Kringle 5 of Plasminogen is a Novel Inhibitor of Endothelial Cell Growth*

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Angiostatin is a potent angiogenesis inhibitor which has been identified as an internal fragment of plasminogen that includes its first four kringle modules. We have recently demonstrated that the anti-endothelial cell proliferative activity of angiostatin is also displayed by the first three kringle structures of plasminogen and marginally so by kringle 4 (Cao, Y., Ji, R.-W., Davidson, D., Schaller, J., Marti, D., Sohndel, S., McCance, S. G., O'Reilly, M. S., Llinás, M., and Folkman, J. (1996) J. Biol. Chem. 271, 29461–29467). We now report that the kringle 5 fragment of human plasminogen is a specific inhibitor for endothelial cell proliferation. Kringle 5 obtained as a proteolytic fragment of human plasminogen displays potent inhibitory effect on bovine capillary endothelial cells with a half-maximal concentration (ED50) of approximately 50 nm. Thus, kringle 5 would appear to be more potent than angiostatin on inhibition of basic fibroblast growth factor-stimulated capillary endothelial cell proliferation. Appropriately folded recombinant mouse kringle 5 protein, expressed in Escherichia coli, exhibits a comparable inhibitory effect as the proteolytic kringle 5 fragment. Thus, kringle 5 domain of human plasminogen is a novel endothelial inhibitor that is sufficiently potent to block the growth factor-stimulated endothelial cell growth.

Angiogenesis is the process of formation of capillaries that sprout from existing blood vessels (1). Blood capillaries are primarily composed of endothelial cells, which are usually quiescent in the adult mammal under physiological conditions (2). In response to appropriate stimuli, quiescent endothelial cells can degrade the surrounding basement membrane, change their morphology, proliferate, migrate, form new lumen, and sprout microtubes (3). This complex process implies the presence of multiple controls, which can be turned on and off within a short period. Outgrowth of new blood vessels under pathological conditions can lead to development and progression of diseases such as tumor growth, diabetic retinopathy, tissue and organ malformation, and cardiovascular disorders (4).

The switch of angiogenesis phenotype depends upon the net balance between angiogenic stimulators and inhibitors, i.e. up-regulation of angiogenic factors and down-regulation of angiogenic suppressors (4, 5). For example, fibroblast growth factors (FGFs) and vascular endothelial growth factor/vascular permeability factor are most commonly overexpressed angiogenic stimulators found in tumors (6–11). Tumors may express one or more of these angiogenic peptides that can synergistically stimulate tumor angiogenesis and promote tumor growth (12). Concomitantly, an angiogenesis inhibitor of thrombospordin-1 has been found to be down-regulated in many tumors with the mutated p53 tumor suppressor gene (13).

Within the family of angiogenesis inhibitors, angiostatin, an endothelial cell-specific inhibitor, has been demonstrated to be the most potent (14–16). Angiostatin contains the first four triple disulfide-linked loops of plasminogen (Pgn) known as kringle domains. Angiostatin isolated from serum and urine of tumor-bearing mice, or the corresponding fragment of human Pgn compatible to murine angiostatin, impairs both primary and metastatic tumor growth without toxicity (14, 15). In vitro, it specifically inhibits endothelial cell growth, but not proliferation of other cell types, including tumor cells (14, 16). In vivo, angiostatin suppresses neovascularization in the chick chorioallantoic membrane assay and in the mouse corneal assay (14, 15).

Smaller fragments of angiostatin have been demonstrated to display differential effects on suppression of endothelial cell growth (16). For example, the kringle 1 (K1) structure of angiostatin is a potent endothelial inhibitor, whereas kringle 4 (K4) exhibits markedly low inhibitory activity (16). In this paper, we report on the anti-endothelial activity of kringle 5 (K5), which, while structurally related to the other four kringles in Pgn, is not present in angiostatin.

