Antiplasmin Activity of a Peptide That Binds to the Receptor-binding Site of Angiogenin*

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It has been suggested that angiogenin binds to an actin-like molecule present on the surface of endothelial cells. Actin inhibits plasmin activity, but the angiogenin-actin complex is not active. In this report, we found that plasmin inhibits the interaction between angiogenin and actin suggesting a possibility that both angiogenin and plasmin may bind to a similar site on actin. Here we report that chANG, an antiangiogenin peptide that binds to the actin-binding site of angiogenin, inhibits the proteolytic activity of plasmin without any apparent effect on the activities of plasminogen activators and matrix metalloproteases. Its antiplasmin activity is comparable with that of actin. chANG inhibits plasmin activity via its binding to plasmin kringle domains while scrambled chANG does not bind to plasmin. chANG also inhibits the invasion of angiogenin-secreting human fibrosarcoma and colorectal carcinoma cells without affecting migration. Furthermore, chANG blocks angiogenesis induced by fibrosarcoma cells and metastasis of colorectal carcinoma cells to the liver. Therefore, the 11-amino acid peptide chANG has both antiangiogenin and antiplasmin activity, and could be useful in the development of anticancer agents.

Angiogenin is one of the most potent tumor-derived angiogenic factors (1). Both the unique ribonucleolytic activity and the cell surface receptor-binding site of angiogenin are essential for its angiogenic activity (2–4). Angiogenin has a cell surface receptor-binding site that is distinct from the catalytic center (5–14). The fact that angiogenin activates phospholipases (7–9) and undergoes nuclear translocation (10) suggests that the biological action of angiogenin is mediated through a cell surface receptor. Human angiogenin can specifically bind to endothelial cells with high affinity (11), and a cell surface actin-like protein was identified as an angiogenin-binding protein (12–14). Several lines of evidence suggest that the interaction of angiogenin with an angiogenin-binding protein or actin occurs through the receptor-binding site of angiogenin. Actin and anti-actin antibody inhibit the internalization of angiogenin into endothelial cells, and block the biological activity of angiogenin in the chick chorioallantoic membrane (CAM) assay (12, 13). Therefore, the interaction of angiogenin with endothelial cell surface actin is an essential step in angiogenin-induced neovascularization.

Although the exact mechanism for angiogenin-induced angiogenesis is still not clear, angiogenin induces migration, invasion, and tubular morphogenesis of endothelial cells via activation of plasminogen activator and plasmin systems (15, 16). The activities of plasminogen activator and plasmin are involved in cell invasion via either facilitating the degradation of extracellular matrix and basement membrane (17) or via activating matrix metalloproteases (MMPs) (18, 19). The invasion of endothelial and tumor cells through extracellular matrix is an essential step for neovascularization and tumor metastasis. Angiogenin stimulates invasion of endothelial cells via activation of cell-associated tissue-type plasminogen activator (tPA) activity (15). An anti-angiogenin antibody and α2-antiplasmin block angiogenin-induced endothelial cell invasion. These results indicate that regulation of plasmin activity is essential for angiogenin-induced angiogenesis. However, the presence of both angiogenin and actin induces increased invasion of endothelial cells, and this observation is consistent with the results from in vitro experiments (20–22). Actin activates tPA activity (20) while it inhibits plasmin activity (21). The overall plasmin activity produced from a mixture of plasminogen and tPA is increased 2-fold by the presence of actin. The overall plasmin activity increases 11-fold in the presence of the angiogenin-actin complex because this complex does not inhibit plasmin activity, but still activates tPA activity (22). These results imply that the plasmin- and the angiogenin-binding sites in actin are similar or even identical.

We previously reported that peptide antagonists of human angiogenin, designated as chANG and chGNA, specifically bind to angiogenin without any visible effect on the ribonucleolytic activity but inhibit the interaction between angiogenin and actin (23). Both chANG and chGNA also inhibit neovascularization induced by angiogenin and by angiogenin-secreting PC3 tumor cells in the chick CAM assay. Although there is no formal evidence that plasmin and angiogenin bind to the same or similar sites in actin, there is a possibility that the peptides we developed might also inhibit plasmin activity. Here we report that chANG inhibits plasmin activity and binds to a region of plasmin that is not related to the active center. chANG inhibits the invasion of tumor cells but does not inhibit...
experiments were carried out in the presence of an appropriate amount of 0.5 mM Chromo PL (Roche Molecular Biochemicals) in the absence or presence of the interaction of chANG with plasmin, 0.4 mM plasminogen (R & D Systems) and incubated for 1 hour at room temperature. The bound biotinylated actin was detected by incubation with streptavidin-horseradish peroxidase followed by a chemiluminescent substrate (Amersham Biosciences Inc.). All assays were carried out in triplicate and repeated at least twice. 

