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# Antiangiogenic and Antitumor Activities of the Cryptic Fragments with Kringle Architecture

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**Abstract** – Various angiogenesis inhibitors target vascular endothelial cells and block tumor angiogenesis. Angiostatin is a specific endogenous angiogenesis inhibitor in clinical trials, which contains only the first four triple loop structures, known as kringle domains. Its generated by proteolytic cleavage of its parent molecule plasminogen, which itself does not exhibit antiangiogenic activity. Kringle domains from prothrombin, apolipoprotein, hepatocyte growth factor, urokinase and tissue-type plasminogen activator also elicit anti-angiogenic or antitumor activities in several model systems, albeit low amino acid sequence identity between angiostatin and each individual kringle. However, the differential effects of each kringle domain on endothelial cell proliferation, and migration observed in these kringle domains, suggest that the amino acid sequence of the primary structure is still important although kringle architecture is essential for anti-angiogenic activity. If it is further studied as to how amino acid sequence and kringle architecture contributes in anti-angiogenic activity, with studies on underlying mechanisms of anti-angiogenesis by kringle-based angiogenesis inhibitors, it will provide basis for the development of new potent anti-angiogenesis inhibitors and improvement of the efficacy of angiogenesis inhibitors.

**Key words** □ angiogenesis, angiostatin, kringle, inhibitor, antitumor

Angiogenesis, the formation of new blood vessel, is essential for tumor growth and metastasis. By targeting genetically stable, activated endothelial cells involved in newly formed vessel, inhibition of angiogenesis have been getting much attention for cancer researchers to overcome drug resistance, since selection of resistant cells during cycled therapy of chemotherapeutic agent targeting genetically unstable cancer cells has become serious obstacle in the treatment of cancer patients. Indeed, increasing body of evidence indicates that angiogenesis inhibitors can be used effectively for suppressing tumor growth (Bergers *et al.*, 1999; Folkman, 1995a; O'Reilly *et al.*, 1997; O'Reilly *et al.*, 1994). Recently, various angiogenesis inhibitors have been developed to target vascular endothelial cells and block tumor angiogenesis. More than 30 endogenous inhibitors have been discovered (see review, (Cao, 2001)). Among angiogenesis inhibitors, angiostatin has been found to be a potent endogenous inhibitor. It was isolated from the urine and blood of tumor-bearing mice in 1994 (O'Reilly *et al.*, 1994) and now in clinical study. Since discovery of this molecule, several other related molecules have been discovered by possessing a characteristic structure, called kringle. In this review, the structure and functional relationships of various kringle fragments are discussed in terms of regulation of angiogenesis and tumor growth.

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## Tumor angiogenesis can be a target for overcoming obstacles of drug resistance and blood brain barrier

The vasculature remains quiescent in the adult mammal, except for transient processes of neovascularization in the female reproductive system and wound repair. It is now believed that a switch of angiogenic phenotype in a tissue is dependent upon the local balance between angiogenic factors and inhibitors. Therefore, the quiescence of the vasculature either lacks angiogenic stimuli or angiogenesis is suppressed by endogenous inhibitors. In response to an angiogenic factor, endothelial cells can degrade the basement membrane locally (Fig. 1). Simultaneously, the quiescent endothelial cells change their morphology, proliferate, migrate, invade into the surrounding stroma tissue, form microtubes, sprout new capillaries, and

