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Endogenous angiogenin in endothelial cells is a general requirement for cell proliferation and angiogenesis

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Angiogenin is an angiogenic protein that undergoes nuclear translocation in endothelial cells where it accumulates in the nucleolus and stimulates rRNA transcription, a rate-limiting step in ribosome biogenesis, protein translation, and cell growth. Here, we report that angiogenin is required for cell proliferation induced by various other angiogenic proteins including acidic and basic fibroblast growth factors (aFGF and bFGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF). Downregulation of angiogenin in endothelial cells by small interfering RNA (siRNA) and antisense results in a decrease in rRNA transcription, ribosome biogenesis, and cell proliferation induced by these angiogenic factors. Inhibitors of the nuclear translocation of angiogenin abolish the angiogenic activities of these factors. Stable angiogenin antisense transfection in HeLa cells reduces tumor angiogenesis in athymic mice despite the elevated expression level of bFGF and VEGF. Thus, nuclear angiogenin assumes an essential role in endothelial cell proliferation and is necessary for angiogenesis induced by other angiogenic factors. Angiogenin-stimulated rRNA transcription in endothelial cells may thus serve as a crossroad in the process of angiogenesis induced by various angiogenic factors.
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Introduction

A growing number of endothelial cell mitogens have been identified and their mitogenic activities are believed to help turn on the 'angiogenic switch' in tumor progression (Hanahan and Folkman, 1996; Bergers and Benjamin, 2003). Numerous angiogenic stimulators have been identified and their expression and distribution have been associated with angiogenesis-based diseases (Papetti and Herman, 2002; Ribatti *et al*., 2002). However, the relationship among various endothelial cell mitogens remains unclear. We hypothesized that angiogenin, an angiogenic ribonuclease (Adams and Subramanian, 1999) that stimulates ribosomal RNA (rRNA) transcription (Xu *et al*., 2002, 2003), may serve as a crossroad in the process of angiogenesis induced by various angiogenic factors.

Angiogenin was originally isolated from the conditioned medium of HT-29 human colon adenocarcinoma cells (Fett *et al*., 1985). It has been shown to play a role in tumor angiogenesis (Olson *et al*., 1995, 2001, 2002). Its expression increases in a variety of cancer cells, which results in an elevation of plasma angiogenin in cancer patients (Li *et al*., 1994; Barton *et al*., 1997; Montero *et al*., 1998; Shimoyama *et al*., 1999; Eberle *et al*., 2000; Kushlinskii *et al*., 2000; Bodner-Adler *et al*., 2001; Ugurel *et al*., 2001; Homer *et al*., 2002; Shimoyama *et al*., 2002). Angiogenin antagonists have been shown to inhibit the establishment, progression, and metastasis of xenografic human tumors in athymic mice (Olson *et al*., 1995, 2001, 2002).

Angiogenin binds to the endothelial cell surface, interacts with a 170 kDa putative receptor (Hu *et al*., 1997) or a 42 kDa binding protein (Hu *et al*., 1993) on the cell surface, induces cell proliferation (Hu *et al*., 1997), activates cell-associated proteases (Hu and Riordan, 1993), and stimulates cell migration and invasion (Hu *et al*., 1994). Angiogenin undergoes nuclear translocation in endothelial cells, which is essential for its biologic activity (Moroianu and Riordan, 1994). When nuclear translocation is inhibited, its angiogenic activity is abolished (Hu, 1998). Recently, we discovered that angiogenin is able to bind to the ribosomal DNA (rDNA) and to stimulate rRNA transcription (Xu *et al*., 2002). An angiogenin-binding DNA sequence has been identified from rDNA and we have shown that this DNA sequence has angiogenin-dependent promoter activity (Xu *et al*., 2003). Since rRNA transcription regulates ribosome production and, consequently, the translation potential of a cell, we hypothesized that angiogenin-induced rRNA transcription is required for endothelial cell proliferation induced by other angiogenic factors.

To evaluate this hypothesis, we used both small interfering RNA (siRNA) and antisense to knockdown

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angiogenin expression in human umbilical vein endothelial cells (HUVEC) and examined the resultant changes in cellular responses toward other angiogenic factors. We found that downregulation of angiogenin expression in HUVEC inhibited angiogenic factor-induced rRNA transcription, ribosome biogenesis, and cell proliferation. We also found that neamine and actin, inhibitors of nuclear translocation of angiogenin (Moroianu and Riordan, 1994; Hu, 1998), inhibited angiogenesis induced by these angiogenic factors on the chick chorioallantoic membrane (CAM). Furthermore, angiogenin antisense-transfected HeLa cells have reduced angiogenesis and tumorigenesis when injected into athymic mice.

Results

Nuclear accumulation of endogenous angiogenin upon growth stimulation

Angiogenin undergoes nuclear translocation in endothelial cells where it stimulates rRNA transcription (Moroianu and Riordan, 1994; Hu *et al*., 2000; Xu *et al*., 2002). Since rRNA is the rate-limiting step for ribosome biogenesis and therefore, for protein translation and cell proliferation (Ruggero and Pandolfi, 2003), we hypothesized that angiogenin-stimulated rRNA transcription might be a general requirement for endothelial cell proliferation in the process of angiogenesis regardless of the nature of the angiogenic stimuli. This hypothesis was first tested by nuclear translocation of angiogenin in HUVEC upon growth stimulation. If nuclear function of angiogenin is essential for other endothelial mitogens to induce cell proliferation, angiogenin should be stimulated by these mitogens to undergo nuclear translocation. Figure 1 shows that this is indeed the case. In unstimulated HUVEC, no nuclear angiogenin was detected by immunofluorescent staining with a human angiogenin-specific monoclonal antibody 26-2F (Figure 1a). However, upon stimulation with bFGF, VEGF, aFGF, EGF, and FBS for 4 h, nuclear accumulation of angiogenin was observed (Figure 1b–f). It is unknown at present whether the nuclear angiogenin comes from those previously distributed in the cytoplasm or from those that have already been secreted. However, because no exogenous angiogenin was added in these experiments, it is clear that the nuclear angiogenin is originated from HUVEC themselves. These results are consistent with our hypothesis that the endogenous angiogenin may participate in endothelial cell proliferation induced by other angiogenic factors.

Downregulation of angiogenin expression inhibited rRNA transcription

If endogenous angiogenin participates in the process of endothelial cell proliferation induced by other angiogenic factors, knocking down its expression should decrease rRNA transcription and ribosome biogenesis, especially when stimulated by other angiogenic factors. We transfected HUVEC with an angiogenin antisense oligo CT-1 (second generation, phosphorothioate-2′-*O*-methyl RNA chimera) to inhibit the synthesis of endogenous angiogenin, and then measured rRNA transcription and ribosome biogenesis induced by bFGF and VEGF.

CT-1 hybridizes with nucleotides 122–139 of the angiogenin mRNA. CT-2 is a control oligo that has the same base composition as that of CT-1 but in the reverse sequence and thus does not hybridize with angiogenin mRNA. We first determined the effect of CT-1 on cell survival. Figure 2a shows that cell survival decreased as the cells were transfected with increasing concentration of CT-1. CT-2 has no effect at concentration up to 1 μM. Since the base compositions in CT-1 and CT-2 are the same, the effect of CT-1 is not because of the nonspecific toxicity, and is probably due to its suppression of angiogenin-induced rRNA transcription. At 0.1 μM, both CT-1 and CT-2 are not toxic to HUVEC. Therefore, this concentration was used for all the experiments in this paper.

