Angiogenesis promotes growth and metastasis of tumor cells. In this study, we have developed two peptide antagonists of human angiogenin by deducting the codes from the antisense RNA sequence corresponding to the receptor-binding site of angiogenin in either 5′→3′ (chANG) or 3′→5′ (chGNA) direction. chANG and chGNA peptides bind to angiogenin with specificity and high affinity (Kd ~ 44 nM) and inhibit the interaction of angiogenin with actin, which is regarded as the angiogenin-binding protein on the surface of endothelial cells. The peptides inhibit the neovascularization induced by angiogenin in the chick chorioallantoic membrane assay. The anti-angiogenic activity of the peptides is specific for angiogenin, and the peptides do not have any apparent effect on embryonic angiogenesis or the preexisting blood vessels. chANG and chGNA also inhibit the angiogenesis induced by the angiogenin-secreting PC 3 human prostate adenocarcinoma cells and have no direct effect on the proliferation as well as the adhesion of PC 3 cells to angiogenin. Therefore, the inhibition of the tumor-induced angiogenesis by the peptides is most likely caused by neutralization of the extracellular angiogenin secreted by PC 3 cells. Based on our results, chANG and chGNA peptides may be effective for treatment of various human tumors which secrete angiogenin.

Angiogenin is the tumor-derived potent angiogenic factor (1). Angiogenin has 35% sequence identity with human pancreatic ribonuclease and belongs to RNase superfamily (2, 3), and its ribonucleolytic activity is essential for the angiogenic activity (4). The exact mechanism for the induction of neovascularization by angiogenin is still not known. However, several lines of evidence suggest that the biological action of angiogenin is mediated through a cell surface receptor (5–7). Angiogenin specifically binds to endothelial cells (8) and a 42-kDa angiogenin-binding protein, a member of actin family has been identified from endothelial cells (9, 10). Actin and anti-actin antibody block the angiogenic activity of angiogenin in the chick chorioallantoic membrane (CAM) assay (10). In addition to its ribonucleolytic activity, the receptor-binding site of angiogenin (residues 58–70) is essential for its angiogenic activity (11).

According to the primary (2, 3) and three-dimensional structure (12, 13), the receptor-binding site of angiogenin is distinct compared with the homologous protein RNase (Fig. 1A). This appears to be the reason for RNase not being angiogenic despite its high ribonucleolytic activity. Recently, it was reported that a hybrid RNase protein in which the receptor-binding site of angiogenin (residues 58–70) replaced the corresponding residues of RNase (residues 59–73) promotes angiogenesis as effectively as angiogenin (14). Consequently, the receptor-binding site of angiogenin is a good target site for development of angiogenin antagonists.

According to the hydrophobic complementarity of amino acids, the amino acid deduced by an antisense code (either 5′→3′ or 3′→5′ direction) is generally antipathic, that is, a hydrophobic amino acid can be deduced from a code for a hydrophilic amino acid, vice versa (15). The peptides, which are designed by the hydrophatic complementary approach, show inverse hydrophatic relationship to the peptides encoded by sense mRNA, and the designed peptide binds target protein with specificity and high affinity (16). There are several examples that demonstrate successful application of this approach. Antagonists of various proteins such as ACTH, ribonuclease S peptide, c-Raf protein, fibronectin, insulin, and α-chain of fibrinogen were developed based on this approach (16–21).

In this report, we show that the two peptides deduced from the antisense RNA corresponding to the receptor-binding site of human angiogenin (residues 58–70; Ref. 11) in either 5′→3′ direction (chANG) or 3′→5′ direction (chGNA) effectively inhibit the interaction of angiogenin with its putative receptor actin in vitro. Furthermore, these peptides block the neovascularization induced by angiogenin and angiogenin-secreting tumor cells in the chick CAM assay. These angiogenin antagonists may be useful for the treatment of human angiogenin-dependent tumors.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—chANG and chGNA peptides with the sequence of NH2-Val-Phε-Ser-Val-Arg-Val-Ser-Val-Arg-Val-Ser-Ile-Leu-Val-Phe-COOH and NH2-Val-Leu-Phε-Leu-Pro-Leu-Pro-Leu-Gly-Val-Ser-Leu-Asp-Ser-COOH, respectively, were synthesized and purified by Bio synthesis, Inc. (Lewisville, TX). Due to the hydrophobicity of these peptides, chANG and chGNA peptides were dissolved in 10% dimethyl sulfoxide (MeSO) at 2 mg/ml. Neurotensin was purchased from Bachem California.

