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Angiogenin Activates Erk1/2 in Human Umbilical Vein Endothelial Cells

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Angiogenin is a potent angiogenic factor that binds to endothelial cells and is endocytosed and rapidly translocated to the nucleus where it is concentrated in the nucleolus and binds to DNA. Angiogenin also activates cell-associated proteases, induces cell invasion and migration, stimulates cell proliferation, and organizes cultured cells to form tubular structures. The intracellular signaling pathways that mediate these various cellular responses are not well understood. Here we report that angiogenin induces transient phosphorylation of extracellular signal-related kinase1/2 (Erk1/2) in cultured human umbilical vein endothelial cells. Angiogenin does not affect the phosphorylation status of stress-associated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p38 mitogen-activated protein (MAP) kinases. PD98059—a specific inhibitor of MAP or Erk kinase 1 (MEK 1), the upstream kinase that phosphorylates Erk1/2—abolishes angiogenin-induced Erk phosphorylation and cell proliferation without affecting nuclear translocation of angiogenin. In contrast, neomycin, a known inhibitor of nuclear translocation and cell proliferation, does not interfere with angiogenin-induced Erk1/2 phosphorylation. These data indicate that both intracellular signaling pathways and direct nuclear functions of angiogenin are required for angiogenin-induced cell proliferation and angiogenesis. © 2001 Academic Press

The angiogenic function of angiogenin is mediated by a number of cellular activities that are stimulated when the protein binds to endothelial cells. Subconfluent endothelial cells express on their surface an angiogenin binding protein (1) that has been identified as an α-smooth muscle type actin (2). Upon binding of angiogenin to actin, the resultant complex dissociates from the cell surface to activate the tissue plasminogen activator/plasmin protease system (3) that, in turn, promotes the invasiveness of endothelial cells (4). When endothelial cells are cultured under sparse density ($<2 \times 10^4$ cells/cm^2), they express a 170 kDa putative cell surface receptor to which angiogenin binds and induces cell proliferation (5). The binding protein and the putative receptor, which mediate different but equally necessary cellular activities for angiogenesis, do not seem to be expressed simultaneously on the cell surface. Their cell density-dependent expression appears to reflect the differential activities of angiogenin that have been related to different phases of the angiogenesis process where cells of different density play different roles (5). However, the intracellular signaling pathways that are responsible for these various cellular activities remain to be understood.

Angiogenin undergoes nuclear translocation in proliferating endothelial cells (6) in a process that is mediated by its nuclear localization sequence (7) but independent of lysosomal processing and the cellular microtubule system (8). Inhibition of nuclear translocation by neomycin (9) or through site-directed mutagenesis (6) abolishes angiogenin-induced angiogenesis indicating that nuclear translocation is essential. Nuclear translocation of angiogenin is also dependent on cell density. The amount of angiogenin that maximally accumulates in the nucleus decreases as the cell density increases (10). Again, it remains to be elucidated whether intracellular signals are required for nuclear translocation and whether nuclear translocation of angiogenin activates some signaling pathways in the cells.

Mitogen-activated protein (MAP) kinases are a class of signaling protein kinases that are activated by diverse extracellular stimuli and by protooncogene prod-

Abbreviations used: bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; Erk, extracellular signal-regulated kinase, FBS, fetal bovine serum; HE-SFM, human endothelial serum-free medium; HUVE, human umbilical vein endothelial; MAP, mitogen-activated protein; MEK, MAP or Erk kinase; PBS, phosphate-buffered saline; SAPK/JNK, stress-associated protein kinase/c-Jun N-terminal kinase.