Figure 2b shows that the mRNA level of angiogenin was decreased after CT-1 transfection, whereas no changes were observed in mock- and CT-2-transfected cells. The level of GAPDH mRNA was not changed in all three samples, indicating a lack of nonspecific toxicity of CT-1 at this concentration (0.1 μM) and an equal loading of the samples. ELISA analysis (Figure 2c) showed that the level of secreted angiogenin in CT-1-transfected cells (0.11 pg/10^3 cells/day) was significantly ($P<0.0001$) lower than that in the mock- or CT-2-transfected cells (0.31 and 0.33 pg/10^3 cells/day, respectively). There is a concomitant reduction of the steady-state level of 45S rRNA (Figure 2b) as determined by Northern blotting.

In order to know whether CT-1-transfected cells have a reduced synthesis rate of 45S rRNA transcription, we carried out a metabolic labeling experiment to measure the effect of angiogenin downregulation on the *de novo* synthesis of the rRNA. HUVEC were transfected with CT-1 or CT-2 and pulse-labeled with ^{32}P-orthophosphate for 30 min. The newly synthesized 45S rRNA was revealed by autoradiography (Figure 2d, top), the level of 28S rRNA was determined by EB staining (Figure 2d, bottom). CT-1-transfected cells (lane 2) have a reduced rate of the *de novo* synthesis of 45S rRNA. Transfection with CT-2 (lane 3) did not cause a significant change in rRNA synthesis as compared with mock transfection (lane 1). EB staining of the 28S rRNA showed that equal amounts of total RNA were applied in each lane. It should be noted that CT-1 transfection reduced the steady-state level of 45S rRNA, which results in a decrease in the cellular levels of 28S rRNA. Therefore, using of 28S rRNA as a loading control actually underestimated the difference in newly synthesized 45S rRNA per cell among samples. Therefore, the actual inhibition of the *de novo* synthesis of 45S rRNA caused by CT-1 should be more than that shown in Figure 2d. In any event, this experiment clearly demonstrated that downregulating angiogenin expression inhibits 45S rRNA transcription.

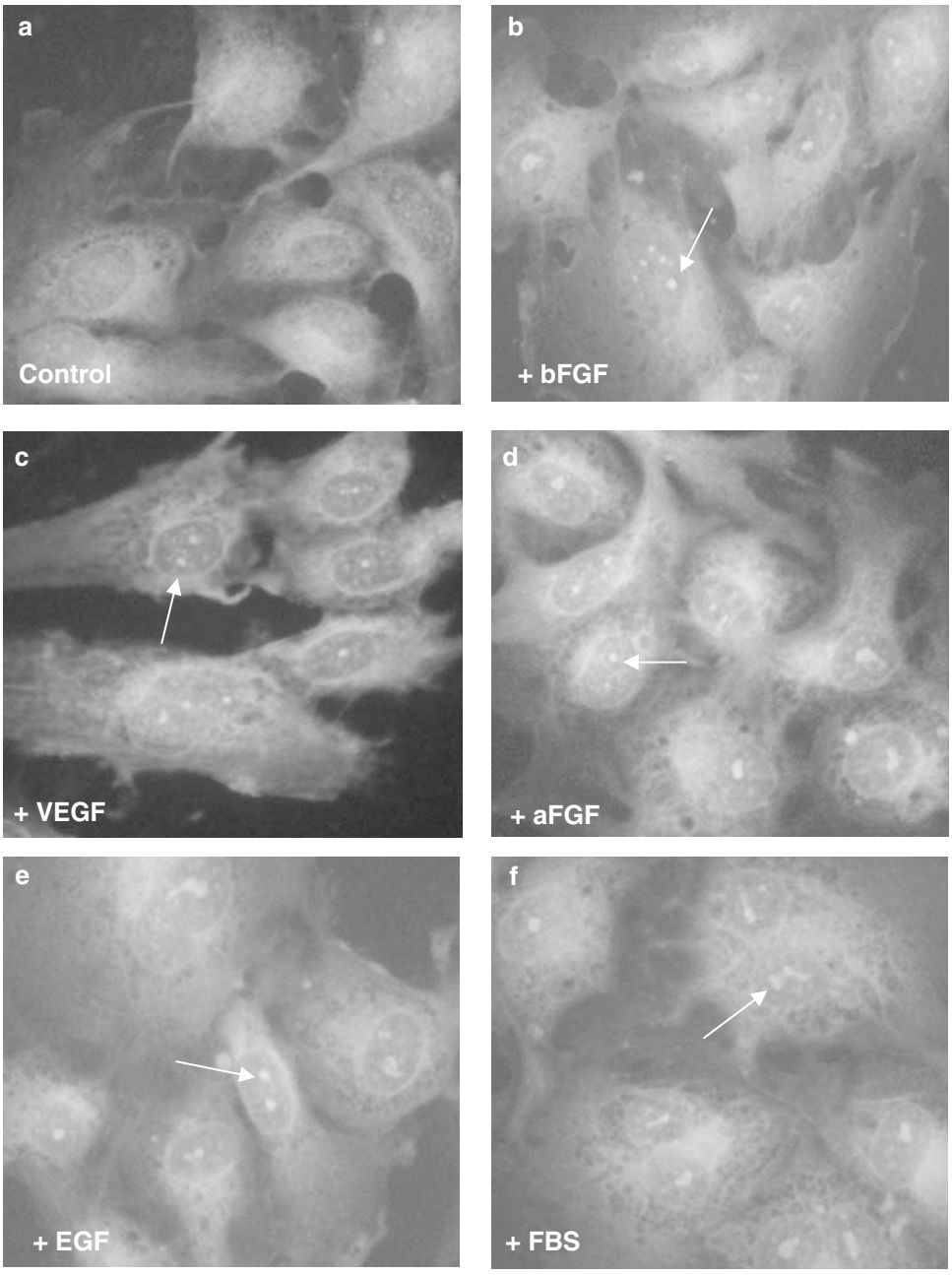
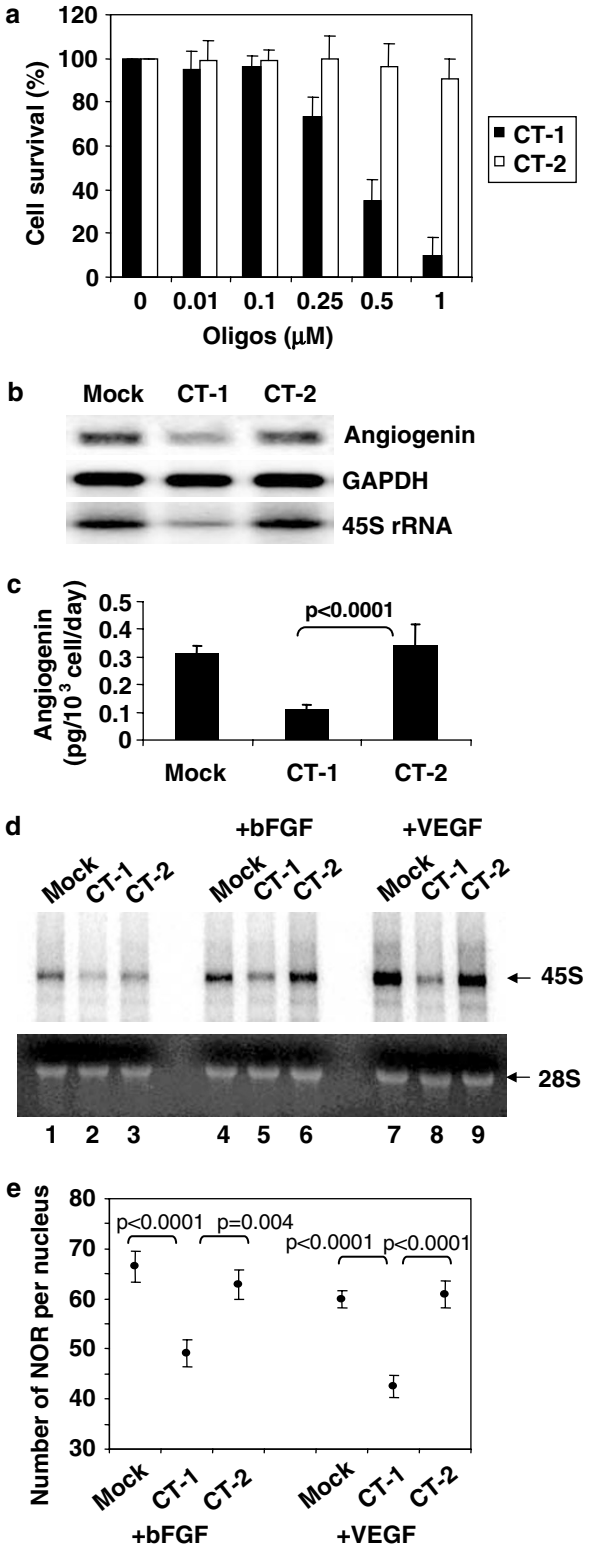