**EXPERIMENTAL PROCEDURES**

Preparation of Proteolytic Fragment of Human Kringle 5 and Angiostatin—Intact, NH2-terminal [Glu1]Pgn was purified from outdated and citrated human blood plasma (Central Blood Bank of Pittsburgh, Pittsburgh, PA) by affinity chromatography on L-Lys-Sepharose (17), and further purified into two isoforms by L-Lys-Sepharose affinity chromatography with a gradient of the ligand, 6-aminohexanoic acid (18). Pgn was subjected to limited digestion with porcine pancreatic elastase to produce Pgn compatible to murine angiostatin, impairs both primary and metastatic tumor growth without toxicity (14, 15). In vitro, it specifically inhibits endothelial cell growth, but not proliferation of other cell types, including tumor cells (14, 16). In vivo, angiostatin suppresses neovascularization in the chick chorioallantoic membrane assay and in the mouse corneal assay (14, 15).

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The abbreviations used are: FGF, fibroblast growth factor; Pgn, plasminogen; K, kringle; AMCHA, trans-aminomethylcyclhexanecarboxylic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; bFGF, basic FGF; BCE cells, bovine capillary endothelial cells.

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elastase (Nutritional Biochemical Corp.). Further chromatography through L-Lys-Seqharose permitted us to obtain miniPgn (K5 + proteinase, Ser41–Asn52) as the flow-through fraction, resolved from the K1–K3 (Try4–Val384) and K4 (Val385–Val558) fragments (19). The flow-through fraction was lyophilized, dialyzed against doubly distilled water, and again lyophilized. MiniPgn was digested with porcine stomach mucous pepsin (Sigma) to obtain the K5 fragment (Val449–Pro542–Phe545), as reported (20) except that the K5-specific ligand trans-aminomethylcyclohexanecarboxylic acid (AMCHA; Sigma) was added to the incubation mixture in 5-fold excess. The reaction mixture was incubated for 75 min at 25 °C with shaking. After dissolving K5 the mixture in 0.1 M ammonium bicarbonate, pH 8.0, K5 was isolated by column chromatography on Sephadex G-75 SF. The intact K5 complexed with AMCHA comprises the largest fraction. K5 was further purified by ion-exchange chromatography on DEAE-Seqharose and was desalted by gel filtration through a G-50 Sephadex column. To remove AMCHA, K5 was dialyzed against doubly distilled water, pH 3.5, for 48 h and lyophilized. The final K5 preparation was stored as a dry powder. Pepsin digestion in the presence of AMCHA raised the yield from 10% (20) to greater than 50%. K5 was identified via 1H NMR spectroscopy at 500 MHz using a Bruker WM-500 instrument, as reported previously (20). NH2-terminal and COOH-terminal sequence analyses indicated that the obtained K5 contains a 40:60% Asp/Asn heterogeneity at site 452 of the [Glu1]Pgn sequence.

Alternatively, human K5 was prepared from the digestion of [Lys78]Pgn (Abbott Laboratories) with porcine elastase (Sigma). Briefly, 1.5 mg of elastase was incubated with 200 mg of [Lys78]Pgn in 5–50 ml of 50 mM Tris-HCl, pH 8.0, and incubated overnight at room temperature. The mixture was dialyzed for 6 h against 4 liters of 50 mM Tris, pH 8.0, followed by changing fresh buffer overnight and lyophilized. The cleaved Pgn was applied a 1.0 cm × 6.5-cm Bio-Rad Mono-S column equilibrated with buffer A (20 mM NaOAc, pH 5.0). The kringle portions, K4, K5, K1–K3, and K1–K4, were eluted at 1.0 ml/min with step gradients of 0–20%, 20–50%, 50–70%, and 70–100% of 20 mM NaOAc, 1 mM KCl, pH 5.0, with 5 min of flow at constant concentration at the end of each step. K5 was eluted within 5 min when the following buffer had reached to 50%. The peak of K5 was dialyzed overnight against 20 mM Tris, pH 8.0. The purity of K5 was analyzed by SDS-PAGE. Purified K5 was confirmed by NEI-terminus-terminal sequencing. Human angiotensin was prepared by digestion of human Pgn with porcine elastase and purified by affinity chromatography on lysine-Seqharose 4B as described previously (16).

