Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 2232–2236, March 1992
Cell Biology

Angiogenin supports endothelial and fibroblast cell adhesion

(angiogenesis/extracellular matrix/receptors)

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Communicated by Bert L. Vallee, December 20, 1991

ABSTRACT **When coated on bacteriological plastic at doses ≥0.1 μg/cm², human and bovine angiogenin support calf pulmonary artery endothelial and Chinese hamster fibroblast cell adhesion and spreading, but do not affect cell adhesion when in solution. The kinetics of endothelial cell attachment to angiogenin are indistinguishable from those in the presence of gelatin. Calcium and/or magnesium ions are critical for cell adhesion or spreading onto angiogenin but protein synthesis and glycoprotein secretion are not necessary. Adhesion to angiogenin is not altered by the addition to the incubation solution of fibronectin, fibrinogen, laminin, collagen I and IV, or vitronectin. The peptide Arg-Gly-Asp-Ser inhibits endothelial cell response to angiogenin whereas the reverse peptide Ser-Asp-Gly-Arg-Gly has no effect. These findings show that angiogenin can serve as an effective substratum for cell adhesion by inducing an interaction similar to but independent from that of other extracellular matrix molecules. Induction of cell adhesion and subsequent migration may be critical steps in the process of angiogenesis.**

Angiogenesis, the formation of new blood vessels, underlies organogenesis in general and tumorigenesis in particular. It occurs by the outgrowth of new capillaries from established blood vessels through a process that involves digestion of the extracellular matrix (ECM) and subsequent migration, proliferation, and differentiation of the endothelial cells into tube-like structures (1). It can be induced by a variety of proteins including basic and acidic fibroblast growth factors (FGFs), transforming growth factor β, tumor necrosis factor α, vascular endothelial cell growth factor, and angiogenin (2, 3). Save for angiogenin, all of these angiogenic proteins are known to modulate endothelial cell growth directly. Angiogenin is a 14-kDa protein first purified from tumor cell-conditioned medium based on its capacity to induce neovascularization on the chicken embryo chorioallantoic membrane. While it is one of the most potent angiogenic proteins (3), it has not been found thus far to promote endothelial cell proliferation (4). It does appear to interact with these cells via a specific receptor(s), since it has been shown to activate their phospholipase pathways (5, 6) and bind specifically to their surface (7). Indeed, an angiogenin-binding protein on the endothelial cell surface was recently identified (8).

Angiogenin, FGFs, and transforming growth factor β have all been shown to bind to ECM components (7, 9, 10). The interrelations between angiogenic proteins and ECM molecules are complex. Thus, active FGF is released by endothelial cells into their ECM, which can serve as a site for its extracellular storage *in vitro* (11–13). Depending on the suitability of the substratum for endothelial cell adhesion and spreading, FGF can act *in vitro* either as a mitogen or as a differentiating agent (14–16). Moreover, FGF can modulate the production of ECM molecules; e.g., the production of fibronectin is diminished when endothelial cells are grown in the presence of FGF (17). In contrast, transforming growth factor β induces fibronectin and collagen expression in various cell types (18). Among ECM molecules, fibronectin, when presented as a substratum, modulates endothelial cell shape and DNA synthesis in the presence of basic FGF *in vitro* (19), and laminin itself induces capillary formation *in vitro* (20).

Thus, the differentiation of capillaries *in vitro* depends on the conditions of endothelial cell growth, adhesion, and spreading, all of which are controlled by the substratum on which the cells are grown (16, 21). Their growth factors can themselves serve as substrata for endothelial cells. Indeed, FGF has been shown to support endothelial cell adhesion (22). Since angiogenin can bind to the ECM (7), the present experiments were undertaken to investigate its role in cell adhesion. The results demonstrate that angiogenin supports endothelial and fibroblast cell adhesion and spreading. In this regard, these properties are found to be similar to but apparently independent from those of the common ECM molecules.

MATERIALS AND METHODS

Materials. Bovine angiogenin (bAng) was purified from milk (23), and recombinant human [Met^{-1}]angiogenin (hAng) was produced in *Escherichia coli* as described (24). Ribonuclease A (RNase A), fibronectin, fibrinogen, laminin, vitronectin, collagens I and IV, gelatin, Arg-Gly-Asp-Ser (RGDS) and Ser-Asp-Gly-Arg-Gly (SDGRG) peptides, cycloheximide, and monensin were from Sigma. Rabbit polyclonal antibodies directed against hAng and rabbit control antibodies (Organon Teknika) were purified from the antiserum by ammonium sulfate precipitation and protein A-Sepharose affinity chromatography.