Figure 1 Exogenous angiogenic factors stimulate nuclear translocation of angiogenin. Cells were unstimulated (**a**) or stimulated with 10 ng/ml of bFGF (**b**), VEGF (**c**), aFGF (**d**), EGF (**e**), or 10% FBS (**f**) for 4 h, fixed with methanol and stained with 10 μg/ml 26-2F and Alexa 488-labeled goat anti-mouse IgG

rRNA syntheses induced by bFGF (Figure 2d, lanes 4–6) and VEGF (Figure 2d, lanes 7–9) were inhibited in CT-1-transfected cells, demonstrating that the decrease in *de novo* synthesis of 45S rRNA caused by CT-1 transfection cannot be compensated by stimulation of strong endothelial cell mitogens. Therefore, these results not only demonstrated that endogenous angiogenin in endothelial cells plays a role in rRNA transcription but also suggested that angiogenin-stimulated rRNA synthesis might be required for other angiogenic factors to stimulate endothelial cell proliferation.

Nuclear organizer regions (NOR) are loops of rDNA that are being actively transcribed for rRNA. These regions are thus involved in the synthesis of ribosomes and are of central importance in the regulation of protein synthesis (Ruschoff *et al*., 1989). The size and number of NOR reflect the capacity of the cell to transcribe rRNA (Trere *et al*., 1989). NOR are associated with a group of argyrophilic proteins and can be visualized by silver staining. Figure 2e shows that bFGF and VEGF failed to induce an increase in NOR in the cells that have been transfected by CT-1. No

significant differences were observed in mock- or CT-2-transfected cells. These results indicated that ribosome biogenesis was inhibited by downregulating angiogenin expression in HUVEC.



Downregulation of angiogenin expression inhibited growth factor-induced cell proliferation

Figure 3 shows that the proliferation activities of bFGF (Figure 3a), VEGF (Figure 3b), aFGF (Figure 3c), and EGF (Figure 3d) in HUVEC were all inhibited by CT-1. In these experiments, cell survival in the absence of exogenous growth factors was not affected by CT-1 transfection at the concentration (0.1 μM) used as has been shown in Figure 2a. The proliferative activities of these angiogenic factors were not completely abolished, probably due to the fact that CT-1 inhibited angiogenin expression only by 65%. The cells were still expressing 0.11 pg angiogenin/10^3 cells/day after transfection with 0.1 μM CT-1. If angiogenin expression was inhibited to a greater extent (<0.01 pg/10^3 cells/day) by increasing CT-1 concentration, massive cell death occurred (Figure 2a).

To confirm that the decreased cell proliferation induced by other angiogenic factors is a consequence of reduced angiogenin expression, we rescued cell proliferation by adding exogenous angiogenin to CT-1-transfected cells. As shown in Figure 3e, exogenous angiogenin reversed the effect of CT-1, indicating that the decrease in cell proliferation was the consequence of reduced angiogenin expression. However, addition of exogenous angiogenin did not increase bFGF-induced cell proliferation in mock- and CT-2-transfected cells (Figure 3e) presumably because the amounts of endogenous angiogenin in these cells are already adequate. Exogenous angiogenin was also able to rescue the inhibitory activity of CT-1 on cell proliferation induced by other angiogenic factors including VEGF, aFGF, and EGF (data not shown).

Angiogenin siRNA inhibited bFGF-induced HUVEC proliferation

To verify the results obtained with angiogenin antisense CT-1, we have also used an angiogenin-specific siRNA HX12 to transiently transfect HUVEC to inhibit angiogenin expression. Figure 3f shows that transfection

Figure 2 Angiogenin antisense CT-1 decreases rRNA transcription and ribosomal biogenesis. (**a**) HUVEC were transfected with CT-1 or CT-2 at the concentrations indicated in the presence of 1.2 μl/ml lipofectin for 5 h and cultured for 48 h. Cell numbers were determined and the percentage of cell survival was calculated based on the cell number in mock transfection. (**b**, **c**) Cells were transfected with 0.1 μM CT-1 or CT-2 in the presence of 1.2 μl/ml lipofectin for 5 h and cultured for 24 h. Total RNA was extracted by Trizol. Levels of angiogenin mRNA and 45S rRNA were determined by Northern blotting (**b**). The amount of secreted angiogenin was determined by ELISA (**c**). (**d**) Cells were transfected for 16 h, and stimulated with 10 ng/ml of bFGF or VEGF for 24 h and pulsed with 25 μCi/ml ^{32}P-orthophosphate for 30 min. Equal amounts of RNA were applied for electrophoresis. The radiolabeled RNA were visualized by autoradiography and the 28S rRNA were shown by ethidium bromide staining. (**e**) Cells were fixed and stained with silver. Silver-stained NOR dots were counted in 30 randomly selected nuclei. All experiments were repeated five times. Data presented in (**a, c**, and **e**) were mean$\pm$s.d. Statistical significance was determined by the two-tailed Student's *t* test. Results shown in (**b** and **d**) were from a representative experiment