Binding of 125I-Angiogenin to the Immobilized Peptides—To immobilize peptides, 500 ng of either chANG or chGNA peptide in 50 μl of 20% acetic acid was added to each well of an ELISA plate (96 well, Maxisorb, Nunc) and dried under air overnight. The peptide-coated wells were washed three times with binding buffer (0.1% bovine serum albumin, 0.05% lysozyme, and 0.05% sodium azide in PBS). Then, 8.1 nM of 125I-Angiogenin (10.5 μCi/μg) in 50 μl of binding buffer was incubated at 37 °C for 1 h in the presence of varying concentrations of recombinant human angiogenin (carrier-free, R & D Systems). After washing three times with 150 μl of binding buffer for 3 min each, the bound 125I-angiogenin was dissolved in 0.1 ml NaOH, and the radioactivity was determined in a γ-counter. Two independent experiments were carried out, and each assay was performed in duplicate.
Biotinylated of Actin—Bovine muscle actin (0.5 mg, Sigma) was biotinylated using 100 µg of N-biotinyl-L-aminocaproic acid N-acetylsuccinimide ester (Boehringer Mannheim) according to the manufacturer’s instruction. After reaction, the mixture was dialyzed against 50 mM Tris-HCl, pH 7.6, 100 mM NaCl at 4 °C. After centrifugation at 12,700×g for 30 min, the supernatant was aliquoted and stored at −20 °C until use.

Binding of Biotinylated Actin to Angiogenin—An ELISA plate (96 well, Maxisorp, Nunc) was coated with 50 ng of human angiogenin and then blocked with 0.1% gelatin in PBS. Biotinylated actin (8.0 nM) in 0.1% Tween 20 was added to the plate and incubated for 1 h at room temperature. Each well was washed five times with PBS containing 0.1% Tween 20. The bound biotinylated actin was detected by incubation with streptavidin-horseradish peroxidase (1: 1,500 dilution with 0.1% gelatin in PBS (PBS + 0.1% Tween 20)) and 3 mg/ml of O-phenylenediamine dihydrochloride (Pierce) in substrate buffer (30 mM citrate, 70 mM NaHPO4, 0.02% H2O2, pH 5.5). After stopping the reaction by addition of an equal volume of 3 N HCl, absorbance at 490 nm was determined in an automated ELISA reader (EL 312e, Bio-Tek Instruments). All assays were carried out in triplicate.

Assay for Ribonucleolytic Activity of Angiogenin—Reaction mixtures consisting of 0.4 µg of TRNA (yeast, type X Sigma), 10 µM of phenylmethanesulfonyl fluoride, 1 µg of human angiogenin in 0.1 ml of 33 mM Hepes, pH 7.0, and 10 µM NaCl in the absence or presence of 100 µg/ml of peptides were incubated for 15 min at 37 °C. The reaction was terminated by the addition of 0.24 ml of 3.4% ice-cold perchloric acid, and the samples were kept on ice for 10 min. The mixtures were centrifuged at 15,000 × g for 10 min, and the absorbance of supernatants was determined at 260 nm (22). Experiments were carried out in duplicate.