Cell Culture. Calf pulmonary artery endothelial (CPAE) cells, CCL 209 from the American Type Culture Collection, were cultured in 75-cm² culture flasks (Nunc) in minimum essential medium (MEM, GIBCO) with 20% heat-inactivated fetal bovine serum (GIBCO), 50 units of penicillin per ml, and 50 μg of streptomycin per ml in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells between passages 21 and 26 were used. At higher passages, they were found to adhere spontaneously to bacteriological plastic. Dede Chinese hamster lung fibroblasts (ATCC CCL 39) were cultured under the same conditions in Dulbecco's modified MEM (DMEM, GIBCO) with 25 mM glucose, 10% fetal bovine serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml.

Coating of Petri Dishes. Bacteriological Petri dishes (35-mm diameter, Falcon no. 1008) were incubated with angiogenin or RNase A in 1 ml or 1.5 ml of Dulbecco's phosphate-buffered saline (DPBS, Whittaker Bioproducts) or as recommended by the manufacturer for coating overnight at 4°C and were

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Abbreviations: bAng, bovine angiogenin; hAng, human angiogenin; BSA, bovine serum albumin; CPAE, calf pulmonary artery endothelial; DPBS, Dulbecco's phosphate-buffered saline; ECM, extracellular matrix; FGF, fibroblast growth factor.

rinsed in DPBS. An estimation of angiogenin coating efficiency estimated by enzyme-linked immunosorbent assay (K. A. Olson, personal communication) gave 99.9% and 93% when the plates were treated with, respectively, 1 μg and 10 μg of hAng. For RNase A, the coating efficiency was estimated by assaying the supernatants for enzymatic activity toward cytidylyl(3',5')adenosine (25). The efficiency was 86% and 36% when the plates were incubated with, respectively, 1 μg and 5 μg of RNase A.

Adhesion Assay. Confluent cell monolayers were harvested with a trypsin/EDTA mixture (Whittaker Bioproducts), suspended in fresh culture medium containing serum, and centrifuged for 5 min at 2000 × *g*. The cell pellet was washed three times in medium containing 0.1% bovine serum albumin (BSA fraction V, low endotoxin, Sigma), without serum (MEM/BSA for CPAE cells, DMEM/BSA for Chinese hamster lung fibroblasts, 5.4 mM KCl/0.1 M NaCl/26.2 mM $NaHCO_3$/5.6 mM glucose/0.1% BSA for the Ca^{2+}/Mg^{2+} experiments). Cells were seeded at 30,000 cells per ml in 1 ml of medium/BSA and incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C for 6 hr, except when stated otherwise. The cells were then washed three times with DPBS, fixed for 15 min in DPBS/3.7% formaldehyde, and stained by the methylene blue method (26). The absorbance was measured at 600 nm with a Gilford model 250 spectrophotometer. It was established that the A_{600} value varied linearly with cell number and that an A_{600} of 0.1 represented ≈14,000 CPAE cells.

RESULTS

Cell Adhesion onto Angiogenin. When CPAE cell monolayers were trypsinized and washed to remove any serum components that could induce cell adhesion, the individualized cells did not adhere to an uncoated bacteriological plastic surface. However, when the surface was precoated with bAng at 1 μg/cm², the cells did adhere and spread on this substratum (Fig. 1). Adherent cells are also referred to "positive" or "responsive" cells. After subtraction of the A_{600} value for uncoated plastic, the number of cells that responded to bAng when the dishes were precoated with bAng solution at ≥1 μg/ml (0.1 μg/cm²) was the same as for adherence to gelatin (Fig. 2). This value was taken as 100%. When plates were precoated with the homologous protein RNase A, up to 0.18 μg/cm² (corrected by the coating efficiency, see *Materials and Methods*), only 20% of the cells were positive (Fig. 2). CPAE cells added to uncoated plastic dishes but in an incubation solution containing either bAng or RNase A at 0.1–5 μg/ml, corresponding to the above coating doses, failed to adhere (Fig. 2).