Binding of Biotinylated Actin to Angiogenin—Binding assay was carried out as described previously (23). Briefly, biotinylated actin (8.0 nM) in 0.5% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) was preincubated with varying concentrations of human serum plasmin (Roche Molecular Biochemicals) and plasminogen (Calbiochem) in the presence of aprotinin (6.2 μg/ml, Roche Molecular Biochemicals) for 30 min. The preincubated samples were added to each well that was coated with 100 ng of human angiogenin (R & D Systems) and incubated for 1 hour at room temperature. The bound biotinylated actin was detected by incubation with streptavidin-horseradish peroxidase followed by a chemiluminescent substrate (Amersham Biosciences Inc.). All assays were carried out in triplicate and repeated at least twice.

Plasmin Activity Assay—Plasmin (59 nM) was added to a reaction mixture containing buffer A (20 mM Tris-HCl, pH 7.5, 130 mM NaCl) and 0.5 mM Chromo PL (Roche Molecular Biochemicals) in the absence or presence of either peptides or bovine muscle actin (Sigma) in a total volume of 0.2 ml. The resulting mixture was incubated at room temperature. The plasmin activity, i.e., initial rate of hydrolysis of Chromo PL (change of absorbance units/min), was determined by monitoring the change in absorbance of the solution at 405 nm in an automated ELISA reader. To investigate the effect of chANG on plasmin activity in a mixture of tPA and plasminogen, human serum plasminogen (54 nM, Roche Molecular Biochemicals) was added to a reaction mixture containing buffer A and 0.5 mM Chromo PL in a total volume of 0.2 ml. The reaction was initiated by addition of 7.7 nM tPA (Calbiochem). Plasmin activity was determined as the initial rate of hydrolysis of Chromo PL. Experiments were performed in duplicate.

Binding Assay—chANG, scrambled chANG, or chGNA (0.5 μg) in 50 μl of 20% acetic acid was added to each well of an ELISA plate and air dried overnight. The peptide-coated wells were then blocked with 3% BSA/PBS. Plasmin (20 μg/ml) in 0.5% BSA/PBS was added to each well and incubated for 1 hour at room temperature. The bound plasmin was detected by incubation with monoclonal anti-plasminogen antibody (Calbiochem) and goat anti-mouse IgG-horseradish peroxidase (Kirekgaard & Perry Laboratories) followed by a chemiluminescent substrate. All assays were carried out in triplicate and repeated at least twice.

Surface Plasmon Resonance Analysis—Surface plasmon resonance measurements (24, 25) were carried out to monitor real-time binding of plasmin to chANG in a BIAcore 2000 (Amersham Biosciences, Inc.). chANG was immobilized onto a CMS biosensor chip surface by standard amine coupling method (26). It was immobilized via the N-terminal primary amine due to the absence of a lysine in chANG. Several concentrations of plasmin in a running buffer (20 mM HEPES, pH 7.4, containing 150 mM NaCl, 5 mM EDTA, and 0.05% surfactant P20) were injected at 10 μl/min. To regenerate the sensor chip, buffer ligands were removed by 4 M urea for 3 min. Dissociation (k_d) and association (k_a) rate constants were estimated using the BIA evaluation program. The dissociation constant (K_d) was calculated from the equation K_d = k_d/k_a. To investigate the effect of previously known inhibitors of plasmin on the interaction of chANG with plasmin, 0.4 μg/ml plasmin in the presence of actin, α2-antiplasmin (Sigma), and aprotinin was injected at 10 μl/min.

Cell Culture—HT1080 human fibrosarcoma, HM7 human colorectal carcinoma, and NIH/3T3 mouse fibroblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine calf serum and antibiotics (amphotericin).