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ucts (11). In endothelial cells, at least three subtypes of MAP kinase are known to be activated under different circumstances. Stress-associated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) has been shown to regulate expression of gene products involved in stress-related events (12). p38 MAP kinase activation mediates remodeling of the actin cytoskeleton, cell adhesion, and migration (13, 14). Extracellular signal-regulated kinase 1 and 2 (Erk1/2) has been shown to be involved in cell proliferation (15). Here we investigated the effect of human angiogenin on MAP kinase activation in cultured human umbilical vein endothelial (HUVE) cells. The data show that angiogenin induces phosphorylation of Erk1/2 in HUVE cells without affecting that of SAPK/JNK and p38 MAP kinase and that angiogenin-induced Erk1/2 phosphorylation is required for cell proliferation but is unrelated to nuclear translocation of angiogenin.

MATERIALS AND METHODS

Materials. Recombinant human angiogenin was expressed and isolated as described (16). Angiogenin variant R33A in which arginine-33 has been replaced by alanine by means of site-directed mutagenesis was provided by R. Shapiro (Harvard Medical School). The anti-human angiogenin monoclonal antibody 26-2F (17) was provided by K. A. Olson (Harvard Medical School). bFGF was from Promega. Human endothelial serum-free medium (HE-SFM) was from Gibco BRL Life Technologies. Fetal bovine serum (FBS) was from BioWhittaker. PD98059 and Western blotting detection kits for phosphorylated Erk1/2, SAPK/JNK, and p38 MAP kinase were from New England Biolabs. Neomycin sulfate was from Sigma. Alexa 488 goat anti-mouse IgG was from Molecular Probes.

Cell culture. HUVE cells were purchased from Cell Systems Corp. (Kirkland, WA) and were cultured in HE-SFM supplemented with 5% FBS and 5 ng/ml bFGF at 37°C under 5% humidified CO_2. Medium was changed every two days. Cells from passages 3 to 11 were used for all experiments.

Cell proliferation assay. Cells were detached by trypsinization and centrifuged at 800*g* for 5 min. Cell pellets were resuspended in serum-free HE-SFM and seeded at a density of 5000 cells/cm^2 in 35-mm dishes that had been coated by attachment factor (Cell Systems Corp.). Test samples were added immediately after the cells were seeded. In experiments with PD98059 and neomycin, these were added 1 h and 10 min, respectively, prior to the addition of angiogenin to the cells. Cell numbers were determined with a Coulter counter after 48 h incubation.

Nuclear translocation. Cells were seeded at a density of 5000 cells/cm^2 on attachment factor-coated cover slips, placed in 35-mm dishes and cultured in HE-SFM supplemented with 10 ng/ml bFGF at 37°C under 5% humidified CO_2 for 24 h. The cells were washed three times with prewarmed (37°C) HE-SFM and incubated with angiogenin (1 μg/ml) at 37°C for 30 min. Immunofluorescent staining of nuclear angiogenin was performed as described (6). Briefly, cells were fixed with −20°C methanol for 10 min and washed three times with phosphate-buffered saline (PBS) containing 30 mg/ml bovine serum albumin (BSA) for 10 min at 37°C and incubated with 50 μg/ml of anti-angiogenin monoclonal antibody 26-2F in PBS containing 5 mg/ml BSA at 37°C for 1 h. The cells were washed five times with PBS + 5 mg/ml BSA at 37°C and incubated with Alexa 488-labeled goat F(ab′)$_2$ anti-mouse IgG at 1:100 dilution in PBS + 5 mg/ml BSA for 1 h at 37°C. After incubation, the cells were washed five times with PBS + 5 mg/ml BSA, once with PBS and then mounted in 50% glycerol. Fluorescence was observed with a Nikon Labphot fluorescent microscope.

Western blot analysis. Cells were cultured in HE-SFM + 5% FBS + 5 ng/ml bFGF at 37°C under 5% CO_2 for 24 h, washed with HE-SFM three times, and serum-starved for another 24 h. The cells were then washed another three times with prewarmed HE-SFM and incubated with 1 μg/ml of angiogenin at 37°C for different lengths of time. Cells were washed once with PBS and lysed in 60 μl of the lysis buffer (20 mM Tris–HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 1 mM NH_4VO_4, 30 mM $Na_4P_2O_7$, 50 mM NaCl, 1% Triton X-100, 1 × complete protease inhibitor cocktail). Protein concentrations were determined chromometrically with a microplate method (Pierce). Samples with equal amounts of protein were subject to SDS–PAGE and Western blotting analyses for phosphorylation of Erk1/2, SAPK/JNK, and p38 MAP kinase per manufacturer's instructions (New England Biolabs). In experiments with PD98059 and neomycin, they were added to the cells 1 h and 10 min, respectively, before addition of angiogenin.