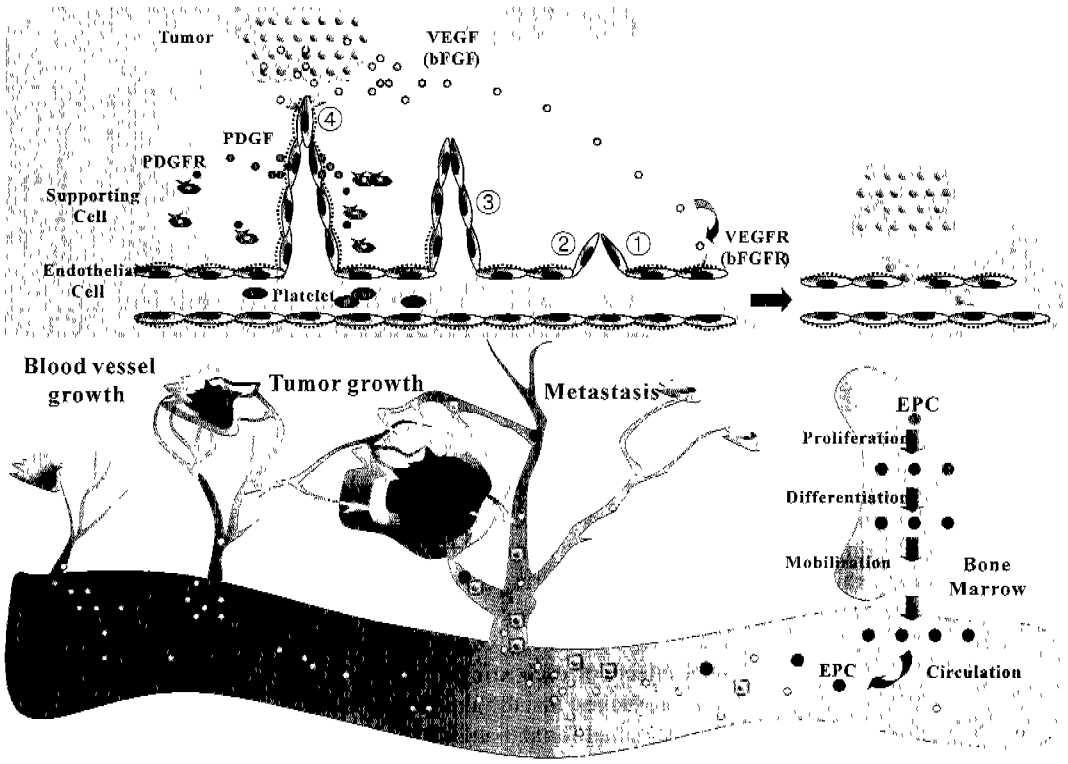


**Fig. 1.** Tumor growth depends on angiogenesis. Hypoxic avascular tumor tissue releases angiogenic factors like bFGF or VEGF and these growth factors bind to their receptors, resulting in endothelial cell activation. As a following event, angiogenesis occurs in sequential steps: 1) basement membrane disintegration opens the way for 2) endothelial cell migration. 3) Cords of cells proliferate and define a new vascular channel. 4) Cessation of cell migration and proliferation coincides with the formation a new basement membrane and vessel maturation. The endothelial cells also secret growth factors such as platelet-derived growth factor (PDGF), which attracts supporting cells to stabilize the new blood vessel. Once neovascularization occurs, tumor grows exponentially. Blood vessel supplies not only nutrients and oxygen, but also provides the route for metastasis of tumor cells. Endothelial progenitor cells (EPC) derived from bone marrow are also recruited to the angiogenic site in tumor.

reconstitute the basement membrane. This complex process implies the presence of multiple controls which can be switched on and off within in a short period.