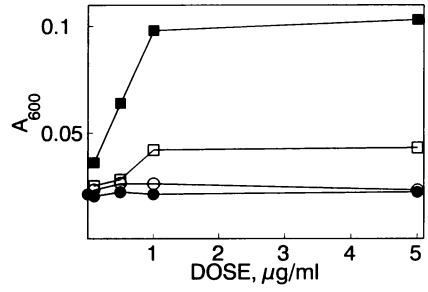

FIG. 2. Effect of bAng and RNase A on cell adhesion. CPAE cells were added to uncoated plastic dishes in the presence of bAng (●) or RNase A (○) in solution or to dishes precoated with bAng (■) or RNase A (□). Adherent cells were fixed and stained with methylene blue, and the absorbance of acid-released dye was determined at 600 nm.

Kinetics of Adhesion onto Angiogenin. Virtually no cells adhered to uncoated plastic after 18 hr of incubation (Fig. 3). In contrast, 50% and 90% of the cells responded to bAng-coated dishes within 2 hr and 5 hr, respectively, compared with the value obtained at 18 hr for gelatin-coated dishes (≈17,000 cells per dish). The kinetics and number of positive cells were identical for both bAng and gelatin. In each case there was a consistent decrease in the number of adherent cells after 6 hr of incubation, which might reflect a temporary modification of the adhesion properties of the cells.

In a parallel experiment, prior to trypsinization, the cell monolayer was exposed to bAng at 1 μg/ml for 1 hr. The cells were then detached for the assay on bAng-coated dishes, but without bAng in the incubation solution. This preexposure did not affect the kinetics of CPAE cell adhesion onto bAng or gelatin but did increase the number of cells attached to bAng (Fig. 3) or gelatin (data not shown). The number of positive cells at 18 hr was close to that initially added.

Effect of Inhibitors. Pretreatment of the cells with 25 μM cycloheximide followed by an assay with bAng at 0.1 μg/cm² in the presence of the inhibitor neither changed the kinetics nor changed the number of adherent and spread cells (A_{600} was 0.115 ± 0.25 and 0.129 ± 0.018 after a 4.5-hr assay in the absence and presence, respectively, of cycloheximide). When assayed in the presence of monensin (1 μg/ml), an inhibitor of glycoprotein secretion (26), the cells still adhered and spread on bAng (A_{600} values for untreated plastic were 0.044 ± 0.006; for bAng, 0.146 ± 0.006; and for bAng with monensin, 0.125 ± 0.002). Hence, CPAE cell adhesion and spreading onto bAng require neither protein synthesis nor glycoprotein secretion.

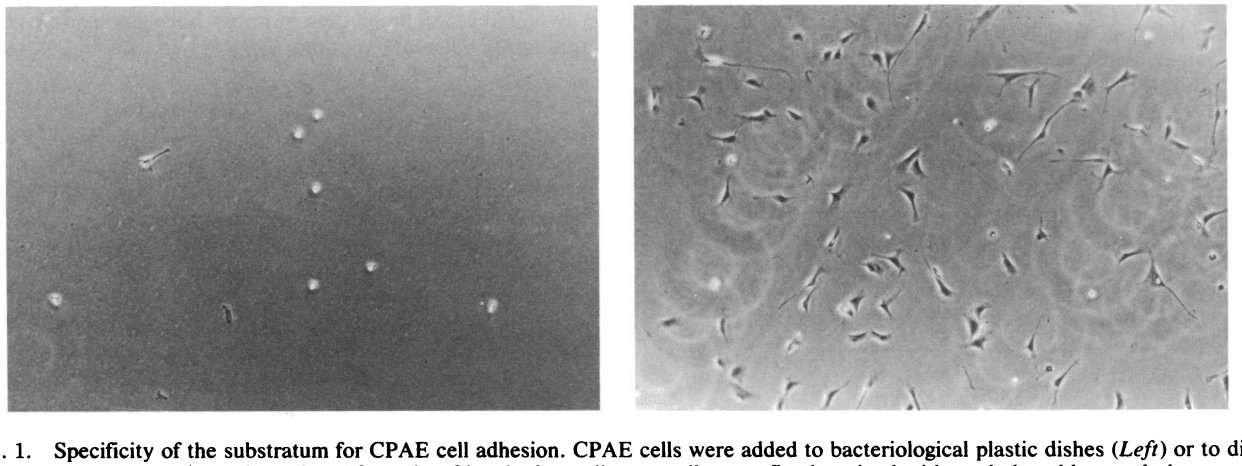

FIG. 1. Specificity of the substratum for CPAE cell adhesion. CPAE cells were added to bacteriological plastic dishes (*Left*) or to dishes coated with bAng, 10 μg/1.5 ml (*Right*). After 6 hr of incubation, adherent cells were fixed, stained with methylene blue, and photographed with a Nikon microscope. (×55.)