RESULTS AND DISCUSSIONS

Angiogenin activates Erk1/2 in HUVE cells. We investigated the effects of exogenous angiogenin on phosphorylation of the three subfamilies of MAP kinases: Erk1/2, SAPK/JNK, and p38 MAP kinase by means of Western blotting with specific antibodies against the phosphorylated forms of these three MAP kinases. Figure 1 shows that exogenous human angiogenin-induced phosphorylation of Erk1/2 in HUVE cells in a time-dependent manner (Fig. 1A). Activation of Erk1/2 by angiogenin was rapid. It reached a maximum at 1 min, remained at the activated status for at least 30 min, and began to decline after 60 min. The other two MAP kinase subfamilies, SAPK/JNK and p38 MAP kinase, were not affected by exogenous angiogenin (Figs. 1B and 1C).

Erk1/2 are ubiquitous components of signal transduction pathways, especially those used by receptor tyrosine kinases (18, 19). Binding of extracellular ligands causes autophosphorylation of the receptors on tyrosine residues. Those phosphorylated tyrosine residues then bind the SH2 domains of adapters such as Grb2 (growth factor receptor-binding protein 2). The SH3 domains of the adapters recruit guanine nucleotide exchange factors and promote association of Ras with GTP. The GTP-bound form of Ras then immobilizes Raf to the membranes where it is phosphorylated by Raf protein kinase. The phosphorylated Raf then activates MAP or Erk kinase 1 (MEK 1) that phosphorylates Erk1/2. We do not know at present whether or not this precise signaling pathway is activated in HUVE cells by angiogenin. An early study has shown that angiogenin activates phospholipase C and induces release of diacylglycerol (20). These early results are not necessarily inconsistent with the present data as it is known that the MAP kinase cascade can also be activated by heterotrimeric G proteins in both Ras-dependent and -independent pathways (21–24). It is also known that receptors that do not contain intrinsic tyrosine kinase activity but that contain binding sites