Angiogenesis is also involved in the development and progression of pathological processes in a variety of disorders, including diabetic retinopathy, psoriasis, rheumatoid arthritis, cardiovascular diseases and cancer (Folkman, 1995a). In 1971, Dr. Judah Folkman proposed that tumor growth and metastasis are dependent on angiogenesis and the suppression of angiogenesis might be used in the treatment of cancer. Since then, a large body of work by a number of laboratories has provided both direct and indirect evidence supporting this idea. At the pre-vascular stage, a solid tumor, rarely grows larger than 2-3 $mm^3$ and may contain a few million cells. Once a tumor is vascularized, it grows exponentially and often reaches an uncontrollable volume (Fig. 1). The infiltration of new blood vessels in tumors not only supplies nutrients and oxygen but also provides the route for metastasis. In addition, endothelial cells can communicate directly with tumor cells by producing tumor growth promoting factors. The angiogenic switch is characterized by oncogene-deriven tumor expression of angiogenic proteins, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), placenta growth factor (PlGF), transforming growth factor-β (TGF-β), platelet-derived endothelial growth factor (PDGF), pleiotrophin and others (Carmeliet *et al.*, 1998; Relf *et al*, 1997). Tumor-associated hypoxic conditions also activate hypoxia-inducible factor, which promotes upregulation of several angiogenic factors (Carmeliet *et al.*, 1998). Fibroblasts in or near the tumor bed also begin to produce angiogenic factors (Fukumura *et al.*, 1998). In addition, tumors recruit endothelial progenitor cells from bone marrow to promote new vessel formation (Asahara *et al.*, 1999; Shi *et al.*, 1998) (Fig. 1). This angiogenic switch also involves down regulation of angiogenesis suppres-

sor proteins, such as thrombospondin (Dameron *et al.*, 1994).

In principle, targeting the tumor vasculature rather than tumor cells has several remarkable advantages. First, the targeted vascular endothelial cells are normal, genetically stable cells, and therefore are less likely to become drug-resistant as compared to tumor cells (Folkman, 1995b). Therefore, it is not likely to cause bone marrow suppression, gastrointestinal symptoms, or hair loss. Second, since all solid tumors (probably many leukemias (Perez-Atayde *et al.*, 1997)) are angiogenesis-dependent, this approach circumvents the need to tailor therapy to the unique genetic make up of an individual tumor. Third, one of most important features is independence of the blood-brain barrier by targeting at proliferating endothelial cells. Since malignant brain tumors, especially malignant gliomas, are among the most vascularized tumors known, angiogenesis inhibitors can be used effectively for the treatment of the brain tumor.

## Discovery of an endogenous angiogenesis inhibitor with kringle architecture, angiostatin

Inhibition of metastatic tumor growth by primary tumor mass has been observed in some clinical malignancies. The removal of certain primary tumors in patients, such as breast and colon carcinomas, can be followed by a rapid growth of distant metastases. In a similar animal metastatic model, angiostatin was isolated from both serum and urine of mice bearing a transplantable murine Lewis lung carcinoma (3LL) in syngeneic C57B16/J mice, thus providing the compelling explanation for this phenomenon (O'Reilly *et al.*, 1994). The activity of angiostatin was monitored by an *in vitro* assay of inhibition of bFGF-stimulated endothelial cell proliferation. Angiostatin is a circulating angiogenesis inhibitor produced in association with primary Lewis lung tumor growth. This 38 kD inhibitor accumulates in the circulation in the presence of a growing primary tumor and disappears from the circulation after removal of the primary tumor. Thus, resection of the primary Lewis lung carcinoma results in depletion of circulating angiostatin and promotes neovascularization and growth of lung micrometastasis.

Angiostatin is not generated by de novo biosynthetic pathway. Rather, the proteolytic cleavage of plasminogen by several proteases generated from tumor cells or macrophage is involved in angiostatin production (Fig. 2A). The mediator of angiostatin production in 3LL-Lewis lung carcinoma has been found to be a tumor-infiltrating macrophage (Dong *et al.*, 1997). Three human prostate carcinoma cell lines (PC-3, DU-145, and LN-CaP) also have been found to produce proteolytic activity that generates angiostatin from plasminogen or plasmin (Gately *et al.*, 1997).