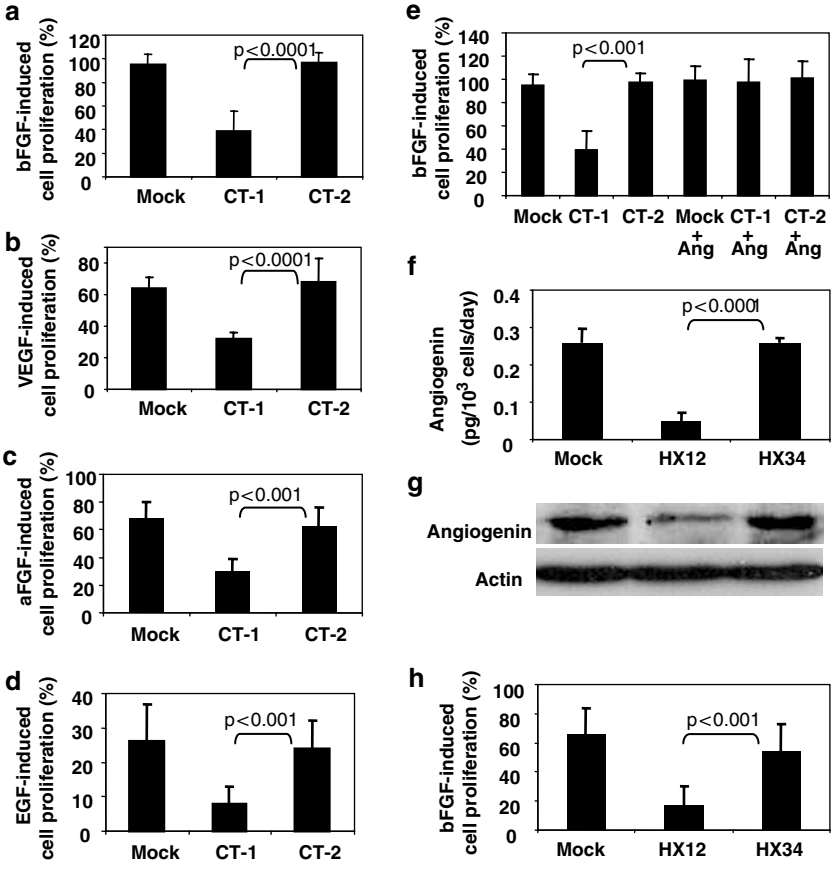


Figure 3 Downregulating angiogenin expression inhibits HUVEC proliferation induced by other angiogenic factors. (**a–d**) HUVEC were transfected with 0.1 μM CT-1 and CT-2 in the presence of 1.2 μl/ml lipofectin for 5 h and stimulated with 5 ng/ml bFGF (**a**), VEGF (**b**), aFGF (**c**), and EGF (**d**) for 48 h. Cell numbers were determined with a Coulter counter. (**e**) Exogenous angiogenin (1 μg/ml) was added together with bFGF to rescue the cells from CT-1 inhibition. (**f–h**) Cells were transfected with 20 nM HX12 or HX34 in the presence of 2 μl/ml Effectene for 5 h and stimulated with 5 ng/ml bFGF for 48 h. Secreted and cellular angiogenin was determined by ELISA (**f**) and Western blotting (**g**), respectively. Cell numbers were determined with a Coulter counter (**h**). The percentage increase in cell number induced by bFGF was calculated based on the corresponding controls in the absence of bFGF. Data shown were means $\pm$ s.d. of five independent experiments

of HX12 that corresponds to the angiogenin mRNA sequence at nt 362–383 inhibited angiogenin expression in HUVEC by 80% (from 0.26 to 0.05 pg/10^{3}cells/day). Transfection of the control double-stranded RNA (HX34) that has the same base composition as that of HX12 but with a reversed sequence had no effect on angiogenin expression. Figure 3g shows that the level of cell-associated angiogenin was also lowered by siRNA transfection. Consistent with the results obtained with CT-1 transfection (Figure 3a), reduced angiogenin expression by HX12 resulted in a decrease in cell proliferation induced by bFGF (Figure 3h). In agreement with a greater degree of reduction in angiogenin expression caused by HX12 than by CT-1, the proliferative activity of bFGF in HX12-transfected cells is lower than that in CT-1-transfected cells. These results further supported our hypothesis that endogenous angiogenin in endothelial cells is required for other angiogenic factors to stimulate cell proliferation. CT-1 and HX12 target nt 122–139, and 362–383 of the angiogenin mRNA, respectively. They both inhibited angiogenin expression but with different mechanisms. However, cell proliferation induced by other angiogenic factors was decreased in both cases, indicating a specific role of endogenous angiogenin in the response of endothelial cell to general proliferative stimuli.

The effect of angiogenin downregulation on endothelial cell proliferation was specific

In order to rule out the possibility that inhibition of expression of any one angiogenic factor will result in a drastic change in cell response toward another, we used antisense oligos to downregulate the expression of endogenous bFGF and VEGF and examined the resultant changes in cell proliferation induced by exogenous factors. The second-generation antisense oligos and the corresponding controls for bFGF (CT-3 and CT-4, respectively) and VEGF (CT-5 and CT-6, respectively) were used to transfect HUVEC and the proliferation response of these cells toward exogenous angiogenic factors was examined. HUVEC are known

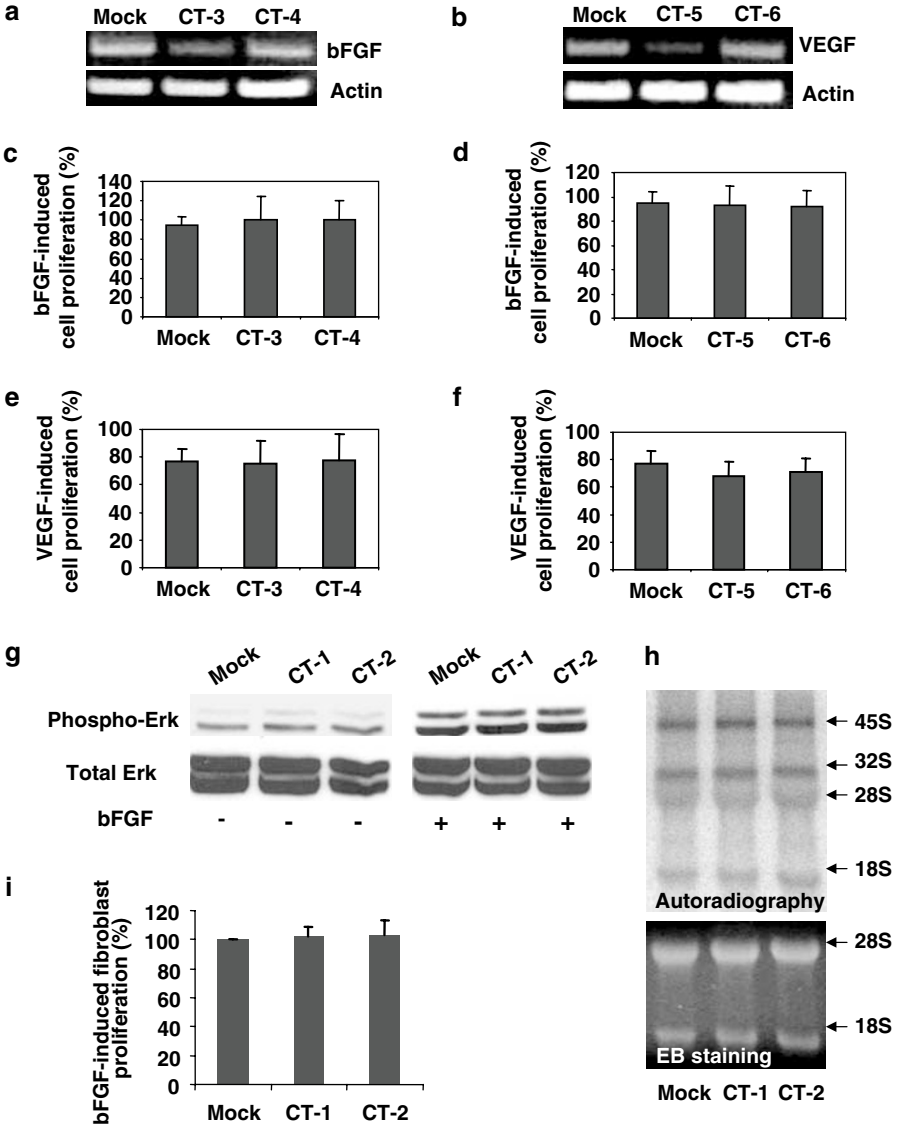


Figure 4 Effect of angiogenin downregulation on induced endothelial cell proliferation is specific. (**a–f**) HUVEC were transfected with 0.1 μM antisense and the corresponding control for bFGF (CT-3 and CT-4), and VEGF (CT-5 and CT-6) in the presence of 1.2 μl/ml Lipofectin for 5 h and cultured for 24 h. The mRNA levels of bFGF (**a**) and VEGF (**b**) were determined by RT–PCR. In cell proliferation assay, HUVEC were transfected for 5 h and stimulated with 5 ng/ml bFGF (**c** and **d**) and VEGF (**e** and **f**) for 48 h. Cell numbers were determined with a Coulter counter. (**g**) Erk1/2 phosphorylation was detected by Western blotting after the cells were transfected with 0.1 μM CT-1 or CT-2 for 5 h and stimulated by bFGF for 30 min. Total Erk was blotted to show that equal amounts of proteins were loaded. (**h** and **i**) Effects of CT-1 and CT-2 on 45S rRNA transcription (**h**) and cell proliferation (**i**) in human dermal fibroblasts were examined under the same conditions as in HUVEC. Data shown in (**c–f**) and (**i**) were means$\pm$s.d. from a representative experiment of three repeats. Results in (**g** and **h**) were from a representative experiment