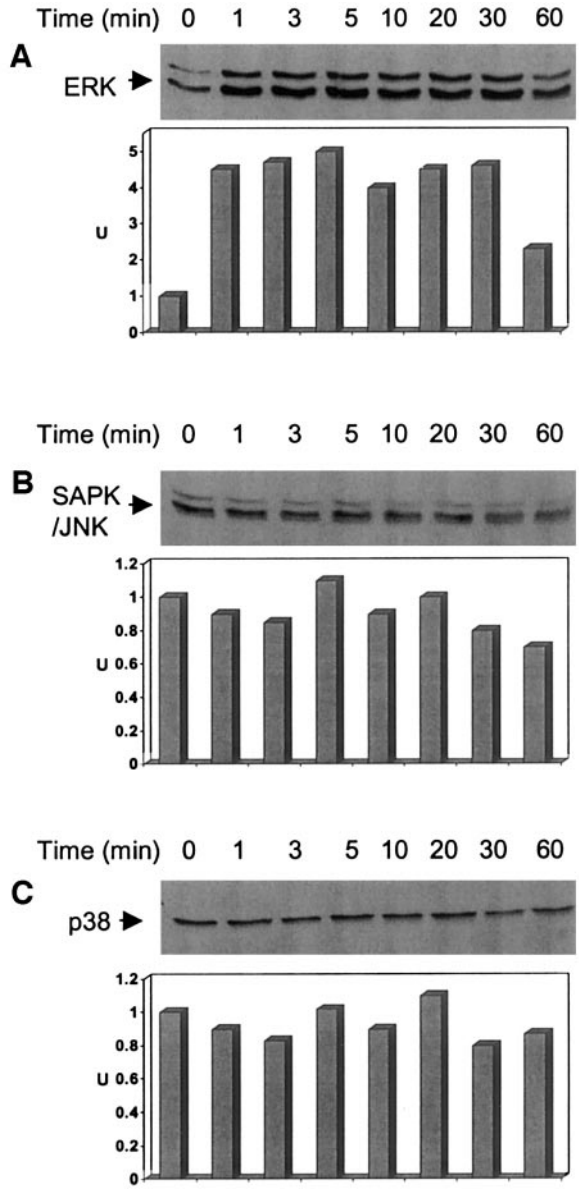

FIG. 1. Angiogenin induces phosphorylation of Erk1/2 in HUVE cells. HUVE cells were cultured at a density of 5000 cells/cm^2 in HE-SFM supplemented with 5% FBS and 5 ng/ml bFGF for 24 h and starved in serum-free HE-SFM for another 24 h. Cells were treated with 1 μg/ml angiogenin for different periods of time, lysed, and subjected to SDS–PAGE and Western blotting analyses for phospho-Erk1/2 (A), phospho-SAPK/JNK (B), and phospho-p38 MAP kinase (C). Equal amounts of protein were applied for each sample. Upper panels are the actual blots and lower panels are densitometry data presented as the sum of both bands in arbitrary units (U).

for tyrosine phosphorylation may also activate MAP kinase via association of phosphotyrosine residues to the receptors (25, 26). We do not yet know the nature of the angiogenin receptor in endothelial cells, but in any event, activation of Erk1/2 in HUVE cells by angiogenin provides a central point in the signal transduction pathway from which its upstream kinases and downstream targets can be elucidated.

Angiogenin-induced Erk1/2 activation is cell density-dependent. Figure 2 shows that activation of Erk1/2 by angiogenin in HUVE cells depends on cell density. Angiogenin-induced Erk1/2 phosphorylation decreases as cell density increases. Thus, 1 μg/ml of angiogenin induced a 4.5- and 3.2-fold increase of Erk1/2 phosphorylation when the cells were cultured at a density of 5×10^3 and 1×10^4 cells/cm^2, respectively (Fig. 2, lanes 1–4). When the cell density reached 2×10^4 per cm^2, angiogenin no longer stimulated Erk1/2 phosphorylation (Fig. 2, lanes 5–8). It is notable that the basal level of Erk1/2 phosphorylation increased considerably when the cell density increased from 1×10^4 to 2×10^4 cells/cm^2. This is probably because of the increased cell–cell contact or the activities of some autocrine and paracrine factors secreted by endothelial cells. It is known that a variety of cellular activities are associated with Erk1/2 activation (15, 27).

We have shown previously that cell density was the most important factor that influenced angiogenin-induced ^{3}H-thymidine incorporation and cell proliferation in endothelial cells (5). Endothelial cells responded to angiogenin in proliferation assays only when the cell density was less than 2×10^4 cells/cm^2 (5). This cell density dependency has been attributed to a 170 kDa putative angiogenin receptor that is expressed only when the cell density is low (5). The present data are consistent with the previous results that cell density determines how endothelial cells respond to angiogenin and suggest that binding of angiogenin to this