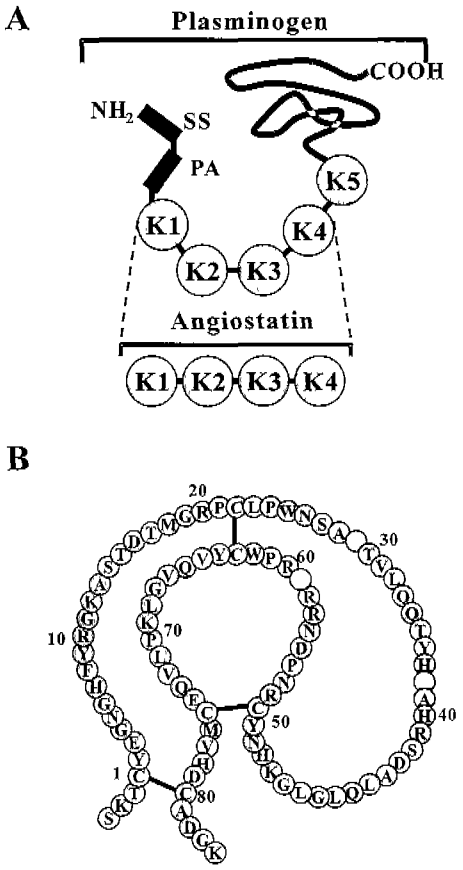


**Fig. 2.** The structures of plasminogen (A), angiostatin (A), and urokinase kringle (B). Angiostatin is generated by proteolytic cleavage of plasminogen, and consists of first four kringle domains. The structures of all the kringle moieties like urokinase kringle retain highly conserved triple disulfide bonds. Such a structure resembles a Scandinavian cookie named "ringla" in Swedish.

Purified angiostatin specifically inhibits proliferation of endothelial lineages, including bovine capillary endothelial (BAE), human umbilical vein endothelial (HUVE) and malignant hemangioendothelioma (EOMA) cells in a dose-dependent manner (O'Reilly *et al.*, 1994). In contrast, concentrations of angiostatin for maximal inhibition of endothelial cell proliferation are not inhibitory on a variety of normal and neoplastic non endothelial cell lines, including 3T3 fibroblasts, bovine arota smooth muscle cells, bovine retinal pigment epithelial cells, human fetal fibroblasts, and 3LL murine Lewis lung carcinoma cells. Thus, angiostatin specifically inhibits endothelial cell proliferation.

In the chick embryo chorioallantoic membrane (CAM), purified angiostatin induces avascular zones over a concentration range of 0.1-100 μg/embryo (O'Reilly *et al.*, 1994). The dose-dependent inhibition reaches saturation at approximately 100 mg/

embryo. Systemic administration of human angiostatin in mice implated with bFGF in corneal micropocket (80-100 ng bFGF/cornea) significantly inhibits corneal neovascularization induced by bFGF. At the concentration of 50 mg/kg/every 12 h, angiostatin inhibits new vessel growth by 85% compared to controls.

Angiostatin suppresses not only neovascularization, but also tumor growth in animals without displaying toxic effects (O'Reilly *et al.*, 1996; O'Reilly *et al.*, 1994). Systemic administration of human angiostatin potently inhibits the growth of transplanted human and murine primary tumors in mice. The growth of three aggressive primary murine tumors (Lewis lung carcinoma, T241 fibrosarcoma, and reticulumn cell sarcoma) is inhibited by an average of 84% at doses of 100 mg/kg/day. These tumors exhibit poor response to other therapies (O'Reilly *et al.*, 1996). Inhibition of primary tumor growth becomes apparent at 10 mg/kg/day and increasing doses of angiostatin correlate with increased antitumor efficacy. Systemic treatment of human tumor growing in immunodeficient mice produces an almost complete suppression of growth of human breast carcinoma (by 95%), colon carcinoma (by 97%), and prostate carcinoma (by almost 100%). In the colon and breast carcinoma bearing mice, tumors re-grow within two weeks after withdrawal of angiostatin treatment. An angiostatin derivative, PK1-3 also was found to inhibit brain tumor growth *in vivo*, supporting that angiogenesis inhibitor is not dependent of blood brain barrier (Joe *et al.*, 1999). Angiostatin treatment does not result in weight loss, or other toxicity in mice even in those that receive 100 mg/kg/day or immunocompromised mice receiving treatment for as long as 60 days. Histological studies reveal that in angiostatin-treated mice, the apoptotic index of tumor cells can increases to five times that of control mice, whereas tumor cell proliferative rate remains at the same level before and after exposure to angiostatin. In addition, angiostatin-induced dormant tumors lack neovascularization as detected by von Willbrand factor (Holmgren *et al.*, 1995).

