of endothelial cells by angiogenin. GM 7373 cells were cultured in the presence of different concentrations of angiogenin for 28 hr. Proteolytic activities in cell lysates were measured spectrophotometrically by using as substrate the tPA-specific chromogenic peptide chromozym tPA and are expressed as milliunits of tPA per μg of protein. Each result is the average of eight measurements.

Induction of Fibrinolytic Activity of Endothelial Cells by Angiogenin. Since angiogenin and its complex with actin enhance the activity of the tPA/plasmin system *in vitro* (18) and stimulate the cell-associated (likely tPA/plasmin-related) proteolytic activity of endothelial cells in culture, we investigated their effect on the fibrinolytic activity of these cells because fibrin is the natural substrate for plasmin. For this purpose, the endothelial cells were grown on a three-dimensional fibrin gel. Fig. 2 shows a typical culture of GM 7373 cells on such gels with or without angiogenin. The cells had a normal, characteristically cuboidal morphology (22) and exhibited significant basal fibrinolytic activity: they could dissolve the fibrin gel even without addition of angiogenin. They formed a monolayer and dissolved the underlying gel homogeneously (Fig. 2 *A* and *C*). Small focal defects of the fibrin gel began to appear at day 3 (Fig. 2*E*), but complete dissolution of the gel required 10 days (not shown). In the presence of 1 μg of angiogenin per ml, dissolution of the gel was more heterogenous and occurred faster as evidenced by progressive reduction in its thickness. Focal defects already formed at day 2 (Fig. 2*D*) and were extensive at day 3 (Fig. 2*F*), and the gel was completely lysed at day 5. The results clearly indicate at least a 2-fold stimulation of the fibrinolytic activity of the GM 7373 cells by exogenous angiogenin.

The angiogenesis that occurs in pathological conditions (23) such as cell-mediated immune response, tumor growth, and wound healing is associated with abundant extravascular fibrin deposition (24). Thus, promotion of endothelial cell fibrinolysis may be one of the physiological functions of angiogenin. To quantify this effect, a fluorescent fibrin gel