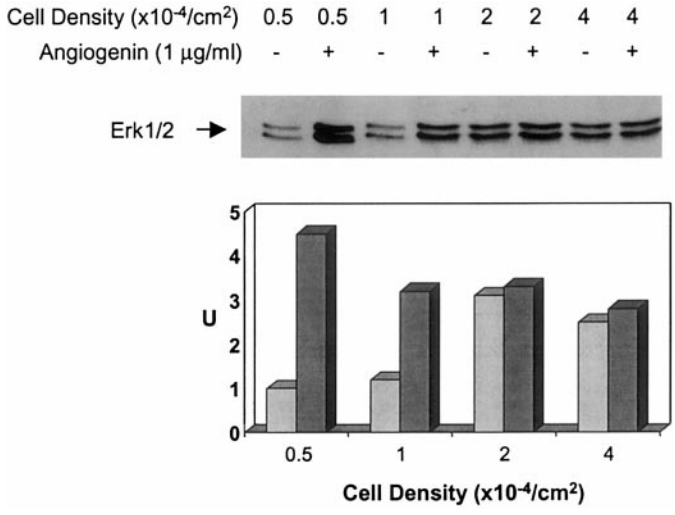

FIG. 2. Angiogenin-induced Erk1/2 phosphorylation depends on cell density. HUVE cells were seeded at various cell densities and cultured at 37°C in HE-SFM + 5% FBS + 5 ng/ml bFGF for 24 h and serum-starved for another 24 h. Cells were treated with and without angiogenin (1 μg/ml) for 30 min and cell lysates were analyzed for Erk1/2 phosphorylation by Western blotting with anti-phosphorylated Erk1/2 antibody. Equal amounts of protein were applied for each sample.

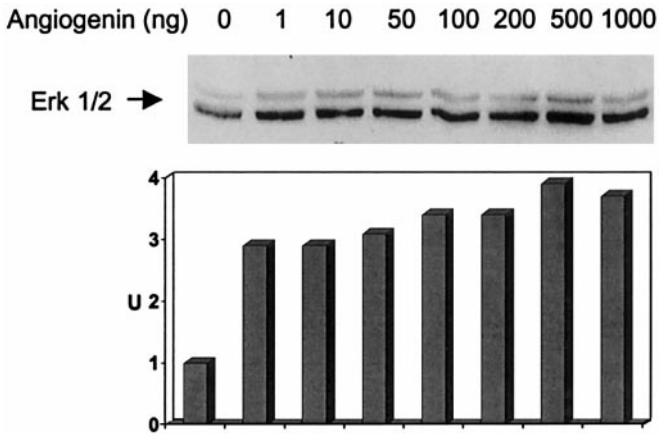

FIG. 3. Angiogenin stimulates Erk1/2 phosphorylation in a concentration-dependent manner. HUVE cells, at a density of 5000 cells/cm^2, were cultured in HE-SFM + 10% FBS + 5 ng/ml bFGF for 24 h and serum-starved for another 24 h. Cells were treated with different concentrations of angiogenin at 37°C for 30 min and cell lysates were analyzed for Erk1/2 phosphorylation by Western blotting. Equal amounts of protein were applied for each sample.

putative receptor is connected to Erk1/2 phosphorylation and cell proliferation.

Angiogenin-stimulated Erk1/2 phosphorylation is concentration-dependent. Figure 3 shows that angiogenin induced Erk1/2 phosphorylation in a concentration-dependent manner. At a concentration as low as 1 ng/ml, angiogenin already stimulates a ~3-fold increase of Erk1/2 phosphorylation. Raising the concentration to 1 μg/ml enhances the phosphorylation by ~3.5-fold, not a significant increase over that obtained at 1 ng/ml angiogenin (Fig. 3). It is interesting that the concentration of angiogenin required to induce Erk1/2 phosphorylation in HUVE cells is so low. Under normal physiological conditions, the concentration of circulating angiogenin in plasma is estimated to be 250–360 ng/ml (28, 29). To avoid unnecessary stimulation by circulating angiogenin, endothelial cells would have to control the expression and manifestation of angiogenin receptor that, in turn, is regulated by cell density. As has been shown in Fig. 2, angiogenin does not stimulate Erk1/2 phosphorylation when the cells are confluent. The relatively high concentration of angiogenin in plasma and the low threshold for Erk1/2 activation may provide for prompt repair of blood vessel damage caused by physical, chemical, and pathological conditions under which the local density of endothelial cells will likely be decreased. On the other hand, the present data may also suggest that Erk1/2 activation is not sufficient for angiogenin to stimulate cell proliferation. Previous results have shown that the optimal concentration of angiogenin to induce cell proliferation is around 1 μg/ml (5). The difference in dose-dependency suggests a more complex regulatory mechanism to control cell proliferation and angiogenesis.