## What is kringle module?

Interestingly, angiostatin consists only of the first four kringle domains of plasminogen (O'Reilly *et al.*, 1994). A kringle (kringla in Swedish) is a type of Scandinavian cookie folded into two rings. This term was originally adopted to describe a

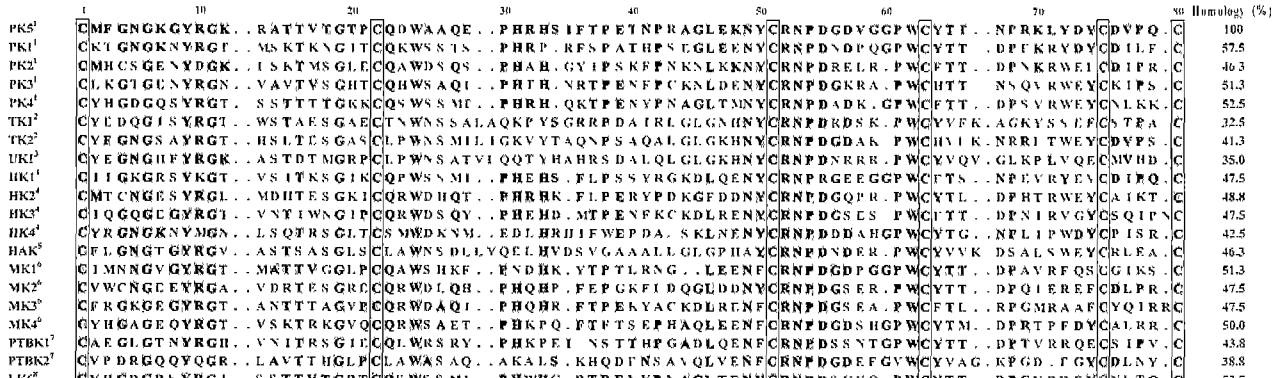

**Fig. 3.** Comparison of the amino acid sequences of kringle proteins. A typical kringle domain contains approximately 80 amino acids of which six cysteine residues are conserved in their predicted positions. In addition to the conserved cysteines, a few amino acids flanking Cys51 and Cys63 are also highly conserved among various kringle proteins. [1]Human plasminogen, [2]human tissue-type plasminogen activator, [3]human urokinase-type plasminogen activator, [4]human hepatocyte growth factor, [5]human hepatocyte growth factor activator, [6]human macrophage-stimulating protein, [7]human prothrombin, [8]human apolipoprotein A, [9]human transmembrane receptor ROR1, [10]human transmembrane receptor ROR2, [11]human factor XII, [12]human plasma hyaluronan binding protein, [13]mouse kremen, [14]murine neurotrypsin, [15]Drosophila neurospecific receptor kinase, [16]Drosophilar receptor tyrosine kinase, [17]Caenorhabditis elegans ROR receptor tyrosine kinase, [18]Xenopus muscle specific tyrosine kinase, and [19]Herdmania momus serine proteinase.

triple loop structure linked by three pairs of disulfide bonds present in prothrombin (Cao, 2001). A typical kringle structure in a protein is shown in Fig. 2B. Kringle domain exists in many proteins (Fig. 3). It is a protein module composed of 78-80 amino acids connected by a characteristic triple disulfide-linked loop. The triple disulfide bonds are strictly conserved between kringles (Castellino and Beals, 1987). However, other amino acids in the primary structure are less conserved among various kringles. Conservation of kringle structures among different proteins suggests possible replications of similar genetic information during evolution. These domains appear to be independent folding units (Castellino *et al.*, 1981; Novokhatny *et al.*, 1984; Trexler and Patthy, 1983), but a general functional role is not clear. However, it is clear that each kringle-containing protein has its specific function like growth factors, proteases, or coagulation factors (Cao *et al.*, 2002). Divergence of biological functions of various kringle-containing proteins might imply that these particular structures may not possess a common biological function although they may assist protein folding and stabilize other regions of proteins.