cDNA Construction, Expression, and Purification of Recombinant Kringle 5—The cDNA coding for K5 of mouse Pgn was amplified by the polymerase chain reaction (PCR) using the mouse plasminogen cDNA as template and oligonucleotides primers (5’–end primer of aagcggagcgactctgagacag and 3’-end primer of aagcggtccgagcgactctgagacag), corresponding to amino acid residues 476–481 and 558–563 of mouse Pgn, was expressed in E. coli. rK5 was obtained as a soluble protein from the periplasmic space, pH 8.0, K5 was isolated by column chromatography on Sephadex G-75 SF. The intact K5 complexed with AMCHA comprises the largest fraction. K5 was further purified by ion-exchange chromatography on DEAE-Seqharose and was desalted by gel filtration through a G-50 Sephadex column. To remove AMCHA, K5 was dialyzed against doubly distilled water, pH 3.5, for 48 h and lyophilized. The final K5 preparation was stored as a dry powder. Pepsin digestion in the presence of AMCHA raised the yield from 10% (20) to greater than 50%. K5 was identified via 1H NMR spectroscopy at 500 MHz using a Bruker WM-500 instrument, as reported previously (20). NH2-terminal and COOH-terminal sequence analyses indicated that the obtained K5 contains a 40:60% Asp/Asn heterogeneity at site 452 of the [Glu1]Pgn sequence.

Fig. 1. Amino acid sequence alignment of kringle regions of human plasminogen. Plasminogen contains five structurally related kringle domains; each carries six conserved cysteine residues (asterisks). The sequences (19) for the five kringle were aligned according to their conserved cysteine residues. Conserved amino acids are shaded. Kringle 5 contains 80 amino acids. In kringle 4, the positively charged lysine pairs adjacent to Cys27 and Cys80 are boxed.

Generation and Expression of Kringle 5—A K5-containing fragment was obtained via porcine pepsin digestion of human miniPgn, as described under “Experimental Procedures” (Fig. 2A). Alternatively, K5 was also generated by overdigestion of Pgn with elastase (Fig. 2A) (see “Experimental Procedures”). The proteolytic fragments containing K5 were purified by gel filtration through a Sephadex G-75 column, followed by ion-exchange chromatography (20). Amino-terminal sequence analysis of the purified protein revealed two elastolytic cleavage sites at the NH2-terminus of K5. The first cleavage site was located to the position between Val448 and Val449 (Fig. 2A) of [Glu1]Pgn. The second site was located three amino acids downstream of the first cleavage position, i.e. between Leu451 and Pro452 (Fig. 2A). Carboxy-terminal sequence analysis showed the elastase cleavage site (between Ala544 and Val545) (Fig. 2A) of [Glu1]Pgn. The second site was located three amino acids downstream of the pepsin cleavage site (between Phe547 and Asp548) (Fig. 2A). SDS-gel electrophoresis (Fig. 3A) verifies purity of the K5 fragment isolated via pepsin digestion of miniPgn. In agreement with amino acid sequencing data, two species of protein migrated with molecular masses of 14.9 and 15.7 kDa, representing [Pro449]- and [Val448]K5, respectively (Fig. 3A, lane 1). Purity of the sample was verified by 1H-NMR spectroscopy, as published (20). Similarly, K5 released by elastolytic cleavage of Pgn was also purified to homogeneity (data not shown).

Recombinant murine K5 (rmK5), fragment Cys77–Ala563 of mouse Pgn, was expressed in E. coli as a soluble periplasmic form (Fig. 2B) and purified to homogeneity (Fig. 3B). Under reducing conditions, rmK5 migrated with the expected molecular mass of 13 kDa (Fig. 3B). Apparently, the molecular...
weights of proteolytic human K5 (Fig. 3A, 14.9 or 15.7 kDa) are higher than that of recombinant mouse plasminogen (Fig. 3B) due to the presence of additional amino acid sequences in both NH₂ and COOH termini of human K5 (Fig. 2A).