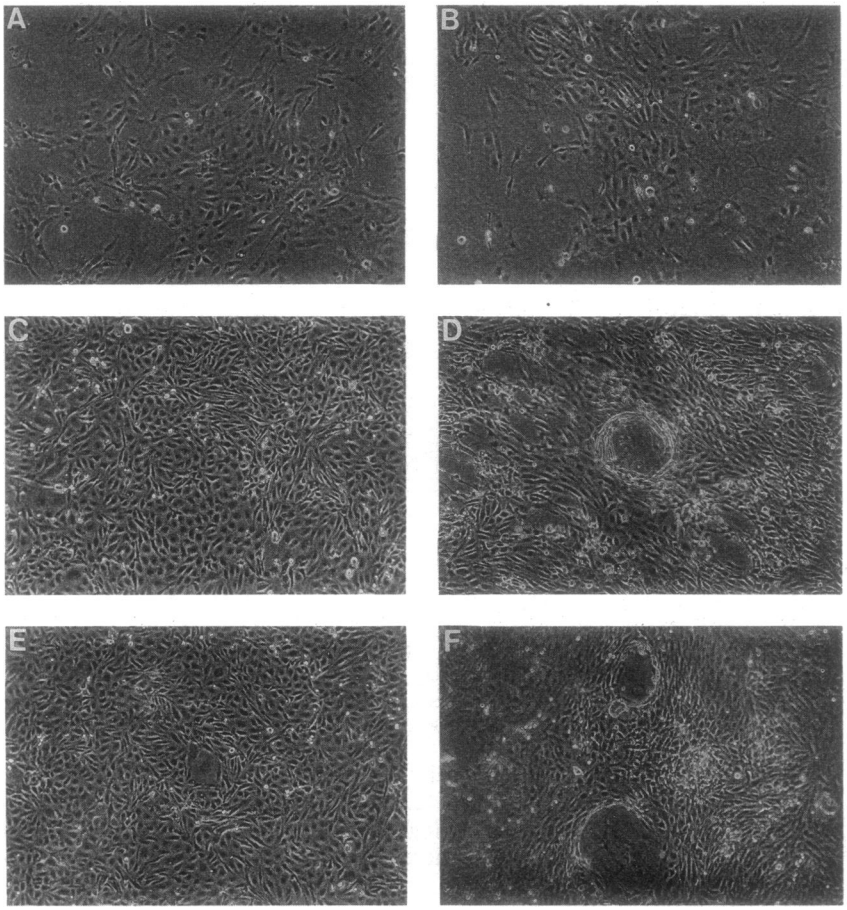

FIG. 2. Effect of angiogenin on endothelial cells cultured on fibrin gels. GM 7373 cells were cultured on three-dimensional fibrin gels in the absence (*A*, *C*, and *E*) or presence (*B*, *D*, and *F*) of 1 μg of angiogenin per ml. Phase-contrast microscopy pictures were taken after 1 (*A* and *B*), 2 (*C* and *D*), and 3 (*E* and *F*) days of incubation, respectively. (Original, ×100; here shown, ×45.)

was prepared by treating fluorescein isothiocyanate-labeled fibrinogen with thrombin. GM 7373 cells cultured on this gel in the presence of different concentrations of angiogenin generated fluorescent fibrin-degradation products and released them into the culture medium. The fluorescein isothiocyanate-labeled fibrin-degradation products were separated by SDS/PAGE, and their fluorescence was recorded with a charge-coupled device camera under UV illumination. They appeared as bright bands at the gel front. Their fluorescence intensities, determined by the fluorophore-assisted carbohydrate electrophoresis system (25), should reflect the fibrinolytic activity of the cells. The fibrinolytic activity of the cells was increased 1.7-, 2.3-, and 2.4-fold in the presence of 50, 100, and 200 ng of angiogenin per ml, respectively (Fig. 3). Maximal stimulation was obtained with angiogenin in the range of 0.4 to 6.4 μg/ml. In contrast to cell-associated proteolytic activity (Fig. 1), the fibrinolytic activity declined when the concentration of angiogenin exceeded 10 μg/ml.

Angiogenin Induces Endothelial Cells To Invade Matrigel. The effect of angiogenin on the invasiveness of endothelial cells was investigated with the use of Matrigel invasion chambers. In each experiment, a suspension of 10^4 cells in 0.2 ml, prepared as described in *Materials and Methods*, was seeded into the invasion chamber, and 0.5 ml of DMEM containing 10% Nuserum was added to the culture well chamber holder. The cells were then incubated in the presence or absence of 1 μg of angiogenin per ml at 37°C under humidified 5% CO_2/95% air. After 28 hr of incubation, an average of 230 cells were found on the angiogenin-treated chambers, whereas an average of 40 invasive cells were found on the bottom side of the membrane in the control invasion chambers. An anti-angiogenin polyclonal antibody inhibited angiogenin-induced endothelial cell invasion. As shown in Table 1, 60 μg of this antibody per ml decreased the amount of angiogenin-induced endothelial invasion to 60 cells. A nonspecific rabbit antibody at the same concentration was much less effective (148 cells). Addition of α_2-antiplasmin also inhibited cell invasion. In the presence of 10 milliunits of α_2-antiplasmin per ml, only 18 cells passed through the Matrigel, even less than in the control, indicating that plasmin is essential for both basal level and angiogenin-induced endothelial cell invasion.

In vitro, the angiogenin–actin complex is a more potent activator of the tPA/plasminogen system than the individual components. Consistent with this, the presence of both angiogenin and actin induced more invasion (292 invasive cells) than did angiogenin alone. Moreover, actin alone stimulated cell invasion only slightly (80 cells). These results imply that binding of angiogenin to cell surface actin and subsequent dissociation of the complex from the cell surface could be physiologically significant by modulating the tPA/plasmin serine protease system.