to express both bFGF and VEGF (Kinoshita and Shimokado, 1999; Imaizumi *et al*., 2000). Transfection with CT-3 and CT-5 effectively decreased the mRNA levels of bFGF and VEGF, respectively (Figure 4a and b). However, this decrease did not affect cell proliferation induced by exogenous growth factors. For examples, cell proliferation induced by bFGF was the same for CT-3- and CT-4-transfected cells (Figure 4c) and for CT-5- and CT-6-transfected cells (Figure 4d). The same was true for VEGF-induced cell proliferation (Figure 4e and f). These results indicated that decreased expression of one angiogenic factor does not necessarily change cell proliferation induced by other angiogenic factors. Thus, endogenous bFGF and VEGF are not required for HUVEC proliferation induced by exogenous growth factors even though they have been shown to stimulate endothelial cell proliferation through an autocrine route (Kinoshita and Shimokado, 1999; Imaizumi *et al*., 2000). Therefore, the effect of CT-1 on the proliferation activity of other angiogenic proteins is related to a unique property of angiogenin: its stimulatory activity toward rRNA transcription. When the supply of rRNA

is inadequate because of downregulation of angiogenin, cell proliferation will be impeded even though they are under stimulation of strong mitogens.

If it is because of the decreased rRNA supply that rendered cells irresponsive to stimulation of exogenous angiogenic factors, CT-1 should not change the signal transduction pathway triggered by receptor binding of these factors. Western blotting analyses showed that bFGF-induced Erk1/2 phosphorylation was unchanged by CT-1 transfection (Figure 4g, top). Reblotting with an anti-total Erk IgG showed that the levels of total Erk were the same (Figure 4g, bottom) when equal amounts of proteins were loaded. These results indicated that the signal transduction pathway of bFGF in HUVEC was not affected by downregulating angiogenin. Although fibroblasts secrete considerable amounts of angiogenin (Moenner *et al*., 1994), it is known that angiogenin does not undergo nuclear translocation in fibroblasts (Moroianu and Riordan, 1994). Therefore, angiogenin should not be involved in rRNA synthesis in fibroblasts and inhibition of angiogenin expression should not affect their proliferation induced by bFGF. Accordingly, transfection of human dermal fibroblasts with CT-1 had no effect on the *de novo* synthesis of 45S rRNA (Figure 4h) and on cell proliferation (Figure 4i) induced by bFGF.

Prevention of nuclear translocation of angiogenin inhibited the angiogenic activity of bFGF and VEGF

To demonstrate that angiogenin is required for the angiogenesis process stimulated by other angiogenic factors, we assessed the angiogenic activities of bFGF and VEGF on the chick CAM (Wilting *et al*., 1991) in the presence of inhibitors of nuclear translocation of angiogenin. Both actin (Moroianu and Riordan, 1994) and neomycin (Hu, 1998) have been shown to prevent nuclear translocation of angiogenin in endothelial cells, thereby inhibiting its angiogenic activity (Olson *et al*., 1995; Hu, 1998). Neamine, a neomycin analog that is virtually nontoxic (Au *et al*., 1986; Williams *et al*., 1987), has the same inhibitory activity toward nuclear translocation of angiogenin (Hu *et al*., unpublished data). Table 1 shows that both actin and neamine inhibit the angiogenic activities of VEGF and bFGF as well as that of angiogenin. Paromomycin, a similar aminoglycoside antibiotic that does not inhibit nuclear translocation of angiogenin (Hu, 1998), did not inhibit angiogenesis induced by all three angiogenic proteins (Table 1). These results demonstrated that blocking nuclear translocation of angiogenin inhibited angiogenesis induced by other angiogenic factors.

Table 1 Effect of actin and neamine on angiogenesis measured on the chick CAM[a]

Samples	*Positive eggs/total eggs*	*% Positive*	*% Inhibition*[b]
Ang (10 ng)	43/68	63	
Ang + actin (40 ng)	22/97	23	98
Ang + neamine (10 ng)	32/126	25	93
Ang + paromomycin (20 ng)	31/48	65	0
bFGF (10 ng)	18/27	67	
bFGF + actin (40 ng)	7/34	21	100
bFGF + neamine (10 ng)	5/30	17	100
bFGF + paromomycin (10 ng)	22/32	69	0
VEGF (10 ng)	22/36	61	
VEGF + actin (40 ng)	9/35	26	90
VEGF + neamine (10 ng)	8/34	24	95
VEGF + paromomycin (10 ng)	17/28	61	0
Actin (40 ng)	11/56	19	
Neamine (10 ng)	10/47	21	
Paromomycin (10 ng)	7/32	22	
Water[c]	15/67	22	

[a]Growth of blood vessels was observed microscopically and recorded as either positive or negative after 68 h of incubation. Data were combined from multiple sets of experiments each using between 10 and 20 eggs. [b]Percent inhibition was calculated from the net increases of % positive of each sample over water control. The net increase of the % positive in the absence of inhibitors was considered as 100% active. [c]A positive response in ~20% of the eggs, typically observed in water controls, is considered as background angiogenesis, which is not affected by inhibitors

Angiogenin antisense HeLa transfectants have reduced angiogenesis and tumor growth in athymic mice

To demonstrate that angiogenin is necessary for other angiogenic factors to induce angiogenesis *in vivo*, we constructed an angiogenin antisense plasmid pCI-Ang(−) and transfected it into HeLa cells. Stable angiogenin under-expressing transfectants were selected and injected into athymic mice. Tumor growth and angiogenesis derived from these cells were examined and compared to that derived from vector control (pCI) transfectants.

Figure 5a shows that the expression level of angiogenin was inhibited by angiogenin antisense transfection in HeLa cells (from 0.68 to 0.35 pg/10^3 cells/day). The appearance, establishment, and growth of HeLa cells in athymic mice were all decreased after angiogenin antisense transfection. At day 13, all of the mice inoculated with vector control transfectants had to be killed because the tumors had grown to an extent that hindered the animals' activity. There was still one tumor-free mouse in the antisense group. When tumors did develop, their average size was about 50% of those in the vector control group (Figure 5b). These results indicate that the tumorigenicity of HeLa cells decreased after transfection with pCI-Ang(−).