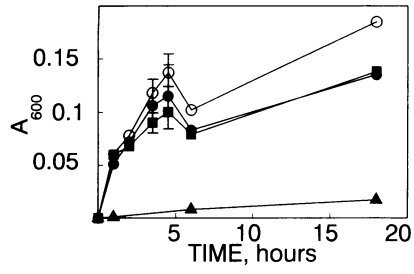

FIG. 3. Kinetics of adhesion of CPAE cells. CPAE cells were added to bAng-coated (1 $\mu g/cm^2$) (●) or 0.2% gelatin-coated (■) dishes or to uncoated plastic (▲). For the pretreatment experiment (○), the CPAE cell monolayers were rinsed three times with DPBS and incubated in MEM/BSA containing bAng (1 $\mu g/ml$) for 1 hr. The cells were then harvested and added to bAng-coated dishes as in Fig. 2.

Specificity of Adhesion. Although hAng promoted the same degree of CPAE cell attachment as bAng, a somewhat higher amount of coating was required for a maximal effect (Fig. 4*A*). The number of positive cells reached a plateau with bAng at 0.1 $\mu g/cm^2$ and with hAng at 0.5 $\mu g/cm^2$. Since in both cases essentially all of the angiogenin adsorbs to the plastic at the coating concentrations used (see *Materials and Methods*), difference in the coating efficiency does not account for this observation.

When added together with CPAE cells to hAng-precoated dishes, an anti-hAng IgG antibody fraction (50 $\mu g/ml$) completely prevented cell adherence. In contrast, at an identical concentration, nonspecific IgG had only a minor effect (A_{600} = 0.137 ± 0.003 in the absence of antibodies, 0.017 ± 0.002 with specific IgG, and 0.107 ± 0.004 with nonspecific IgG).

Chinese hamster lung fibroblasts lack high-affinity binding sites for hAng (7). However, both bAng and hAng supported adhesion of these cells whereas RNase A did not (Fig. 4*B*). Moreover, while CPAE cells prefer bAng, hAng was more effective for Chinese hamster fibroblasts.

Factors Influencing Cell Adhesion onto bAng. Laminin and fibronectin enhance carcinoma cell adhesion to collagen IV (27), but fibronectin, laminin, fibrinogen, collagen I and IV, or vitronectin in solutions at 1, 5, or 10 $\mu g/ml$ neither increased nor decreased the number of bAng-responsive cells (A_{600} = 0.129 ± 0.009 on bAng, at 0.1 $\mu g/cm^2$, mean A_{600} = 0.134 ± 0.020 on bAng in the presence of different concentrations of ECM molecules). On the other hand, all of them supported CPAE cell attachment when precoated onto plastic dishes at 1 $\mu g/cm^2$ (mean A_{600} = 0.170 ± 0.013).

bAng contains an RGD sequence (23), considered to be a major cell–matrix molecule interaction site (28). The peptide RGDS at ≥0.05 mM almost completely inhibited cell adhesion onto bAng-coated dishes (Fig. 5). The control peptide SDGRG, used in the same concentration range, had no effect.

Ca^{2+} and Mg^{2+} are generally thought to be critical for cell adhesion (28). In the experiments described thus far, Ca^{2+} and Mg^{2+} were present in the incubation solutions (MEM/BSA). When CPAE cells were added to bAng-coated dishes in medium prepared without addition of these metal ions, the cells did not respond (Fig. 6). However, when either calcium (1.4 mM) or magnesium (1.8 mM) was added to the incubation solution, cell adherence was restored to equal that in MEM/BSA.

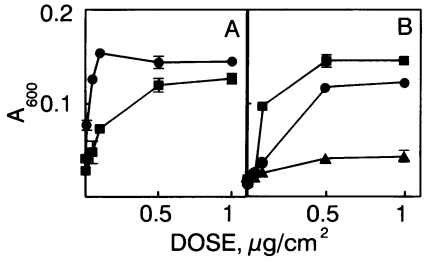

FIG. 4. Dose effect of precoated bAng, hAng, and RNase A on cell adhesion. CPAE (*A*) or hamster fibroblast (*B*) cells were added to plastic wells precoated with bAng (●), hAng (■), or RNase A (▲).