Since angiogenin was added only to the invasion chamber, invasion and accumulation of endothelial cells at the bottom of the membrane would not appear to require the presence of chemoattractants in the culture well. Addition of angiogenin to the culture well instead also stimulated endothelial cell invasion although less efficiently (data not shown). No additive effect was observed when angiogenin was added to both the invasion chamber and the culture well.

The stimulatory effect of angiogenin on endothelial cell invasion was also investigated with [^{3}H]thymidine-labeled GM 7373 cells. In this case an 8-hr incubation was found to give the clearest difference between treated and untreated cells. At longer periods of time, the background radioactivity became too high. Under these conditions we found that 1 μg of angiogenin per ml doubled the invasiveness of ^{3}H-labeled GM 7373 cells as measured by radioactivity (592 cpm in the presence of angiogenin and 291 cpm in the absence of angiogenin).

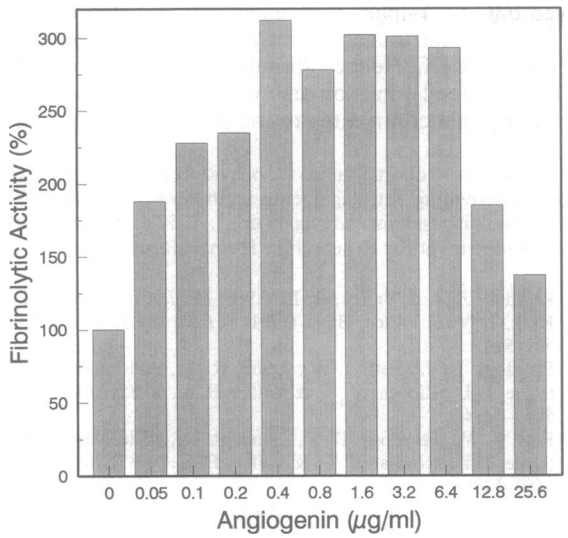

FIG. 3. Stimulation of fibrinolytic activity of endothelial cells by angiogenin. A 7-ml sample of fibrinogen (5 mg/ml, 0.82 μM) in phosphate-buffered saline was mixed with 390 μl of fluorescein isothiocyanate (1 mg/ml in dimethyl sulfoxide) and incubated at room temperature in the dark for 1 hr. The reaction mixture was dialyzed and filtered through a 0.8-μm membrane. Fluorescent fibrin gels were prepared by treating the fluorescein isothiocyanate-labeled fibrinogen with thrombin. GM 7373 cells were cultured on those gels in the presence of different concentrations of angiogenin. A 5-μl aliquot of cell culture medium was taken from each sample after 24 hr of incubation and subjected to SDS/PAGE analysis. The relative fluorescence intensities of the fibrin-degradation-product bands at the gel front, which were generated by the cells and should reflect their fibrinolytic activities, were measured by fluorophore-assisted carbohydrate electrophoresis instrumentation as described (25).

DISCUSSION

The process of angiogenesis is complex and consists, among others, of endothelial cell migration, proliferation, and differentiation (26). Repetitive cycling of these steps ultimately forms new vascular capillaries (27, 28). Under normal circumstances, endothelial cells are quiescent and are surrounded by basement membrane (a dense, impenetrable meshwork of collagen IV, laminin, fibronectin, entactin, and heparan sulfate proteoglycans), which normally is devoid of pores that would allow passive migration of cells. It is a prerequisite of angiogenesis, therefore, that endothelial cells acquire an invasive phenotype so that they can penetrate the basement membrane and interstitial matrix. This involves proteolytic degradation and can be accomplished by activation of cellular proteases. Numerous studies have attempted to correlate the angiogenic potential of endothelial cells with their capacity to degrade the ECM. Proteolytic enzymes of all four classes (serine, cysteine, aspartyl, and metallo) have been implicated in the process, and among these, central attention has focused on the plasmin/plasminogen activator system, which has been the subject of intensive research for the past decade (29, 30).