Quantification of Angiogenin in Conditioned Media—Subconfluent cultures of PC 3 and NIH/3T3 cells in 100-mm culture dishes were rinsed twice with 10 ml of PBS and further washed twice with 10 ml of serum-free RPMI 1640 and Dulbecco’s modified Eagle’s medium, respectively. Fresh serum-free medium (10 ml/100-mm dish) was added and incubated at 37 °C. The medium was collected following 1 and 3 days of culture and centrifuged at 1,000 × g for 10 min to remove any cell debris. The resulting conditioned media were stored at −70 °C until use. Cell densities were determined at the end of experiments using a hemocytometer. Quantikine™ human angiogenin immunoassay (R & D Systems) was carried out to determine the amount of human angiogenin in conditioned medium according to the manufacturer’s instructions. Experiments were carried out with two independently prepared conditioned media, and each assay was performed in duplicate. Serum-free medium was used to determine the blank value. An angiogenin standard curve was constructed using recombinant human angiogenin (R & D Systems) in duplicate.

Tumor-induced Angiogenesis—To investigate the tumor-induced angiogenesis, modified CAM assay was carried out. Confluent cultures of PC 3 cells were trypsinized, washed once with RPMI 1640-supplemented 10% serum, and then washed twice with serum-free RPMI 1640. The resulting pelleted cells were resuspended at 2 × 107 cells/ml in serum-free RPMI 1640, which contained 1.5 mg/ml rat tail type 1 collagen (Collagen Systems) in the absence or presence of peptides. Five microliters of the mixture was loaded onto 1/4 piece of 15-mm-Thermomax discs and permitted to polymerize. The discs were applied to the CAMs of 10-day-old embryos. After 6 h ± 2-h incubation, discs and the surrounding CAMs were photographed. For quantitative analysis, the number of blood vessels entering the collagen sponges was counted under a microscope by two observers in a double-blind manner. Each experiment included 12 eggs.

PC 3 Cell Adhesion Assay—The adhesion of PC 3 cells to the immobilized angiogenin was assayed according to Soncin (23) with slight modification. Briefly, an ELISA plate (96 well, Maxisorp, Nunc) was coated with recombinant human angiogenin (carrier-free, R & D Systems) or bovine pancreatic RNase A (Sigma) in 0.1 ml of PBS by incubation overnight at 4 °C. The plate was washed three times with PBS before cell seeding. The coating efficiency of angiogenin was estimated using iodinated angiogenin as a tracer. For RNase A, the efficiency was determined by assaying the ribonucleolytic activity of the RNase remaining after coating (22).

After trypsinization of confluent PC 3 cells, cells were washed once with complete medium (RPMI 1640 with 10% bovine serum albumin) and twice with serum-free RPMI 1640 containing 0.1% bovine serum albumin (fraction V, low endotoxin, Sigma). The final pelleted cells were resuspended in serum-free RPMI 1640 containing 0.1% bovine serum albumin, and cells were seeded at 108 cells in 0.1 ml onto the angiogenin- or RNase A-coated wells of ELISA plates. After 2-h incubation at 37 °C, the plates were washed three times with 0.2 nl of PBS, and the adhered cells were fixed and stained with methylene blue (24). The amount of 0.1 N HCl-released methylene blue was determined by its absorbance at 600 nm in an automated ELISA reader (EL 312e, Bio-Tek Instruments). To construct a standard curve for PC 3 cells, varying numbers of cells were seeded onto 0.1% gelatin-coated cell culture plate and incubated at 37 °C for 6 h. Plates were washed, and the adhered cells were fixed and stained with methylene blue. The amount of the methylene blue incorporated into the cells was determined. All experiments were carried out in triplicate.

Cytotoxicity Assay—PC 3 cells (7 × 104) were plated to each well of a tissue culture plate (96 well, Nunc). After 24-h incubation at 37 °C, 100 µl of varying amounts of actin, chANG, and chGNA samples diluted in culture medium was added to each well. Following incubation for 3 additional days, the number of viable cells was determined using cell titer 96 non-radioactive proliferation assay kit (Promega) according to the manufacturer’s instructions. Each experiment was carried out in triplicate, and the average value was used to determine percent viability.