Inhibition of Endothelial Cell Proliferation by K5—K5 obtained from pepsin digestion of human miniPgn was assayed for its inhibitory activity on BCE cell proliferation stimulated by bFGF (1 ng/ml). The inhibitory effect was determined using ligand-free K5 as well as K5 complexed with AMCHA, a zwiterionic ligand analogue of lysine of comparatively high affinity for K5 (association constant $K_a = 44 \text{ M}^{-1}$) (39). Both ligand-free and ligand-bound K5 inhibited BCE cell proliferation in a dose-dependent manner (Fig. 4A), suggesting that the lysine-binding site of K5 is not involved in its inhibitory activity. Likewise, the proteolytic K5 obtained via elastase digestion of Pgn yielded a similar inhibitory effect (not shown). In the presence of K5, the morphology of BCE cells appeared similar to that of control cells. In addition, cell proliferation can be rescued with bFGF stimulation after removal of K5. These results indicate that K5 is not cytotoxic to capillary endothelial cells. Furthermore, the inhibitory activity of K5 would appear to be specific for endothelial cells, as assays on other cell types, such as 3T3 fibroblasts, bovine smooth muscle cells, and murine T241 fibrosarcoma cells failed to show inhibition (data not shown).

As is the case for proteolytic human K5, the inhibitory effect of rmK5 on BCE cells occurred in a dose-dependent fashion (Fig. 4B). The inhibitory effect on BCE cells was reversible after removal of rmK5 from the culture medium. In addition, the morphological appearance of cells treated with rmK5 was indistinguishable from that of non-treated cells. To study whether the appropriate folding of rmK5 is required for the inhibitory activity, folded rmK5 was reduced with dithiothreitol, alkylated with iodoacetamide, and assayed on BCE cells. The anti-endothelial proliferation activity was markedly abolished (greater than 80%) after reduction/alkylation (data not shown), suggesting that the formation of appropriate disulfide bridges in rmK5 is essential to maintain its anti-endothelial activity.

Comparison of Inhibitory Efficacy of Human Proteolytic K5, rmK5 with Angiostatin—To directly compare the inhibitory efficacy, human K5, rmK5, and human angiostatin (K1–K4) were assayed on suppression of bFGF-stimulated endothelial cell proliferation (Fig. 5). The concentration of half-maximal inhibition $(ED_{50})$ for human K5 is about 50 nM, i.e. lower than that for angiostatin $(ED_{50} = 140 \text{ nM})$. The concentration of murine rK5 required to elicit 50% inhibition $(ED_{50} < 20 \text{ nM})$ turned out to be comparable to that of proteolytic human Pgn K5 (Fig. 5). The inhibitory effects of human K5 and rmK5 are also more potent than those of human kringles 1, 2, and 4 (data not shown).

**DISCUSSION**

These data demonstrate that the Pgn K5 module is a specific inhibitor of endothelial cell proliferation. K5 obtained as a proteolytic fragment of human Pgn suppresses the bFGF-stimulated endothelial cell growth with a half-maximal concentration between 50 and 60 nM, which is lower than that of proteolytic human angiostatin (about 140 nM). Similarly, recombinant mouse Pgn K5 elicits a potent inhibitory effect that is comparable to the human proteolytic fragment.