PD98059 inhibits angiogenin-induced Erk1/2 phosphorylation and cell proliferation but not nuclear translocation. PD98059 is a highly selective inhibitor of MEK 1, the upstream kinase that specifically phosphorylates Erk1/2. This compound has been successfully used to inhibit Erk1/2 phosphorylation and to terminate signal transduction through this pathway in various cells (30). Figure 4 shows that PD98059 completely abolishes angiogenin-induced Erk1/2 phosphorylation (Fig. 4A). It also decreases the basal level of Erk1/2 phosphorylation in the absence of exogenous

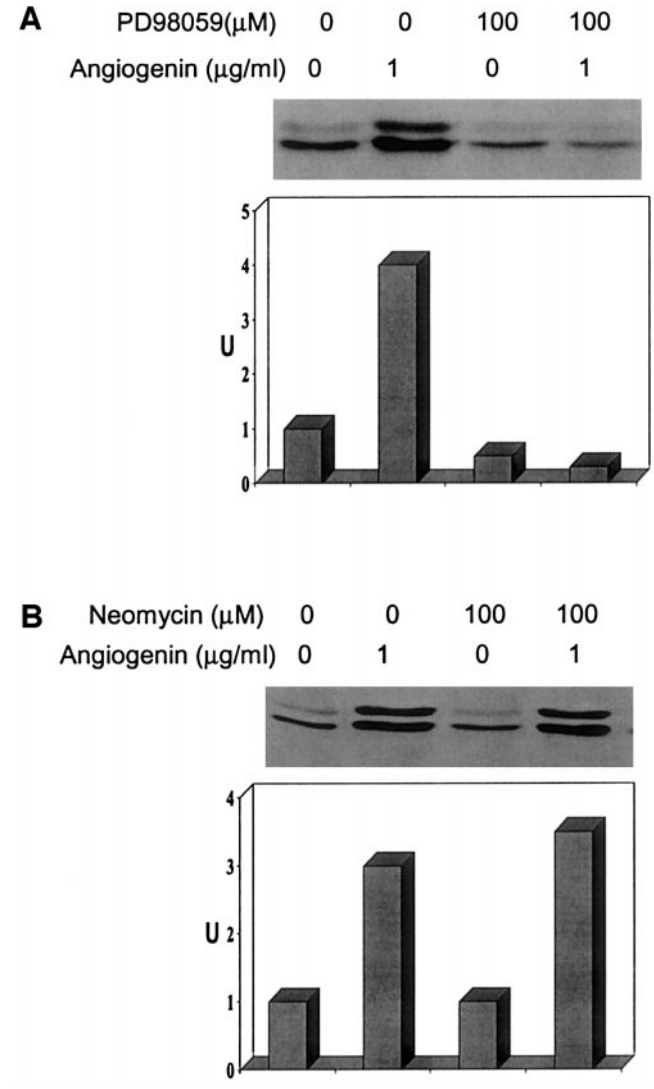

FIG. 4. Effects of PD98059 and neomycin on angiogenin-induced phosphorylation of Erk1/2 in HUVE cells. Cells were cultured at a density of 5000 cells/cm^2 in HE-SFM + 10% FBS + 5 ng/ml bFGF for 24 h and serum-starved for another 24 h. Cells were washed in prewarmed (37°C) HE-SFM three times and incubated in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 10 μM PD98059 at 37°C for 1 h (A) or of 100 μM neomycin for 10 min (B), respectively. Angiogenin, 1 μg/ml, was added (lanes 2 and 4) and incubated at 37°C for 30 min. Cells were lysed and analysed for Erk1/2 phosphorylation by Western blotting.