RESULTS

Design of Anti-angiogenin Peptides, chANG and chGNA—We designed two peptides that are complementary to the receptor-binding site of human angiogenin (residues 58–70) and that of corresponding region of human RNase are shown (2, 3). Disulfide bonds in the human RNase are shown by connecting lines. B, shown are the nucleotide sequences of antisense mRNA in either 5′→3′ (chANG) or 3′→5′ (chGNA) direction corresponding to the receptor-binding site of human angiogenin (residues 58–70) and the deduced amino acid sequences.

Design of Anti-angiogenin Peptides using hydrophilic complementary approach, A, the amino acid sequences of the receptor-binding site of human angiogenin (residues 58–70) and that of corresponding region of human RNase are shown (2, 3). Disulfide bonds in the human RNase are shown by connecting lines. B, shown are the nucleotide sequences of antisense mRNA in either 5′→3′ (chANG) or 3′→5′ (chGNA) direction corresponding to the receptor-binding site of human angiogenin (residues 58–70) and the deduced amino acid sequences.
Angiogenin Binds to the Immobilized chANG and chGNA Peptides—Angiogenin binds to the immobilized chANG and chGNA peptides (Fig. 3). Unlabeled angiogenin inhibited the binding of $^{125}$I-angiogenin to the immobilized chANG and chGNA peptides with similar IC$_{50}$ values of 52 nM for both peptides. The $K_d$ value for the interaction of angiogenin with peptides was calculated from the IC$_{50}$ values determined using the equation: $K_d = IC_{50} \div [125I$-angiogenin]. The equation is based on the assumptions that both unlabeled and labeled angiogenin have identical affinities for the peptides and that only one class of binding sites exists (25). $K_d$ value determined by this method was 44 nM for both peptides.

chANG and chGNA Peptides Inhibit the Interaction of Angiogenin with Actin—chANG and chGNA peptides inhibited the binding of biotinylated actin to the immobilized angiogenin in a concentration-dependent manner (Fig. 4). Half-maximal inhibition (IC$_{50}$) occurred at 31 μM for chANG and at 9.7 μM for chGNA. However, the presence of about 100-fold molar excess of peptides showed little effect on the ribonucleolytic activity of angiogenin (data not shown). These results suggest that the peptides bind to the receptor-binding site of angiogenin and do not affect the ribonucleolytic activity of angiogenin.

chANG and chGNA Peptides Inhibit the Angiogenesis Induced by Angiogenin—Angiogenin (0.69 pmol/egg) in 0.2% Me$_2$SO induced neovascularization in 42.1% of the eggs in five sets of assays ($p < 0.05$) compared with 0.2% Me$_2$SO control (14 pmol). However, chANG and chGNA peptides showed little effect on the ribonucleolytic activity of angiogenin and the residues 58–70 of human angiogenin.

chANG and chGNA (87 pmol) 8/43 18.6 (2.6) 0.893
chGNA (8.7 pmol) 10/48 20.8 (3.9) 0.429
chGNA (87 pmol) 8/43 18.6 (2.6) 0.893
Angiogenin + chANG (8.7 pmol) 12/49 24.4 (3.1) 0.119
Angiogenin + chGNA (87 pmol) 10/52 19.2 (4.3) 0.165
Angiogenin + actin (14 pmol) 13/47 27.7 (4.5) 0.030

$^a$ p values from student’s t test based on comparisons with 0.2% Me$_2$SO control samples tested at the same time as other protein samples. $p < 0.05$ means significant difference from 0.2% Me$_2$SO control.

$^b$ S.D.
effect on the preexisting blood vessels (data not shown). Actin (14 pmol; 20-fold excess of angiogenin) also inhibited about 60% of the angiogenic activity of angiogenin (27.7% positive response, \( p = 0.030 \)). The anti-angiogenin activity observed with actin is comparable with that reported previously (10).

