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Direct binding of recombinant plasminogen kringle 1–3 to angiogenin inhibits angiogenin-induced angiogenesis in the chick embryo CAM ☆

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Abstract

Angiogenin is one of the most potent angiogenesis-inducing proteins. Angiostatin is one of the most potent angiogenesis inhibitors, and it contains the first four kringle domains of plasminogen (K1–4). Recombinant human plasminogen kringle 1–3 (rK1–3) was expressed in *Escherichia coli* and purified to homogeneity. The binding of *t*-4-aminomethylcyclohexanecarboxylic acid with the purified kringle 1–3 was determined by changes in intrinsic fluorescence. rK1–3 exhibits comparable ligand-binding properties as native human plasminogen kringle 1–3. The purified rK1–3 inhibits neovascularization in the chick embryo chorioallantoic membrane (CAM) assay. Interaction of angiogenin with rK1–3 was examined by immunological binding assay and surface plasmon resonance kinetic analysis, and the equilibrium dissociation constants for the complex, K_d, are 0.89 and 0.18 μM, respectively. rK1–3 inhibits angiogenin-induced angiogenesis in the chick embryo CAM in a concentration-dependent manner. These results indicate that rK1–3 directly binds to angiogenin and thus rK1–3 inhibits the angiogenic activity of angiogenin.

Keywords: Angiogenin; Angiostatin; Recombinant plasminogen kringle 1–3; Interaction

Angiogenin is a potent blood vessel-inducing protein originally purified from the conditioned media of cultured colon adenocarcinoma (HT-29) cells [1]. It induces in vivo angiogenesis in the chorioallantoic membrane of the chick embryo [1] and in the meniscus of the knee and cornea of the rabbit [2]. It also induces in vitro angiogenesis by stimulating the proliferation of human endothelial cells in sparse cultures, and a 170-kDa putative angiogenin receptor has been detected on the endothelial cells [3]. Angiogenin inhibits proliferation of aortic smooth muscle cells, and it has been suggested that cultured aortic smooth muscle cells express specific receptors for angiogenin [4]. It binds to human placental ribonuclease inhibitor (PRI) [5] and to a smooth muscle type of α-actin [6–8]. Chimeric anti-angiogenin antibody inhibits the formation of human breast xenografts in athymic mice [9]. Epithelial cells and secretory cells are major sites of angiogenin synthesis, and angiogenin is involved in morphological changes and angiogenesis in the ovary [10,11]. Angiogenin was also purified

☆ *Abbreviations:* nK1–3, native human plasminogen kringle 1–3; rK1–3, recombinant human plasminogen kringle 1–3; *t*-AMCHA, *t*-4-aminomethylcyclohexanecarboxylic acid; CAM, chorioallantoic membrane; Ang, angiogenin.

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from bovine serum [12,13] and milk [8,14,15]. Some enzymatic and biological activities of bovine angiogenin are identical with those of human angiogenin [12], and the crystal structure of bovine angiogenin is closely similar to that of human angiogenin [16]. Extensive reviews of angiogenin on its enzymatic and biological activities, interaction with cells, and structure have been described [17].

Angiostatin is a 38-kDa plasminogen fragment which inhibits the neovascularization and growth of metastases [18]. It was originally purified from serum and urine of tumor-bearing mice. It has also been reported that systemic administration of human angiostatin potently inhibits the growth of human and murine primary carcinomas in mice, and was demonstrated that angiostatin increases tumor cell apoptosis and sustains dormancy of human carcinoma [19].

In this study, the interaction between angiogenin and recombinant human plasminogen kringle (rK1–3) was examined. We demonstrated direct binding of rK1–3 to angiogenin by use of two different assay systems, immunological binding assay and surface plasmon resonance kinetic analysis. In addition, we showed that angiogenin-induced angiogenesis in chick embryo chorioallantoic membrane (CAM) was inhibited by rK1–3.

Materials and methods

Materials

Human plasminogen kringle 1–3 (nK1–3) was produced from human plasminogen by a limited proteolytic digest as described elsewhere [20], and purified by affinity chromatography on a Lysine-Sepharose column. *t*-4-Aminomethylcyclohexanecarboxylic acid (*t*-AMCHA) was from Sigma. All other chemicals used were of analytical grade.

Purification of angiogenin

Angiogenin was isolated from bovine milk by a three-step procedure, essentially similar to the procedure described for its purification from bovine plasma: SP-Sepharose ion-exchange chromatography, Mono S fast-flow liquid chromatography (FPLC), and C18 high-performance liquid chromatography (HPLC) [8,12]. Amino acid and automated sequence analysis of the final purification was carried out at the Korea Basic Science Institute (Taejon, Korea), confirming the identity as bovine angiogenin. The concentration of bovine angiogenin was determined spectrophotometrically using a molar absorptivity of 8575 M^{-1} cm^{-1} at 280 nm [8].