Tumor Cell Invasion and Migration Assay—The tumor cell invasion assay was carried out using a biocoated Matrigel invasion chamber (Becton Dickinson). Briefly, 3T3 conditioned medium (0.5 ml) prepared as described in a previous report (27) was added to the plate well as a chemotactrant. A cell suspension (0.2 ml) containing 10^6 HT1080 cells in 0.1% BSA/DMEM, in the presence of chANG was added to the chamber. After 16 h of incubation, the cells on the upper side of the filter were removed with cotton swabs and the number of invaded cells was determined by the MTT assay (28). For the migration assay, Tissue Culture Inserts (10 mm, Nunc) with a polycarbonate membrane containing 8-μm pores were coated with gelatin. Experiments were performed in triplicate.

The invasion assay was performed as described previously (27) with the HM7 cells in a 48-well microchemotaxis chamber (Neuro Probe Inc.). Briefly, polyester membranes with 8-μm pores were coated with 0.5 mg/ml Matrigel (Becton Dickinson) in double distilled water and then dried overnight. HM7 cells were harvested using Versene (Invitrogen) and resuspended in 0.1% BSA/DMEM. The bottom wells were loaded with 3T3 conditioned medium as a chemotactrant and the upper wells were then loaded with the HM7 cells (3.0 × 10^4) in 0.1% BSA/DMEM, in the presence of chANG. The chamber was then incubated at 37 °C for 4 h, and the filters were fixed and stained using Diff-Quick (Baxter Healthcare Corp.). The number of cells that invaded through the filter was counted under a microscope. Experiments were performed in triplicate.

Tumor-induced Angiogenesis—The modified CAM assay was carried out as described previously (23). Briefly, 5 μl of mixture that contained 2 × 10^6 HT1080 cells/ml in serum-free DMEM and 1.5 mg/ml rat tail type I collagen (Becton Dickinson) in the presence of test sample was loaded onto Thermanox discs (Nunc) and polymerized by warming. The discs were applied to the CAMs of 9-day-old embryos. After a 68 ± 2-h incubation, the negative or positive response was assessed as described. Assays for each test sample were carried out twice, and each experiment contained 12 to 18 eggs per data point.

Liver Metastasis—The effect of chANG on liver metastasis was determined using splenic-portal injection of tumor cells in nude mice (29). Briefly, 10^6 HM7 cells in 0.1 ml of serum-free DMEM in the absence or presence of chANG (2.5 μg) were injected to the spleen of anesthetized mice, followed by splenectomy 1 min later. Experimental mice were given 0.1 ml of PBS or chANG (2.5 μg) in PBS by daily subcutaneous injection for 21 days. After 35 days, all animals were killed and their livers were excised and weighed. The number of liver nodules was determined using a dissecting microscope.

RESULTS

Inhibition of the Interaction between Angiogenin and Actin by Plasmin—Angiogenin binds to an actin-like protein on the surface of endothelial cells (12–14), and actin inhibits plasmin activity (21). We hypothesize that plasmin and angiogenin share a similar binding site on actin. To investigate this possibility, we examined the effect of plasmin on the binding of actin to immobilized angiogenin. Plasmin inhibited binding of actin to angiogenin in a concentration-dependent manner (Fig. 1). Plasminogen also blocked the interaction between actin and angiogenin, and its inhibitory activity was similar to that of plasmin. To ensure that the proteolytic activity of plasmin did not affect the assay, the binding assay was carried out in the presence of severalfold molar excess of aprotinin, an inhibitor of plasmin (30). Aprotinin did not affect the interaction between angiogenin and actin. These results suggest that the
plasmin-binding and the angiogenin-binding sites on actin could be similar or identical.

Inhibition of Plasmin Activity by chANG—Since the antiangiogenin peptides that were targeted to the actin-binding site of angiogenin (23) may also have antiplasmin activity, we tested the antiplasmin activities of these peptides. Interestingly, chANG inhibited plasmin activity in a concentration-dependent manner (Fig. 2A) with half-maximal inhibition at $\sim 4.2 \mu M$. The antiplasmin activity of chANG is comparable with that of actin ($K_i = 2.8 \mu M$, Ref. 21). However, scrambled chANG and the other antiangiogenin peptide, chGNA, showed no significant effect on plasmin activity (data not shown). chANG did not affect the activities of other proteases, including tPA, urokinase-type plasminogen activator (uPA), MMP2, and MMP9 (data not shown). chANG also inhibited the plasmin activity generated from the mixture of tPA and plasminogen (Fig. 2B). As previously reported (20), the plasmin activity of this mixture was increased by the presence of actin. These results indicate that chANG shows specific antiplasmin activity.