**Synthesis of Angiogenin by PC 3 Cells**—Although human angiogenin was first identified from the conditioned medium of human adenocarcinoma cell line HT-29 (1), several types of human tumor cells, such as HT-1080 fibrosarcoma and A 549 lung carcinoma, and some normal cells, including endothelial, smooth muscle, and fibroblast cells, can also produce angiogenin (26). To investigate whether human prostate adenocarcinoma cells (PC 3) secrete angiogenin, the amount of angiogenin present in the serum-free medium taken from PC 3 culture was estimated by immunoassay using Quantikine™ assay kit (R & D Systems). A large amount of angiogenin was present in conditioned medium, and the amount increased with culture days. About 5 and 13 ng of angiogenin were produced by 10^6 PC 3 cells in 1 and 3 days, respectively (Fig. 5). Neither serum-free RPMI 1640 nor the conditioned medium prepared from the culture of NIH/3T3 had any detectable angiogenin (data not shown). The results clearly indicate that PC 3 cells secrete angiogenin into media, and the angiogenin was not derived from either serum-free media (RPMI 1640) or the bovine calf serum used to maintain the culture of PC 3 before the preparation of serum-free conditioned medium.

**chANG and chGNA Peptides Inhibit the Tumor-induced Angiogenesis**—When PC 3 cells (10^6 cells) were entrapped in type I collagen sponges and loaded directly on the CAMs of 10-day-old embryos, detectable infiltration of blood vessels into collagen sponges was observed within 3 days (Fig. 6), but the control sponges without tumor cells did not induce neovascularization. Most of the infiltration of vessels into the collagen sponges was abolished by the presence of chANG and chGNA without any visible effect on the preexisting blood vessels. However, neurotensin (250 pmol) used as a negative control peptide that has no relationship with angiogenin, did not inhibit the tumor-induced angiogenesis (data not shown). Quantitative analysis revealed that 79 pmol of chANG and 72 pmol of chGNA abolished about 90 and 73% of the blood vessels entering the collagen sponges, respectively (Fig. 6).

**chANG and chGNA Do Not Affect the Adhesion of PC 3 Cells to Angiogenin**—Angiogenin supports adhesion of endothelial and tumor cells (23, 27). Thus, angiogenin as an adhesion molecule may play an important role in the process of angiogenesis and tumor metastasis. When PC 3 cells were seeded to an ELISA plate in the absence of serum, most of the cells did not adhere to the plastic wells even after 6-h incubation. In contrast, angiogenin induced attachment of 63% of the cells within 2 h, and the number of adhered cells was dependent on the amount of coated angiogenin, reaching saturation after 0.16 \( \mu \)g/well (Fig. 7A). PC 3 cells also adhered to the RNase-coated wells, but the ability of RNase A as an adhesion molecule was weaker than that of angiogenin. Gelatin, which is one of the well known adhesion molecules, induced attachment of 35% of the cells within 2 h (data not shown). Thus, angiogenin appears to be a more potent adhesive substrate than gelatin.

As shown in Fig. 7B, actin, which is an antagonist of angiogenin, inhibited the adhesion of PC 3 cells to immobilized angiogenin in a concentration-dependent manner. In contrast, peptide antagonists of angiogenin (chANG and chGNA) did not.

**chANG and chGNA Do Not Show Anti-tumor Activity in Vitro**—To investigate whether the inhibition of PC 3 cell-induced angiogenesis by the peptide antagonists was due to direct cytotoxic effect against the tumor cells, effect of the peptide antagonists on the proliferation of PC 3 cells grown in culture was investigated. Actin, chANG, and chGNA did not show any cytotoxicity against PC 3 cells (Fig. 8).

**DISCUSSION**

Since neovascularization is indispensable for tumor growth and metastasis (28, 29), development of inhibitors that block
Peptide Antagonists of Angiogenin

The formation of new blood vessels can be an attractive therapeutic approach for the treatment of both primary and metastatic cancer. Among the tumor-associated angiogenic factors (30), angiogenin may be the most important angiogenic factor, because angiogenin has been known as one of the most potent angiogenic factors secreted by tumor cells (1) and has an essential role for the establishment of different histological types of human tumor cells (31, 32). Therefore, development of angiogenin antagonists that can block the angiogenic activity of this protein is worthwhile.