Vessel staining (Figure 5c) showed that the HeLa tumors grown from pCI-Ang(−) transfectants have only 38% of the blood vessel density (5.9 *vs* 15.3) compared to that grown from vector control transfectants. Staining with 26-2F confirmed that angiogenin expression in pCI-Ang(−) tumor tissue was lower than that in control tumor tissue (Figure 6a and b). It is interesting to observe that pCI-Ang(−) HeLa tumor actually expressed a higher amount of bFGF and VEGF (Figure 6d and f) than did the pCI-vector control HeLa tumor (Figure 6c and e). The elevated expression of

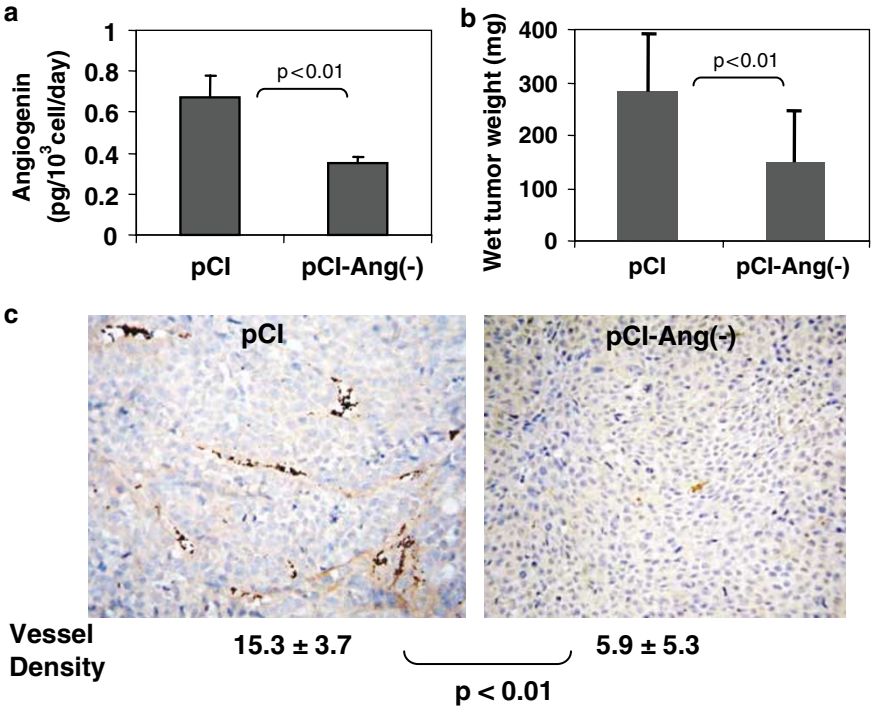


Figure 5 Angiogenesis and tumor growth in athymic mice were inhibited in an angiogenin antisense HeLa transfectants. HeLa cells were transfected with angiogenin antisense construct pCI-Ang(−) or vector control pCI. Stable transfectants were screened with G418. Angiogenin secretion levels of these cells were determined by ELISA (**a**). Tumor growth in nude mice was carried out by injecting 8×10^5 cells s.c. into the left shoulder of each mouse. Mice were killed on day 13 and the wet tumor weights were recorded (**b**). Data shown were means$\pm$s.d. from eight mice. Immunohistochemical staining for blood vessels (**c**) was performed on formalin-fixed, paraffin-embedded tissue with an anti-vWF IgG as the primary antibody

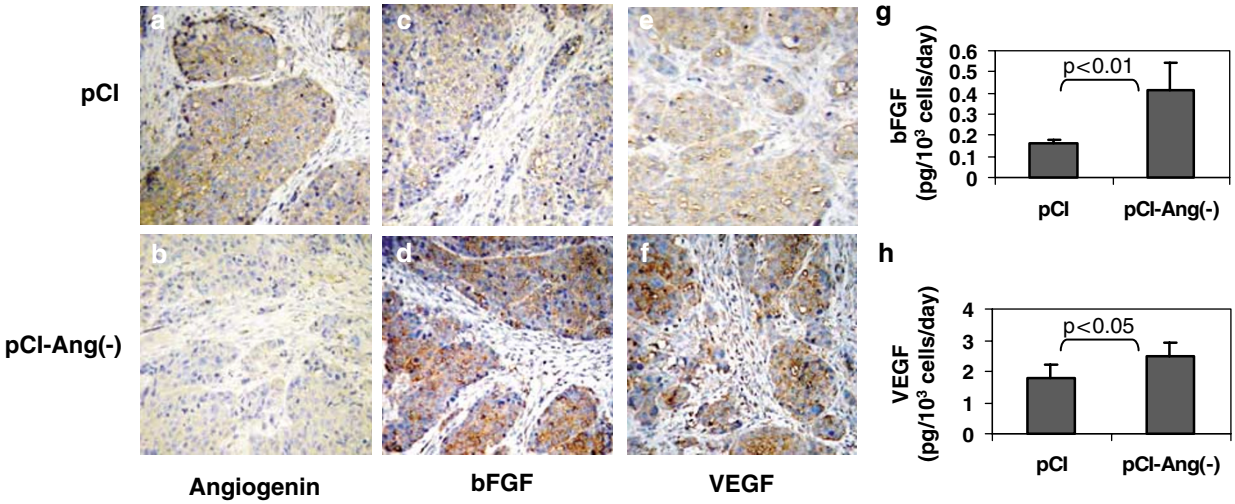


Figure 6 Expression levels of bFGF and VEGF in pCI-Ang(−) Hela cells and tumor tissues are upregulated. (**a–f**) Immunohistochemistry. Thin sections (4 μm) from formalin-fixed, paraffin-embedded tumor tissues derived from pCI vector-transfected HeLa cells (**a**, **c**, and **e**) and from angiogenin antisense pCI-Ang(−)-transfected HeLa cells (**b**, **d**, and **f**) were stained with an anti-angiogenin (**a** and **b**), anti-bFGF (**c** and **d**), and anti-VEGF (**e** and **f**) mAb, respectively. The bound primary antibodies were visualized with DAKO's Envision system. All experiments have been repeated at least three times and similar results were obtained in each repeat. Data shown were from a representative sample. (**g**, **h**) The secretion levels of bFGF and VEGF. Cell culture media were collected at the exponentially growing phase and the amount of bFGF and VEGF were determined by ELISA kits from R and D Systems

bFGF and VEGF was confirmed by ELISA analyses of the cell culture media of the pCI-Neo and the pCI-Ang(−) transfectants (Figure 6g and h, respectively). These results demonstrated that *in vivo* angiogenesis in mice induced by implanted HeLa cells decreased as a consequence of angiogenin downregulation even though

the expression levels of bFGF and VEGF were actually increased. These data also implicated that angiogenin secreted from the implanted human cancer cells acts on mouse endothelial cells of the hosts, consistent with the finding that human angiogenin-specific mAb 26-2F inhibited the ecotopic growth of human tumor cells in mice (Olson *et al*., 1995).

Discussion

Nuclear function of angiogenin is related to rRNA transcription

Angiogenin is one of the earliest tumor angiogenic factors to be isolated based solely on its angiogenic activity on CAM assay (Fett *et al*., 1985). However, its mode of action remained elusive mainly because of its relatively weak activity toward endothelial cells in various biological assays. Under certain conditions, angiogenin is able to stimulate endothelial cell migration (Hu *et al*., 1994) and proliferation (Hu *et al*., 1997). However, these activities are much lower than that of a typical endothelial cell growth factor, such as VEGF and bFGF, suggesting that angiogenin is not a classical endothelial cell mitogen. Another unsolved problem is that of the cellular receptor for angiogenin even though a number of binding proteins have been identified (Hu *et al*., 1991, 1993, 1997) and angiogenin has been shown to activate phospholipase C (Bicknell and Vallee, 1988) and Erk 1/2 (Liu *et al*., 2001) pathways. However, the effects were not robust and the downstream events are unknown. On the other hand, angiogenin has potent angiogenic activity in various *in vivo* angiogenesis assays including chick CAM, rabbit cornea (Fett *et al*., 1985), and rat cremaster muscle (Fu *et al*., 1999) models. These results suggest that angiogenin is involved in angiogenesis but with an unknown mechanism.