TABLE 1

Effect of PD98059 and Neomycin on Angiogenin-Induced Cell Proliferation

	Cell number		
Inhibitor	Control	Angiogenin (1 μg/ml)	% increase
None	45,000 ± 1,500	61,000 ± 1,200	36
PD98059 (10 μM)	38,000 ± 1,900	40,000 ± 1,700	5
Neomycin (100 μM)	46,000 ± 2,000	45,000 ± 2,100	0

Note. HUVE cells were seeded at a density of 5000 cells/cm^2 in HE-SFM on attachment factor-coated 35-mm dishes. PD98059 and neomycin were added to the cells and incubated at 37°C for 1 h and 10 min, respectively, before angiogenin was added and incubated for 48 h. Data shown are the means and standard deviations of quadruplicates of each sample from a representative experiment.

angiogenin. Endothelial cells are known to secrete a number of angiogenic and growth factors (31) that might be responsible for the basal level phosphorylation of Erk1/2. Sensitivity to PD98058 indicates that angiogenin-induced Erk1/2 phosphorylation occurs through MEK 1 and is therefore likely mediated by the putative angiogenin receptor.

PD98059 also significantly inhibited angiogenin-induced proliferation of HUVE cells (Table 1) indicating that Erk1/2 phosphorylation is necessary for the proliferative activity of angiogenin. When 50,000 cells were cultured in HE-SFM at 37°C for 48 h, 45,000 and 61,000 cells were recovered in the absence and presence of 1 μg/ml angiogenin, respectively, representing a 36% increase of cell numbers stimulated by angiogenin. This angiogenin-stimulated increase in cell number was diminished to 5% in the presence of 10 μM PD98059 (Table 1). It is notable that the number of cells recovered in the presence of PD98059 was lower than that in its absence (38,000 vs 45,000) indicating that PD98059 was somewhat toxic to the cells possibly because of its inhibition of the basal level phosphorylation of Erk1/2 (Fig. 4A).

To determine whether Erk1/2 phosphorylation is involved in nuclear translocation of angiogenin, we examined the effect of PD98059 on nuclear accumulation of exogenous angiogenin in HUVE cells by means of immunofluorescence. As shown in Fig. 5, bright nucleolar staining was obtained after 30 min incubation with 1 μg/ml angiogenin both in the absence (Fig. 5A) and presence (Fig. 5B) of PD98059. This lack of inhibition indicated that Erk1/2 phosphorylation is not required for angiogenin to be translocated to the nucleus. It suggested that although angiogenin-induced Erk1/2 phosphorylation is necessary for cell proliferation, it is not related to nuclear translocation of angiogenin, a process also essential for cell proliferation.

Neomycin does not inhibit angiogenin-induced Erk1/2 phosphorylation. By means of subcellular fractionation and with the use of iodinated angiogenin, we have shown previously that neomycin inhibits nu-