## Other molecules conserving kringle architecture

The sequences of the kringle domains known until now were aligned according to their six conserved cysteine residues and by reference to the homologous 80-residue PK5 (Fig. 3). In addition to the conserved 6 cysteines, a few amino acids flanking Cys51 and Cys63 are highly conserved among various kringle proteins. Since PK5 was found to be most potent in inhibition of BCE proliferation, we compared with amino acid sequence between PK5 and other individual kringle. Amino acid sequence alignment of the individual kringle domain of human plasminogen, PK1, PK2, PK3, PK4, and PK5 display considerable sequence similarity to each other (46-57%) (Cao *et al.*, 1997; Cao *et al.*, 1996). Kringle domains of tissue-type plasminogen activator also display high sequence similarity (51%), compared to the alignment between t-PA kringle and plasminogen kringle sequences (26-39%) (Kim *et al.*, 2003b). The highest sequence identity appear in apolipoprotein kringle V (LK8, 83.8%) and the lowest in neurotrypsin kringle (NTSK, 31.3%). Interestingly, all the tested kringles among them, were found to inhibit endothelial cell proliferation when added as natural fragment or recombinant proteins (Cao *et al.*, 2002; Kim *et al.*, 2003a; Kim *et al.*, 2003c). Their sequence identities to PK5 reside in a wide range between 32.5% and 83.8%. In addition, there are some different levels of inhibition observed between kringles in endothelial cell proliferation and migration. It will be discussed in the following section. Therefore, we presume that other amino acid sequence in the primary structure is also important for antiangiogenic activity, although basic kringle architecture is essential for their antiangiogenic activities.

**Table I.** Summary of inhibitory activities $I_{50}$ of kringle proteins on endothelial cell proliferation (nM)

| Kringle | BCE Cell[a] | HUVEC[b] | | | Other EC | References |
|---|---|---|---|---|---|---|
| | bFGF | bFGF | VEGF | EGF | bFGF | |
| Angiostatin (PK1-4) | 140 | | | | | Cao *et al.*, 1997 |
| PK5 | 50 | | | | | |
| PK5 | | | | | 10 (C-PAE)[c] | Lu *et al.*, 1999 |
| PK1 | 320 | | | | | Cao *et al.*, 1996 |
| PK3 | 460 | | | | | |
| PK1-3 | 70 | | | | | |
| PK1-4 | 135 | | | | | |
| PK1-5 | 0.05 | | | | | Cao *et al.*, 1999 |
| PTBK1 | 100 | | | | | Rhim *et al.*, 1998 |
| PTBK2 | 120 | | | | | |
| PTBK2 | 91 | | | | | Lee *et al.*, 1998 |
| UK1 | 80 | 80-320 | 20-80 | 80-320 | | Kim *et al.*, 2003d<br>Kim *et al.*, 2003a |
| TK1-2 | 40 | 80-320 | 20-80 | 80-320 | | Kim *et al.*, 2003b |
| LK6-8 | | 90 | | | | Kim *et al.*, 2003c |
| HGF/NK4 | | | | | 300 (HDMEC)[d] | Kuba *et al.*, 2000 |
| HK1 | | | | | 32 (BAE cell)[e] | Xin *et al.*, 2000 |

[a]Bovine capillary endothelial cell, [b]human umbilical vein endothelial cell, [c]calf pulmonary arterial endothelial cell, [d]human adult dermal microvascular endothelial cell, and [e]bovine aortic endothelial cell.