Construction, expression, and purification of recombinant kringle 1–3 of human plasminogen

Recombinant plasminogen kringle 1–3 (rK1–3), amino acid residues from S82 to S335 of human plasminogen, was amplified by polymerase chain reaction (PCR) using the following synthetic oligonucleotide primers 1 and 2 on a template consisting of the cDNA of human plasminogen:

1: 5′-CGGGATCCCATATGTCAGAGTGCAAGACTGGGA-3′
2: 3′-CATTCTATGGCAGGACACTGAGGATCATTCCTAGGG C-5′.

A *Nde*I site was included in the 5′-PCR primer 1 while a *Bam*HI site and stop codon (ATC, ATT) were incorporated into the 3′-PCR primer 2. The cDNA encoding rK1–3 was ligated into the *Nde*I/*Bam*HI site of the *Escherichia coli* (*E. coli*) expression vector, pET 11a, resulting in expression plasmid pMETK1. The *E. coli* BL21 clone containing the highest number of pMETK1 plasmids was selected and rK1–3 expression was induced by 1 mM isopropyl thio-β-D-galactopyranoside. The insoluble body (IB) was collected by centrifugation and washed with lipophilic buffers until 95% pure. The IB was then solubilized in 6 M guanidine–HCl containing 50 mM β-mercaptoethanol and refolding was accomplished in Tris buffer, pH 8.6, containing basic amino acids and reducing agents. The refolded rK1–3 was purified to homogeneity utilizing SP-Sepharose and Lysine-Sepharose columns (Pharmacia, NJ).

Characterization of purified recombinant plasminogen kringle 1–3

Ligand binding properties. Small aliquots of a stock *t*-4-aminomethylcyclohexanecarboxylic acid (*t*-AMCHA) solution were added to 1 μM rK1–3 in 50 mM sodium phosphate, pH 7.4, and 50 mM NaCl at 25 °C. Concentrations of rK1–3 were determined spectrophotometrically using the molar extinction coefficient of $A^{1\%} = 18.5$ at 280 nm. Fluorescence measurements were performed using the Bio-Logic MOS-200 with the standard SCI cuvette holder and stirrer. Excitation was at 285 nm, and emission was monitored at 340 nm with a 320-nm cut-off filter.

Anti-angiogenic activities. Anti-angiogenic activity of rK1–3 was assessed by the chick embryo chorioallantoic membrane (CAM) assay. Briefly, fertilized chick embryos were received from Pullmuwon, Inc. (Emsung, Chungbuk), placed in a humidified 37 °C incubator, and designated as day 0 eggs. Albumin was aspirated from the embryos on day 2, and 3-cm "windows" were cut through the shell on day 3. Aqueous, salt-free samples containing rK1–3 (0.5–50 μg) were applied to sterile Thermonox 13-mm disks (Nunc Inc., Naperville, IL) and allowed to dry under sterile conditions. The loaded disks were inverted and applied to the CAM surface of 4.5-day-old embryos through the windows. The CAM was visualized after injection of 10% fat emulsion. Negative and positive responses were microscopically assessed at 48 h after implantation and recorded as the number of positive anti-angiogenic responses per number of eggs surviving per sample dilution.

Immunological binding assays

Angiogenin was adsorbed to the wells of an ELISA plate by incubation at 37 °C for 3 h, and the remaining binding capacity of the wells was eliminated by overnight incubation with 3% bovine serum albumin in phosphate-buffered saline (PBS). Biotin labeled-K1–3 was then applied to the plate and incubated at 37 °C for 1 h. Significant amplification and detection of the interaction was achieved with the subsequent addition of three layers of enzyme complex amplification (ECA) solutions [30]. This was followed by the addition of substrate solution (2 mg/mL *o*-phenylenediamine in 0.05 M sodium citrate, 0.1 M NaH_2PO_4, pH 5, containing 0.03% (w/v) H_2O_2) in order to detect plate-bound horseradish peroxidase (HRP). After allowing 2–5 min for color development, the reaction was stopped with 2 M H_2SO_4 and the absorbance was measured at 490 nm using a Thermomak P/N 0200-0601 Microplate Reader (Molecular Devices Corp.). Color development is directly proportional to the concentration of bound HRP which in turn is directly proportional to the concentration of biotin labeled-K1–3 bound to the angiogenin.

Surface plasmon resonance (SPR) kinetic analysis

A binding assay of angiogenin and rK1–3 was performed by real-time interaction analysis using a BIACORE 2000™ apparatus (Biacore AB, Uppsala, Sweden). All experiments were performed at 25 °C using HBS-EP buffer. Angiogenin (10 μg/ml in 10 mM sodium acetate buffer, pH 5.0) was immobilized on a CM-5 type sensor chip using the reagents and procedures supplied with the Amine Coupling Kit (Biacore AB). The samples of rK1–3 were diluted in the running buffer before injection. The kinetics of binding was measured at a buffer flow of 30 μl/min, including an injection phase of 3 min followed by dissociation for 5 min. Sensor chips were regenerated at the end of each run by the injection of 10 nM Glycine, pH 3.0, 0.1 M NaCl. The BIAevaluation™ software was used for