Binding of Plasmin to chANG—Two methods were used to assess the direct interaction between chANG and plasmin. First, plasmin was added to the wells of an ELISA plate coated with chANG and then the bound plasmin was detected using anti-plasminogen antibody. Plasmin bound to immobilized chANG but not to either scrambled chANG or chGNA that does not have antiplasmin activity (Fig. 3A). The binding of plasmin to chANG was not blocked by either the addition of scrambled chANG or chGNA to the binding buffer (data not shown). e-Aminocaproic acid efficiently blocked the interaction of plasmin with both chANG and actin (Fig. 3B). These results suggest that the binding of plasmin to chANG and actin are mediated through the kringle domains of plasmin.

We next investigated the kinetics of plasmin binding to immobilized chANG in real-time using BIACore. Plasmin bound to immobilized chANG with a $k_d = 4.21 \times 10^9 M^{-1} s^{-1}$, $k_d = 4.96 \times 10^{-3} s^{-1}$, and $K_d = 1.40 \mu M$ (Fig. 4A). This $K_d$ value is in the same range with the IC$_{50}$ value of chANG for inhibition of plasmin activity. We also investigated the effect of a previously known inhibitor of plasmin on the interaction between chANG and plasmin (Fig. 4B). Actin and $\alpha_2$-antiplasmin, which do not bind directly to the active site of plasmin (21, 31), inhibited the binding of plasmin to chANG. However, aprotinin, which interacts with plasmin’s active site (31), did not affect the interaction between chANG and plasmin. These results suggest that chANG interacts with the region of plasmin that is not related to the active center. $\alpha_2$-Antiplasmin blocked binding of plasmin to actin but there was no direct interaction between $\alpha_2$-antiplasmin and angiogenin (data not shown).

Inhibition of Tumor Cell Invasion by chANG—The effect of chANG on the invasive activity of HT1080 and HM7 cells was investigated. chANG reduced cell invasion in a dose-dependent manner (Fig. 5). The presence of $\alpha_2$-antiplasmin also inhibited the invasion of tumor cells, indicating that plasmin is essential for HT1080 and HM7 cell invasion. $\alpha_2$-Antiplasmin, a specific inhibitor of plasmin, did not affect uPA, MMP2, or MMP9 activities (data not shown). However, chANG and $\alpha_2$-antiplasmin did not affect the migration of these tumor cells (data not shown). Therefore, chANG has anti-invasion activity and its antiplasmin activity may be responsible for the inhibition of tumor cell invasion. chANG did not show any cytotoxicity against these tumor cells (data not shown). We also found that HT1080 and HM7 cells secreted angiogenin but not plasminogen into the culture medium (data not shown).

Inhibition of the Tumor-induced Angiogenesis by chANG—When HT1080 cells were entrapped in type I collagen gels and loaded directly on the CAMs, tumor cells induced neovascularization in 66.5% of the eggs (Table 1). This value is significantly higher than the values obtained with medium alone (29.0%
positive response, \( p = 0.004 \)). The tumor-induced angiogenesis was inhibited by the presence of either chANG (35.5% positive response, \( p = 0.015 \)) or \( \alpha_2 \)-antiplasmin (40.5% positive response, \( p = 0.021 \)).

**Inhibition of Hepatic Metastasis by chANG**—To investigate the effect of chANG on tumor metastasis, we used a previously established model of experimental liver metastasis: splenic-portal injection of HM7 human colorectal carcinoma into nude mice (29). After tumor inoculation, animals were treated daily with subcutaneous injections of chANG until day 21 and were sacrificed on day 35. Without chANG treatment, all mice showed evidence of hepatic tumors and increased liver weight (Fig. 6 and Table II). However, the animals treated with chANG showed a dramatic reduction in the number of liver metastasis (\( p = 0.02 \)) and in the weight of the liver (\( p = 0.05 \)).