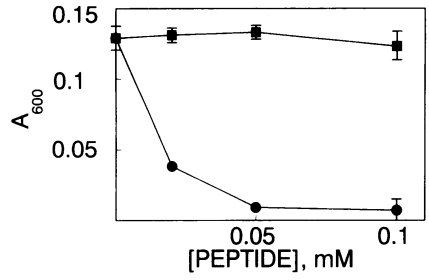

FIG. 5. Effect of RGDS and SDGRG peptides on CPAE cell adhesion. CPAE cells were added to bAng-coated wells (0.1 $\mu g/cm^2$) in 1 ml of MEM/BSA alone or with SDGRG (■) or RGDS (●) peptides.

DISCUSSION

Angiogenin is a potent inducer of neovascularization, but details as to its mechanism of action are sparse. The present results demonstrate that although trypsinized endothelial cells are unable to adhere to uncoated bacteriological plastic surfaces, they can fully adhere and spread on dishes precoated with angiogenin. This raises three important questions: Is angiogenin directly responsible for cell adhesion? What is the nature of the corresponding receptors present on endothelial cells? How does this relate to angiogenesis?

Cell adhesion may occur by direct interaction of endothelial cells with angiogenin as the substratum or through angiogenin-induced synthesis and deposition of some other adhesive molecules that in turn mediate the attachment of the cells. If angiogenin acts by the latter mechanism, its effect should be the same when it is present in the incubation medium and when it forms a substratum. However, angiogenin did not induce endothelial cell response when dissolved. Only when presented as a substratum did it support cell adhesion. Moreover, the cells responded to angiogenin even in the presence of protein synthesis or glycoprotein secretion inhibitors, indicating that expression of new molecules on the cell surface is not required. Further, this suggests that the cell receptors can recognize immobilized angiogenin and that these structures are expressed on the cell monolayer before it is harvested.

Not all ECM molecules support both adhesion and cell spreading. Thrombospondin supports endothelial as well as

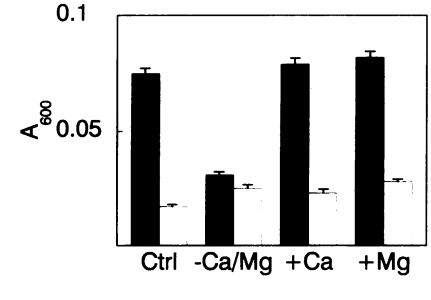

FIG. 6. Effect of Ca^{2+} and Mg^{2+} ions on CPAE adhesion. CPAE cells were incubated in MEM salts/BSA without Ca^{2+} or Mg^{2+} or with 1.4 mM $CaCl_2$, or 1.8 mM $MgCl_2$, or in MEM/BSA (control, Ctrl). Filled bars, bAng-precoated plates (0.1 $\mu g/cm^2$); open bars, uncoated plastic.

smooth muscle cell adhesion, but <5% of these cells were able to spread on this substratum (29). Moreover, although endothelial cell adhesion onto fibronectin or type IV collagen does not require protein synthesis, cycloheximide prevents spreading on both molecules (30). This inhibitor did not, however, affect the spreading of CPAE cells on bAng under our conditions.

The cell membrane structures that recognize angiogenin adsorbed on plastic are not known. Cell adhesion to ECM molecules is mediated by specific receptors that are present on the cell surface (see ref. 31 for review). Two types of receptors for angiogenin, with high and low affinity, have been shown to be present on CPAE cell monolayers (7). Since Chinese hamster lung fibroblasts, which lack high-affinity sites for angiogenin (7), respond to angiogenin, these sites are not necessary for cell adhesion. It may well be that it is the low-affinity/high-capacity sites present on both endothelial and fibroblast cells that are responsible for these interactions. The increased number of bAng-responsive cells after pretreatment of monolayers with angiogenin (Fig. 3) may reflect an increased number of receptors, perhaps due to stimulated expression. In addition, angiogenin pretreatment also increased the number of cells adherent to gelatin, and this effect might reflect a role of angiogenin in cell adhesion on other substrates.

Adhesion molecules such as fibronectin (32), laminin (33), and collagens (34) are present in the ECM of endothelial cells. The adhesion of CPAE cells onto all these molecules was seen in the present study, consistent with the previously observed adhesion of bovine adrenal endothelial cells to the ECM and to purified ECM molecules (30). Many of these molecules interact both with the cell surface, through integrins, and with each other, reflecting the presence of cell surface integrins that can bind multiple ligands (31). Such a common structure may not mediate CPAE adhesion onto angiogenin, because these molecules do not affect cell binding to angiogenin.