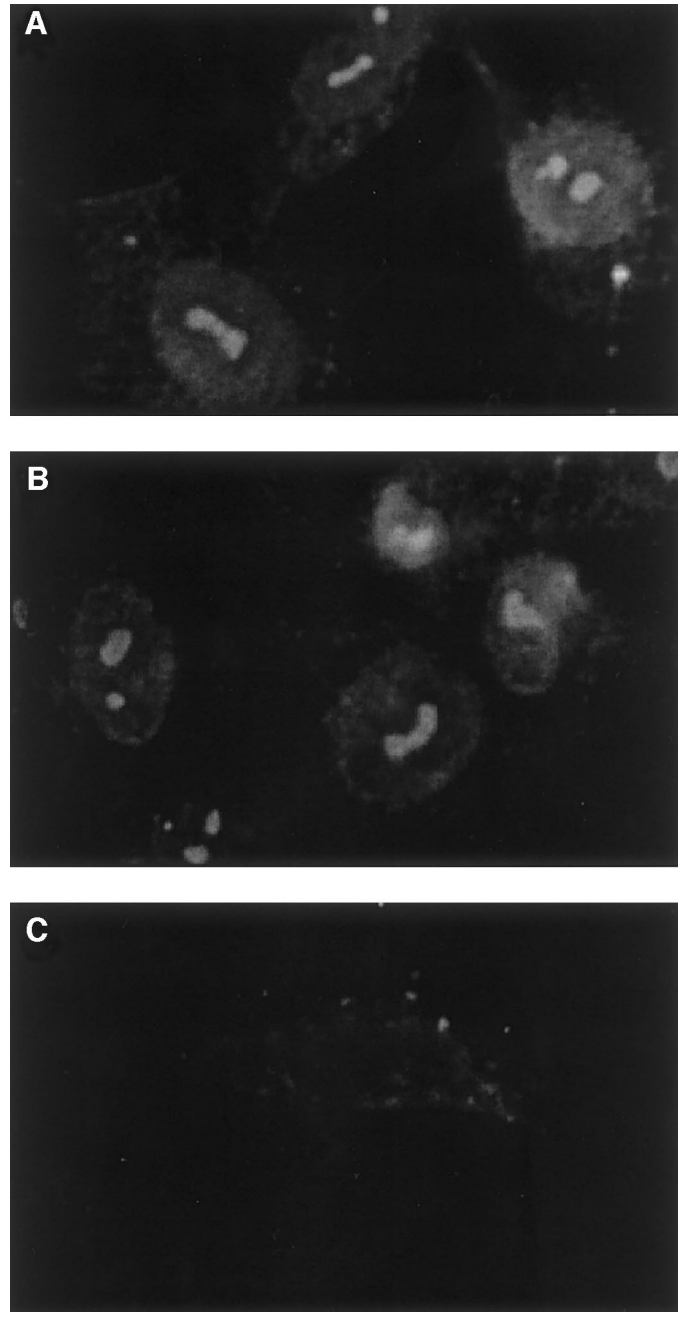

FIG. 5. Effects of PD98059 and neomycin on nuclear translocation of angiogenin. Nuclear angiogenin was visualized with anti-angiogenin monoclonal antibody 26-2F and Alexa 488 goat anti-mouse IgG. (A) Positive control in the absence of inhibitors, 1 μg/ml angiogenin. (B) Effect of PD98059. Cells were preincubated with 10 μM 98059 for 1 h before angiogenin (1 μg/ml) was added. (C) Effect of neomycin. Cells were pretreated with 100 μM neomycin for 10 min before angiogenin (1 μg/ml) was added. Original magnification: 100×.

clear translocation of angiogenin in endothelial cells thereby abolishing its proliferative and angiogenic activities (9). In the present study, we used immunofluorescence to confirm that nuclear translocation is inhibited by neomycin. Figure 5C shows that nuclear translocation of angiogenin was substantially decreased and that nucleolar accumulation was markedly diminished by 100 μM neomycin. Table 1 shows that this concentration of neomycin also abolished angiogenin-induced cell proliferation, consistent with previous results (9). However, neomycin has no effect on Erk1/2 phosphorylation. It neither inhibited nor stimulated the basal level as well as angiogenin-induced Erk1/2 phosphorylation (Fig. 4B). These results indicate that while nuclear translocation of angiogenin is essential for cell proliferation, it is not required for the signal transduction pathway involving Erk1/2 phosphorylation.

Thus, it appears that both Erk1/2 activation and nuclear translocation are necessary for angiogenin to induce cell proliferation but neither of them alone is sufficient. Both events depend on cell density and are probably mediated by the cell surface receptor but they are independent each other. Angiogenin-induced Erk1/2 phosphorylation is neither the cause nor the effect of nuclear translocation of angiogenin and vice versa. Both PD98059 and neomycin inhibit angiogenin-induced cell proliferation but by different mechanisms. PD98059 inhibits Erk1/2 phosphorylation but does not affect nuclear translocation of angiogenin. On the other hand, neomycin does not interfere with Erk1/2 phosphorylation but significantly inhibits nuclear translocation of angiogenin. The present data suggest that angiogenin-stimulated proliferation of endothelial cells requires both activation of Erk1/2 and nuclear translocation of the ligand.

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