Table 1. Endothelial cell invasion of Matrigel basement membrane

Samples	Invasive cells,* no.
Control	40
Angiogenin (1 μg/ml)	230
+ anti-angiogenin IgG (60 μg/ml)†	60
+ nonspecific IgG (60 μg/ml)†	148
+ α_2-antiplasmin (20 μg/ml)†	18
+ actin (5 μg/ml)†	292
Actin (5 μg/ml)	80

*The invasive cells were stained and counted as described in *Materials and Methods*.
†Angiogenin was mixed with the indicated substance immediately before adding to the cells.

We have previously reported that angiogenin binds to cell-surface angiogenin-binding protein/actin and is then released from the cell surface as an angiogenin–actin complex (15, 16). Exogenous actin does not seem to inhibit angiogenin-induced invasion of endothelial cells into Matrigel. In fact, it increases the number of invasive cells somewhat, likely the result of its activation of tPA to produce plasmin from plasminogen (31). Its effect is limited, however, because actin simultaneously inhibits the resultant plasmin activity (32). *In vitro*, it only increases the overall proteolytic activity of a tPA/plasminogen mixture by 2-fold (18).

We have also reported that the angiogenin–actin complex similarly activates tPA to produce plasmin, but it does not inhibit plasmin activity. In this case the net increase in proteolytic activity of the tPA/plasminogen mixture induced by the complex is 12-fold higher than in its absence and 6-fold higher than in the presence of actin alone. The angiogenin–actin complex induces more cells to invade the Matrigel and migrate through the invasion chamber membrane than does angiogenin or actin alone. Thus, binding of angiogenin to cell-surface actin and subsequent dissociation of the angiogenin–actin complex could be an important step in angiogenin-stimulated endothelial cell invasion. Moreover, enzymatic activation of the tPA/plasmin system by angiogenin, actin, and the angiogenin–actin complex could contribute, at least in part, to the enhancement of cell-associated proteolytic activity.

In general, cell-associated protease activity reflects total proteolytic activity, and an increase could be a measure of induced protease synthesis (30). However, it has been shown that stimulation of enzyme activity does not necessarily mean activation of gene expression. For example, Gross *et al.* (33) reported that while phorbol 12-myristate 13-acetate stimulated the activity of endothelial cell-associated plasminogen activator by 2- to 10-fold, it inhibited the incorporation of [^{3}H]uridine into acid-soluble material by 25%. A more plausible mechanism for stimulation of cell-associated proteolytic activity is direct activation of the endogenous enzymes by angiogenin or the angiogenin–actin complex, since it is known to occur *in vitro* (18). Such direct activation likely accounts for at least part of the increased proteolytic activity of endothelial cells. Additional, preliminary experiments have shown that the effect of angiogenin on cell-associated tPA activity may be either stimulatory or inhibitory, depending on cell stage and confluence (data not shown). The reason for this variation and the mechanism by which angiogenin modulates cell-associated proteolytic activity are under investigation.

Actin and an anti-actin antibody inhibit both angiogenin-induced neovascularization in the chorioallantoic membrane and nuclear translocation of angiogenin by endothelial cells (14, 16). This is not inconsistent with our finding that the angiogenin–actin complex stimulates cell invasion here. Angiogenesis is the consequence of multiple cellular actions among which basement membrane degradation and cell invasion might be necessary but not sufficient for angiogenin-induced angiogenesis. The distinct ribonucleolytic activity of angiogenin (34) and its other known functions including stimulation of second messengers (10, 11), nuclear translocation and nucleolar accumulation (16), and promotion of cell adhesion (13) suggest that it serves multiple roles.