Angiostatin contains the first four kringles structures of plasminogen (14). K1 has been characterized as the most potent inhibitory segment of angiostatin on endothelial cell proliferation, whereas fragments containing K4 solely are comparatively inefficient in the suppression of endothelial cell growth (16). Amino acid sequence alignment of the kringles domains of
human plasminogen reveals that K5 displays the highest sequence identity with K1 (57.5%). Thus, the similarity in the primary structure of K1 and K5 may relate to the potent inhibitory activity of these two kringles on endothelial cell growth. Although K5 also shares a high degree of sequence identity with K4 (52.5%), it lacks two sets of positively charged lysine pairs (adjacent to Cys22 and Cys80, respectively) in K4, which might be connected to the latter’s low anti-endothelial cell activity (16).

As previously discussed (16), the potency of angiostatin kringles to inhibit bFGF-stimulated endothelial cell growth does not correlate with their lysine binding affinities. In the case of K5, the growth inhibition experiments in the presence of the K5-AMCHA complex, where the ligand AMCHA is highly selective for the K5 lysine-binding site, yield essentially the same results as those in the presence of ligand-free K5 (Fig. 4A). This further supports the hypothesis that the kringles’ lysine-binding site is unlikely to be responsible for the observed inhibitory effects.

Angiostatin was initially isolated from tumors of a murine Lewis lung carcinoma (14). In addition, several different types of human tumors growing in mice have been found to produce angiogenesis inhibitors (26). Whether these inhibitors are angiostatin or angiostatin-related molecules remains to be established. A recent study shows that tumors can produce an angiogenesis inhibitor different from angiostatin (27). Thus, tumors not only overexpress angiogenic factors, but also retain their abilities of production of angiogenesis inhibitors. It is not yet clear how expression of these angiogenesis inhibitors is regulated. It seems that tumor cells do not express angiostatin molecules per se.2 However, they produce protease(s) that subsequently cleaves circulating Pgn to generate angiostatin (37). It appears that protease(s) released by tumor cells is not the only source for generation of angiostatin. Metalloelastase produced by tumor-infiltrating macrophages has been found to be responsible for angiostatin production by a murine Lewis lung carcinoma (38). Thus, different types of tumors may use different strategies for generation of angiostatin.

It appears that the generation of endogenous angiogenesis inhibitors in vivo from large precursor proteins with distinct functions is a recurrent theme in the inhibition of angiogenesis. A number of angiogenesis inhibitors have been characterized as proteolytic fragments. In addition to angiostatin and K5, examples are endostatin, which is a carboxyl fragment of collagen XVIII, the 16-kDa NH2-terminal fragment of prolactin (28), an NH2-terminally truncated fragment of platelet factor 4 (29), internal fragments of thrombospondin (30, 31), fragments of laminin (32), a fragment of SPARC (33), and a fragment of fibronectin (34). The parental large molecules, namely Pgn, collagen XVIII, and prolactin, which generate angiostatin, endostatin, and 16-kDa prolactin, respectively, do not suppress angiogenesis (14, 27, 28). Thus, proteolytic processing plays critical dual roles in the control of angiogenesis. When angio-
genesis is initiated, proteolytic degradation of the basement membrane surrounding endothelial cells is a prerequisite for endothelial cell proliferation. Once new blood vessels have been formed, they may require angiogenic inhibitors, such as the proteolytic fragments mentioned above, to control the overgrowth of blood vessels. Further studies are required to elucidate the regulation of protease activity and the substrate specificity.

The molecular mechanisms of how angiostatin and its related kringles fragments specifically suppress neovascularization remain uncharacterized. It may be speculated that angiostatin could interact with a specific receptor that is exclusively expressed in the proliferating endothelial cell compartment. However, the concentrations of angiostatin to reach effective suppression of endothelial cell proliferation seem too high to interact with cell surface sensitive receptors. The endothelial specific receptor may be a cell adhesion molecule such as α5β3 and α6β4 (35, 36), thus blocking the integrin-mediated signals for endothelial cell proliferation. Alternatively, angiostatin may block the signal pathways stimulated by angiogenic factors of vascular endothelial growth factor and FGF. Whatever the underlying mechanism might be, angiostatin and its related fragments have been shown to be one of the most potent endogenous molecules for suppression of angiogenesis and tumor growth.

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