Angiogenin undergoes nuclear translocation in endothelial cells. It occurs rapidly in sparse endothelial cell cultures, decreases when cell density increases and ceases when cells are confluent, suggesting that the function of nuclear angiogenin is related to cell proliferation. We have shown that angiogenin is involved in the process of ribosome biogenesis and have identified an angiogenin-binding element from the promoter region of rRNA gene (Xu *et al*., 2003). Here, we used siRNA and antisense to inhibit angiogenin expression in HUVEC and showed that downregulation of angiogenin resulted in a marked decrease in 45S rRNA synthesis (Figure 2b and d). However, the inhibition was not complete. Attempts to obtain a greater degree of inhibition of angiogenin expression by increasing CT-1 concentration were unsuccessful because massive cell death occurred when CT-1 reached 0.5 μM (Figure 2a). It was also unsuccessful to generate stable angiogenin under-expressing cells because they are primary cultures and failed the selection procedure. Further studies would be necessary to determine whether a critical basal level of angiogenin in endothelial cells is necessary to prevent apoptosis.

Essential role of angiogenin in HUVEC proliferation

Both CT-1 and HX12 inhibited angiogenin expression in HUVEC and resulted in a decrease in rRNA transcription and cell proliferation induced by other angiogenic factors, indicating an essential role of endogenous angiogenin in these cellular activities. Consistently, we observed that angiogenin can be induced to undergo nuclear translocation in HUVEC by exogenous angiogenic factors. Therefore, there is a good correlation between the angiogenin level and the activity of exogenous angiogenic proteins in stimulating rRNA transcription, ribosome biogenesis, and cell proliferation.

Oppositely, endogenous bFGF and VEGF in HUVEC were not required for cell proliferation induced by exogenous angiogenic factors. Downregulation of endogenous bFGF and VEGF by their corresponding antisense compounds did not change the responsiveness of cells toward exogenous factors (Figure 4a–f). Therefore, inhibition of one angiogenic factor does not necessarily result in a decrease in cell proliferation induced by other angiogenic factors. It should be noticed that total cell proliferation did decrease after CT-3 and CT-5 transfection, indicating that the endogenous bFGF and VEGF do contribute to the overall cell proliferation. However, the percentage increase induced by exogenous factors over the respective controls did not change after the expression of endogenous bFGF or VEGF was downregulated.

Exogenous angiogenin rescued CT-1-mediated inhibition of bFGF-induced cell proliferation (Figure 3e), indicating that the effect of CT-1 was specific and that the reduction of bFGF-induced cell proliferation was indeed because of the decreased expression of angiogenin. Another line of evidence came from the lack of effect of CT-1 on fibroblasts (Figure 4h and i). The *de novo* synthesis of 45S rRNA and the proliferation of fibroblasts induced by bFGF were not inhibited by CT-1 transfection. This is consistent with the fact that angiogenin does not undergo nuclear translocation in fibroblasts (Moroianu and Riordan, 1994). These results indicated that downregulation of angiogenin only affect cells in which nuclear translocation of angiogenin occurs.

bFGF-stimulated Erk1/2 phosphorylation remained unchanged in angiogenin under-expressing cells, indicating that the function of angiogenin is downstream of the signal transduction pathway triggered by receptor binding. This is consistent with our hypothesis that angiogenin is required for other angiogenic factors to induce cell proliferation by stimulating rRNA transcription that is in higher demand when cells are growing and proliferating.

Angiogenin is required for bFGF and VEGF to stimulate angiogenesis

Stable transfection of pCI-Ang(−) in HeLa cells reduced angiogenin expression but actually increased the levels of bFGF and VEGF both in cells under culture and in those grown in athymic mice (Figure 6). However, despite of the elevated bFGF and VEGF levels, these

cells displayed a reduced capacity to induce tumor growth and tumor angiogenesis in mice, further demonstrating the importance of angiogenin in bFGF- and VEGF-mediated angiogenesis.

Many of the known angiogenic factors are endothelial cell mitogens (Risau, 1996). They induce endothelial cell proliferation thereby contributing in the process of angiogenesis (Papetti and Herman, 2002; Folkman, 2003). However, the interrelationships among these factors are still unclear (Folkman, 2002). How do these factors coordinate to induce angiogenesis? Are they simultaneously involved in all steps in the angiogenesis process or individually responsible for different steps? Are they interdependent or are their actions additive or synergistic? The results presented in this paper may have shed lights on these issues and suggested that some of the angiogenic factors go through a crossroad in the process of angiogenesis: angiogenin-stimulated rRNA transcription in endothelial cells.

It has been reported that ribonuclease inhibitor (RNasin), a high-affinity angiogenin-binding protein ($K_d < 10^{-15}$ M), inhibited bFGF-dependent angiogenesis and tumor growth in mice by an unknown mechanism (Polakowski *et al*., 1993). It may well be that RNasin inhibited bFGF-induced angiogenesis and tumor growth via inhibition of angiogenin. Consistent with these observations, neamine and actin were found to inhibit the angiogenic activity of bFGF and VEGF in CAM (Table 1). Actin is an angiogenin-binding protein that blocks the nuclear translocation of angiogenin and neutralizes the angiogenic activity (Hu *et al*., 1993). Neomycin is an aminoglycoside antibiotic that has been shown to inhibit nuclear translocation of angiogenin thereby abolishing its angiogenic activity (Hu, 1998). However, the nephro- and ototoxicity of neomycin prevents it from being developed as an antiangiogenesis agent for clinical use. We have therefore been searching for neomycin analogues and derivatives that retain the inhibitory activity toward nuclear translocation of angiogenin but are less toxic. We have found that paromomycin, an aminoglycoside antibiotic that differs from neomycin only at the C6 position of the first aminohexose where the $-NH_2$ is substituted with $-OH$, does not inhibit nuclear translocation of angiogenin. However, the half molecule of neomycin, neamine, is virtually nontoxic (Au *et al*., 1986; Williams *et al*., 1987), but is an equally effective inhibitor for nuclear translocation of angiogenin (Hu *et al*., unpublished data). Neamine inhibited angiogenesis induced by angiogenin, bFGF, and VEGF, whereas paromomycin had no effect (Table 1). These results further demonstrated the essential role of angiogenin in angiogenesis in general, and suggested that the nuclear function of angiogenin can serve as an effective target for antiangiogenesis therapy.

Materials and methods

Cell culture

HUVEC (Cell Systems Corp.) were cultured in human endothelial SFM basal medium (HEM, Invitrogen) + 5% FBS and 5 ng/ml bFGF. Cells were seeded at a density of 5×10^3 cells/cm^2 and cultured in the above medium for 24 h, washed three times with serum-free medium prior to various treatments.

Immunofluorescence

Cells were incubated with or without exogenous growth factors at 37°C for 4 h, fixed with −20°C methanol for 10 min, washed with PBS containing 30 mg/ml BSA, and incubated with 50 μg/ml of 26-2F for 1 h. The cells were then washed and incubated with Alexa 488-labeled goat F(ab')$_2$ anti-mouse IgG (Molecular Probes) at 1 : 100 dilution for 1 h.