The complete inhibition of angiogenin-induced endothelial cell invasion by α_2-antiplasmin indicates that plasmin is essential for this process and that angiogenin acts via the plasminogen activator/plasmin protease system. The sub-

strate specificity of plasmin is high. It can digest the major components of basement membrane either by acting directly on fibronectin and laminin or by activating procollagenase (35). Further, it can convert transforming growth factor β (TGF-β) from the latent to the active TGF-β form (36). TGF-β is known to promote a series of cellular events in a variety of cell lines including monocytes and endothelial cells and has a significant role in the process of cell migration and ECM degradation. It stimulates the synthesis of both 92-kDa and 72-kDa gelatinase/type IV collagenases, increases the expression of α and β integrin subunits, and modulates cell binding to extracellular matrix constituents (37). Therefore, plasmin generation and activation could be crucial steps in the process of angiogenesis.

The angiogenin stimulation of both cell-associated proteolytic and fibrinolytic activities is dose-dependent. Activation of proteolytic activity can be detected readily at an angiogenin concentration of 1 ng/ml, reaches maximum at about 250 ng/ml, and remains unchanged up to at least 50 μg/ml. Activation of fibrinolytic activity, on the other hand, is seen at 50 ng/ml, is maximal at about 400 ng/ml, and begins to decrease above 6.4 μg/ml. The difference in dose dependence may simply reflect the fact that the two assays measure different enzymes. Alternatively, it may reflect the *in vivo* protease/antiprotease balance. It has been shown that the plasminogen activator/plasmin system is regulated at multiple levels including gene activation and transcription, mRNA stability, proenzyme translation and secretion, zymogen activation, localization and receptor binding of both proenzymes and enzymes on the cell surface or ECM components, inhibition by specific inhibitors, and degradation or removal of active enzymes. Among these, the specific plasminogen activator inhibitors (PAIs, which include PAI-1, PAI-2, and protease nexin-1) provide the fastest response. They bind covalently to the active site of plasminogen activators, thus neutralizing their activities (38). Both basic fibroblast growth factor and TGF-β_1 increase the synthesis of urokinase-type plasminogen activator as well as PAI-1 (39). However, while basic fibroblast growth factor stimulates more urokinase-type plasminogen activator than PAI-1, thus increasing net proteolytic activity and hence endothelial cell invasion, TGF-β_1 induces more PAI-1 than urokinase-type plasminogen activator and hence inhibits basic fibroblast growth factor-induced formation of tube-like structures in endothelial cell cultures. The simultaneous induction of PAI-1 may serve a protective function against nonspecific proteolysis. It is not as yet known if angiogenin stimulates both PA and PAI activities. If it does, there may be a concentration-dependence with lower concentrations favoring PA and higher concentrations favoring PAI. The cell-associated tPA assay may fail to discriminate the difference because of the conditions used to assay the cell lysates. It is known that PAI-1 is synthesized as an active molecule but is unstable and decays rapidly to an inactive form in solution. Therefore, PAI activity may not be detectable in cell lysates. The assay for fibrinolytic activity measures fluorescein isothiocyanate-labeled fibrin-degradation products generated by viable cultured cells and may more truly reflect cell-associated proteolytic/fibrinolytic activity.

Cell invasion itself is a multistep process involving attachment, local proteolysis, and migration. For invasion to take place, cyclic attachment to the ECM components and subsequent release from its degradation fragments must occur in a directed and controlled manner. Angiogenin supports adhesion of both endothelial and tumor cells when it is coated on a plastic surface. It does not mediate cell attachment when present only in solution (13). This behavior of angiogenin not only suggests that it may play a role in tumor metastasis where specific cell–cell and cell–matrix interactions are involved but also implies that angiogenin-induced endothelial cell invasion can occur in certain directions governed by angiogenin itself. Hence, stimulation of cell invasion and mediation of cell adhesion are two closely related functions that angiogenin contributes to the process of angiogenesis.

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