**DISCUSSION**

Invasion of endothelial and tumor cells through extracellular matrix is an essential step for neovascularization and tumor metastasis. Endothelial and tumor cells secrete MMPs, tPA, and uPA (32, 33). Plasmin generated from plasminogen by the action of tPA and uPA can digest the extracellular matrix directly or via activation of MMP2 and MMP9 (18, 19). These degradative enzymes facilitate the invasion of endothelial and tumor cells through the extracellular matrix. Therefore, inhibitors of plasmin may be useful as antiangiogenic and antimetastatic agents. Angiogenin promotes the invasiveness of endothelial cells *in vitro* by the indirect activation of tPA and plasmin activity through interaction with cell surface actin (15, 22). Also, \( \alpha_2 \)-antiplasmin, a specific inhibitor of plasmin, inhibits angiogenin-induced endothelial cell invasion (15). These results indicate that plasmin is essential for angiogenin-induced endothelial cell invasion and neovascularization.

The peptides that bind to the receptor-binding site of angiogenin were investigated for possible inhibition of plasmin activity. This was based on the following rationale. Actin binds to the receptor-binding site of angiogenin (13) and plasmin inhibits the interaction of actin and angiogenin (Fig. 1). Therefore, there is a possibility that both angiogenin and plasmin bind to the same region of actin and may have a similar actin-binding pocket. Thus, peptides targeted to the actin-binding site of angiogenin might also bind to plasmin and inhibit plasmin.
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activity. Of the two antiangiogenin peptides (chANG and chGNA) developed in our laboratory (23), only chANG inhibited the proteolytic activity of plasmin. Similar to actin (21), chANG binds to a site of plasmin not related to the active site. There was a direct interaction between chANG and plasmin, and this interaction was blocked by actin and α-antiplasmin which do not directly bind to the active site of plasmin. Furthermore, aprotinin that interacts with the active site of plasmin, did not have a lysine residue, the recognition mechanism involved in the interaction with chANG may not be similar to that of the other plasmin-binding proteins. The antiplasmin activity of this peptide is not simply derived from the presence of plasmin’s putative substrate, the arginine at the fifth position, since preincubation of plasmin with chANG for 6 h suppressed the activity of the preincubated plasmin. Furthermore, chANG N-5, with the sequence of NH₂-Val-Ser-Ile-Leu-Val-Phe-COOH, lacks 5 amino acids, including the sole arginine from the N terminus of chANG, has stronger antiplasmin activity than chANG. The antiplasmin activity of chANG is specific since it did not affect the activity of other proteases including tPA, uPA, MMP2, and MMP9. Furthermore, scrambled chANG and chGNA showed little antiplasmin activity and did not bind to plasmin. It is possible that the structure of the actin-binding pockets of angiogenin and plasmin may not be identical. This may explain why α-antiplasmin does not bind to angiogenin although it blocks binding of plasmin to chANG and actin. It may also explain why chGNA, the short peptide that binds to the actin-binding pocket of angiogenin, does not bind to plasmin or interfere with plasmin activity.

Previously (23) we showed that chANG binds to angiogenin, inhibits the interaction of angiogenin and actin, and blocks the neovascularization induced by angiogenin and angiogenin-secreting human prostate adenocarcinoma cells in the chick CAM assay. Here we find that chANG also inhibits the invasion of angiogenin-secreting human fibrosarcoma and colorectal carcinoma cells through the Matrigel matrix without affecting migration. Furthermore, chANG blocks tumour-induced angiogenesis in vivo and metastasis of colorectal carcinoma cells into the liver. Angiogenin induces endothelial cell invasion and neovascularization via plasmin activation (15, 16), and plasmin activity is essential for tumor invasion and tumor-induced angiogenesis (17–19). Therefore, the decrease of tumor invasion, tumor-induced angiogenesis, and liver metastasis by chANG may result from inhibition of both angiogenin and plasmin generated by tumor cells. It is important to note that HT1080 and HM7 themselves do not produce plasminogen, but endothelial and tumor cells produce plasminogen activating proclase, and plasminogen is present in Matrigel and blood (34). Taken together, chANG has both antiangiogenic and antimetastatic activities. These dual inhibitory activities should be advantageous for development of chANG as an effective anticancer agent.

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