Our goal in this work was development of the peptide antagonists of angiogenin using hydrophobic complementary approach (15, 16). chANG and chGNA peptides, which are antipathic to the receptor-binding site of angiogenin, interact directly with angiogenin with somewhat high affinity ($K_d \approx 44$ nM) and also show anti-angiogenin activities without any effect on the ribonucleolytic activity of angiogenin. Both peptides inhibit the interaction of angiogenin with the angiogenin-binding protein, actin, in a concentration-dependent manner and block the neovascularization induced by angiogenin in the chick CAM assay. The anti-angiogenic activity of these peptides is specific for angiogenin, because the presence of these peptides does not have any effect on embryonic angiogenesis or the preexisting blood vessels. Both peptides inhibit the angiogenin-induced neovascularization more effectively than actin, which is previously shown to be an angiogenin antagonist (10). Furthermore, chANG and chGNA inhibit the neovascularization induced by the angiogenin-secreting PC 3 tumor cells without any effect on the preexisting blood vessels. However, the peptides do not affect the adhesion of PC 3 cells to angiogenin and proliferation of PC 3 cells. Therefore, the inhibition of the tumor-induced angiogenesis by these peptides is not caused by cytotoxicity toward tumor cells, but is most likely caused by neutralization of the extracellular angiogenin secreted by PC 3 cells. Our results also provide additional evidence that the interaction of angiogenin with its putative receptor actin via the receptor-binding site of angiogenin (residues 58–70) is essential for angiogenic activity of angiogenin (11).

The results presented in this work clearly demonstrate that small peptides such as chANG (11 amino acid) and chGNA (13 amino acid) bind to the receptor-binding site of angiogenin, and the binding of these peptides to angiogenin inhibits the interaction of angiogenin with actin. The interaction of these peptides with angiogenin is not sequence- or conformation-directed, but rather dependent on the hydrophobic profile of these peptides for the following reason. Although both chANG and chGNA peptides have similar affinity for angiogenin and similar anti-angiogenin activities, the primary amino acid sequence of the chANG peptide is completely different from that of chGNA. However, the hydrophobic profiles of both peptides are almost the same. According to the previous reports, the interactions between hydrophobic and hydrophilic amino acids is feasible and may provide the driving force for the binding of complementary peptides to target protein (17–19). Three-dimensional structure of the cocrystals of angiogenin and these peptides will provide direct information on how these peptides interact with angiogenin.

Actin and noncytotoxic monoclonal antibodies against human angiogenin have been shown to prevent or delay the establishment of the angiogenin-secreting human tumor cell lines such as HT-29 colon adenocarcinoma, HT-1080 fibrosarcoma, and A 549 lung carcinoma in nude mice (31, 32). The antitumor activity of the antibodies and actin is caused by neutralization of the extracellular angiogenin secreted by the tumor cells. chANG and chGNA peptide antagonists of angiogenin may inhibit the growth and metastasis of the angiogenin-secreting human cancer as effectively as the angiogenin antibodies and actin in animal studies and may be directly used for treatment of some types of human cancer.

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FIG. 7. Effect of chANG and chGNA on the adhesion of PC 3 cells to immobilized human angiogenin. A, adhesion of PC 3 cells to immobilized human angiogenin ($\square$) and bovine pancreatic RNase A ($\square$) was determined. Amount of coated protein was corrected by the coating efficiency as described under “Experimental Procedures.” The degree of cell attachment was expressed as percent of the attached cells relative to the number of seeded cells ($10^4$/well). The number of adhered cells was calculated from the absorbance of acid-extracted methylene blue dye using the standard curve constructed with the known number of PC 3 cells as described under “Experimental Procedures.” B, assay for adhesion of PC 3 cells to angiogenin was performed in the presence of varying concentrations of actin ($\triangle$), chANG ($\square$), or chGNA ($\square$) as described under “Experimental Procedures.”

FIG. 8. Cytotoxicity of actin, chANG, or chGNA against PC 3 cells. PC 3 cells grown in culture were treated with varying concentrations of actin ($\diamond$), chANG ($\square$), or chGNA ($\triangle$) for 3 days, and percent viability was determined as described under “Experimental Procedures.”
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