Antisense treatment

Cells were washed with opti-MEM (Invitrogen) three times and transfected with 0.1 μM antisense in the presence of 1.2 μl/ml lipofectin in opti-MEM for 5 h. CT-1 (5′-CAACAAA ACGCCCAGGCC-3′), CT-3 (5′-GCTCCCGGCTGCCATG GT-3′), and CT-5 (5′-CAGCAGAAAGTTCATGGT-3′) are antisense compounds for angiogenin, bFGF, and VEGF, hybridizing with nucleotides 122–139, 464–481, and 14–31 of the angiogenin, bFGF, and VEGF mRNA, respectively. CT-2 (5′-CCGGACCCGCAAAACAAC-3′), CT-4 (5′-TGGTACC GTCGGCCCTCG-3′), and CT-6 (5′-TGGTACTTGAAAGA CGAC-3′) are controls for CT-1, CT-3, and CT-5, respectively. A transfection efficiency of ~70% was obtained under these conditions as been determined by plasmid encoding green fluorescence protein.

siRNA treatment

HX-1 (5′-AAGAAUGGAAACCCUCACAGA-3′) and HX-2 (5′-CUUACCUUUGGGAGUGUCUCU-3′) were annealed to form HX12, a double-stranded RNA with 2-nt overhangs at 3′. The sequence of HX12 corresponds to nucleotides 362–383 of the angiogenin mRNA. HX-3 (5′-AGACACUCC CAAAGGUAAGAA-3′) and HX-4 (5′-CUUACCUUUGG GAGUGUCUCU-3′) were annealed to form HX34 that has the same base composition as that of HX12 but does not correspond to any known sequences and is therefore a control for HX12. HUVEC were transfected with 20 nM HX12 or HX34 in the presence of 2 μl/ml Effectene (Qiagen) for 5 h.

Northern hybridization

RNA was extracted with the use of Trizol (Invitrogen), separated on an agarose/formaldehyde gel, and transferred onto a nylon membrane. The probe used for 45S rRNA is 5′-GGTCGCCAGAGGACAGCGTGTCAG-3′ that hybridizes with the first 25 nucleotides of the 45S rRNA precursor. The probe used for GAPDH is a 40-nt oligonucleotide derived from the N-terminus of the coding region. The probe used for angiogenin was a 569 bp DNA generated by *Apa*I digestion from pAngC. This fragment contains the complete 441 bp angiogenin-coding sequence.

RT–PCR

RNA was extracted by Trizol reagent. RT–PCR was carried out with a set of primers 5′-TCTGAATTCATGAACTTT CTGCTGTCTTG-3′ and 5′-TCTGGTCGACGGCTCACCG CCTCGGCTTG-3′ for VEGF; 5′-TCTGAATTCATGGCA GCCGGGAGCATCAC-3′ and 5′-TCTGGTCGAAAAAT CAGCTCTTAGCAGAC-3′ for bFGF; and 5′-ACAATGAG CTGCGTGTGGCT-3′ and 5′-TCTCCTTAATG TCACGC ACGA-3′ for actin.

Western blotting

Cells were lysed with RIPA buffer. Protein concentrations were determined chromometrically with a microplate method (Pierce). Samples of equal amounts of protein (50 μg) were subjected to Western blotting analysis for angiogenin, actin, phosphorylated Erk1/2, and total Erk1/2 with 26-2F, an anti-actin mAb AC-15 (Sigma), anti-phosphorylated Erk, and anti-total Erk antibodies (New England Biolabs), respectively. The bands were visualized with the Super signal west pico chemiluminescent system (Pierce).

ELISA detection of angiogenin

ELISA plates were coated with 100 μl of 10 μg/ml 26-2F and blocked with 5 mg/ml BSA in PBS. Samples were added in triplicates (100 μl) and the plates were incubated at 4°C overnight, washed with PBS five times and incubated with 100 μl/well anti-angiogenin polyclonal antibody R112 (1 : 4000) at RT for 2 h. After washing four times with PBS, an alkaline phosphatase-labeled goat anti-rabbit IgG (1.25 μg/ml) was added and incubated at RT for 1 h. The plates were washed four times with PBS and *p*-nitrophenyl phosphate (5 mg/ml, 100 μl/well) dissolved in 0.1 M diethanolamine containing 10 mM $MgCl_2$, pH 9.8, was added. After 1 h incubation at RT, the reaction was stopped with 3 N NaOH (50 μl/well) and the absorbance was measured at 630 nm. A standard curve of recombinant human Ang at concentrations ranging from 50 to 1000 pg/well was performed each time on every plate.

Silver staining of NOR

Cells cultured on a cover-slip were fixed with methanol : acetic acid (9 : 1, v/v) for 10 min at RT. The cover-slip was air-dried and incubated in a solution containing 0.8 g/ml $AgNO_3$, 60 mM NaAc, pH 4.1, 15% formaldehyde, and 3% methanol at 37°C in the dark for 30 min. The slip was washed three times with water, mounted on a glass slide and observed at $\times 1000$ magnification on a photonic microscope using oil immersion. Silver-stained NOR dots were counted in 30 randomly selected nuclei.

^{32}P labeling of newly synthesized RNA

Cells were pulse-labeled with 25 μCi/ml ^{32}P-orthophosphate for 30 min and washed three times with PBS. Total cellular RNA was extracted with Trizol reagent. Equal amounts of RNA were applied for agarose/formaldehyde gel electrophoresis and transferred onto a nylon membrane. Radiolabeled RNA was visualized by autoradiography.

Preparation of angiogenin under-expressing HeLa cells

The entire coding region of the human angiogenin cDNA (369 bp) was amplified from the pAngC plasmid. Primers for the PCR reaction are 5′-TCTGAATTCGTTACGGACGA CGG-3′ containing an *Eco*RI site, and 5′-TCTGGTCGACT GATGCAGGATAACTCCAGGTACAC-3′ containing a *Sal*I site. The PCR amplicon was cloned into the pCI-neo mammalian expression vector (Promega) in the antisense-orientation. The pCI-Ang(−) plasmid was transfected into HeLa cells using Lipofectamine reagent (Invitrogen). Stable transfectants were selected in the presence of 2 mg/ml G418 for 2 weeks. Integration of the transfected gene into the chromosomes was confirmed by genomic DNA PCR with the forward primer (5′-AGTACTTAATACGACTCACTA TAGGC-3′) from the T7 sequence of the pCI vector and the reverse primer (5′-ATGCAGGATAACTCCAGGTACAC-3′) from the inserted human angiogenin sequence. Transcription of antisense human angiogenin mRNA from the transfected expression cassette was confirmed by RT–PCR using the same set of primers for genomic DNA PCR. Angiogenin level was determined by ELISA.

Xenografic growth of HeLa tumor in athymic mice

Outbred male athymic mice were obtained at 5 weeks of age from Charles River Laboratories. They were used for experiments when at 6–7 weeks of age. Vector-transfected (pCI) or angiogenin antisense-transfected (pCI-Ang(−)) HeLa cells, 8×10^5 per mouse, were injected s.c. on the left shoulder. Eight mice were used per group. Mice were killed on day 13 and the wet weight of the HeLa tumor was recorded.

Immunohistochemistry

Tissue specimens were fixed in 10% formalin and embedded in paraffin. The sections (4 μm) were deparaffinized with xylene, rehydrated in ethanol, and microwaved for 15 min in 10 mM citrate buffer, pH 6.0. Endogenous peroxidase was blocked by treatment with 0.3% H_2O_2 in methanol for 30 min. The sections were blocked in 5% dry milk for 10 min, and incubated with the primary antibodies at 4°C for 16 h and visualized with Dako's Envision system. The sections were counterstained with hematoxylin. Negative controls were obtained by omission of the primary antibodies. Neovessels were stained with an anti-von Willebrand's factor (vWf) polyclonal antibody A-0082 at a 1 : 200 dilution (Dako Corp.). vWf-positive vessels in each tumor were counted in five most vascularized areas at $\times 200$ magnification (i.e. $\times 20$ objective lens and $\times 10$ ocular lens; 0.785 mm^2/field). Vessel density (vessels/field) was shown as mean $\pm$ s.d. for each group. Angiogenin, bFGF, and VEGF were stained with their respective monoclonal antibodies.

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