Ca^{2+} and Mg^{2+} are essential for cell adhesion to ECM molecules (see ref. 28 for review). EDTA and EGTA inhibit endothelial cell response to fibronectin and vitronectin (35) and to thrombospondin (29), respectively, whereas Ca^{2+} is necessary both for the interaction of thrombospondin with the glycoprotein IIb/IIIa integrin (36) and for endothelial cell adhesion onto thrombin (26). To obviate the uncertainty associated with the use of nonspecific metal chelators such as EDTA and EGTA, assays were performed in media with or without Ca^{2+} or Mg^{2+}. In the absence of both ions there was little CPAE cell response to angiogenin, in contrast with full attachment in the presence of either metal ion. Thus Ca^{2+} and/or Mg^{2+} have an important role in the CPAE–angiogenin interaction much as they do in other cell–matrix molecule interactions.

The RGD sequence is essential for the interaction between ECM molecules and their cell receptors (28), based on inhibition of endothelial cell adhesion to fibronectin, laminin, and fibrinogen (37), to thrombin (26), or to thrombospondin (29) when oligopeptides containing this sequence are present at submillimolar concentrations in solution, as is also the case for inhibition of CPAE cell adhesion to bAng. However, angiogenins from species other than bovine, including hAng (38), do not contain an RGD sequence. Nevertheless, RGDS inhibits CPAE adhesion onto hAng (data not shown).

Angiogenin shares 35% sequence identity with RNase A (38), which neither is angiogenic nor specifically binds to endothelial cells (7). This, together with the present observation that CPAE cells are unable to adhere to dishes precoated with RNase at doses where angiogenin is effective, suggests a specific interaction of endothelial cells with angiogenin. Moreover, the dramatic inhibition of cell adhesion observed in the presence of hAng-specific antibodies confirms that the cells interact directly with angiogenin. Angiogenin exhibits ribonucleolytic activity (39) that is a requisite for its angiogenic activity (36, 40). The enzymatic active site and receptor binding site are at least partially distinct (41), but the enzymatic capacity is not known to be necessary for cell adhesion.

Endothelial cell surfaces are polarized both *in vivo* and *in vitro* (21, 42). Angiogenin specifically binds to confluent endothelial cells *in vitro* and induces second-messenger pathways (5–7), but it is not known whether this protein is active on the luminal surface of cells *in vivo*. Angiogenin is present in circulating blood plasma and, hence, is continually in contact with the luminal face of endothelial cells (43). The activity of an angiogenic molecule that is abundant in the circulation must be well controlled to preclude spurious angiogenesis and endothelial cell stimulation, but thus far, there is no information about the manner in which such inhibition and/or activation of angiogenin in blood is achieved. Angiogenin is fully active when purified from plasma (43) and does not appear to be tightly associated with any carrier or inhibiting molecule in the circulation. Alternatively, the action of angiogenin in the circulation could be limited by the absence of functional receptors on the luminal cell surface under normal circumstances—i.e., when the endothelium is intact. Angiogenin also binds to the ECM (7) and, as shown here, the molecule itself can act as a matrix component. Angiogenin can come in contact with the endothelium ECM *in vivo* only when the endothelium is denuded accidentally. It may then bind to this ECM and induce endothelial repair by providing a substrate for cell adhesion and migration, which are particularly important in the process of angiogenesis.

Effectors of angiogenesis could also modulate the endothelial cell–matrix interaction. FGF, an angiogenic molecule, and thrombospondin, an inhibitor of angiogenesis *in vivo*, support endothelial cell adhesion (22, 44, 45). Laminin, which supports cell adhesion, also induces the formation of capillaries *in vitro* (20). A role of the adhesive property of angiogenin in endothelial cell stimulation and differentiation during angiogenesis has not been demonstrated. The localization of the sites for cell adhesion and their relation to the ribonucleolytic activity of the molecule as well as the characterization of the receptors involved in this effect are of great importance for understanding the role of angiogenin *in vivo* and remain to be investigated.

I thank Dr. Karen A. Olson for performing ELISAs and Dr. Robert Shapiro for ribonuclease assays. I thank Drs. James W. Fett and Robert Shapiro for many valuable discussions. The support and advice of Dr. Bert L. Vallee are gratefully appreciated. This work was supported by funds from Hoechst under agreements with Harvard University.

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