**Antiangiogenic and antitumor activity of kringle-based inhibitors**

The individual kringles of plasminogen have been shown to exhibit different effects on endothelial cell proliferation and migration, probably related to their molecular diversity (Cao *et al.*, 1996; Ji *et al.*, 1998b; Rhim *et al.*, 1998; Xin *et al.*, 2000). For example, plasminogen kringles 1, 2, 4 and 5, but not 3, have lysine binding capability (Cao *et al.*, 1997; Cao *et al.*, 1996). Kringle 4 showed a marked functional difference from other kringles in terms of anti-endothelial cell proliferation and migration activities. It is comparatively inefficient in suppressing endothelial cell growth (Cao *et al.*, 1996), but potent in inhibiting endothelial cell migration (Ji *et al.*, 1998b). In addition, the lack of lysine binding ability of UK1 does not appear to influence its anti-angiogenic activity (Kim *et al.*, 2003d). Therefore, it could be concluded that the lysine-binding site existing in some kringles might not be related to anti-proliferative activity (Cao *et al.*, 1996; Lee *et al.*, 2000).

As shown in Table I, various kringle domains elicit anti-proliferative activity against several types of endothelial cells, although different levels of inhibition were observed (Cao *et al.*, 1996; Kim *et al.*, 2003a; Kim *et al.*, 2003c; Lee *et al.*, 1998). PK1-5 was found to be most potent at $IC_{50}$ of 0.05 nM (Cao *et al.*, 1999). Kringles, PK5 and TK1-2 are also potent in inhibition of BCE cell proliferation. Interestingly, the number of kringles does not coincide with the potency of anti-proliferative activity, since PK1-3 with three kringles is more potent than angiostatin with four kringles and less potent than one-kringle domain, PK5, whereas PK1-5 with five kringles is most potent. In addition, two-kringle domains, TK1-2 elicits more potent inhibitory activity than PK1-3 and PK5. Besides angiostatin, endothelial cell-specific inhibition was also observed in other kringles (Kim *et al.*, 2003b; Kim *et al.*, 2003d; Kuba *et al.*, 2000). Two-fold high concentration of TK1-2 for maximal inhibition of endothelial cell proliferation is not inhibitory on a variety of non-endothelial cells including tumor cells.

The individual kringles also have been shown to exhibit different effects on endothelial migration, as in endothelial proliferation as mentioned above (Table II). Surprisingly, in the inhibition of endothelial cell migration, angiostatin is most potent in plasminogen kringles, which is different from the potency in inhibitory activity of endothelial cell proliferation (Ji *et al.*, 1998b). In this study, no observable inhibition by PK1-3 was detected in endothelial cell migration. However, other group reported the contradictory result that PK1-3 is as potent as angiostatin in bFGF-induced HUVE cell migration (MacDonald *et al.*, 1999). PK5 is also less potent in the inhibition of endothelial cell migration compared to anti-proliferative activity (Ji *et al.*, 1998a). Inhibitory concentration is different, depending on assay systems using different growth factors or chemotactic factors. UK1 is also potent in VEGF induced HUVE cell migration with $IC_{50}$ of 1 nM. Unlike plasminogen and apolipoprotein kringles, the inhibitory activity of HGF-derived kringles (NK1-4) is more potent in endothelial cell migration than endothelial cell proliferation when induced by the same growth factor, bFGF, since it inhibits HDMEC migration at 10 times lower concentration than $IC_{50}$ (300 nM) for inhibition of cell proliferation. At the present, more studies on anti-migratory

**Table II.** Summary of inhibitory activities $IC_{50}$ of kringle proteins on endothelial cell migration (nM)

| Kringle | BCE Cell[a] | HUVEC[b] | | Other EC | | References |
|---|---|---|---|---|---|---|
| | bFGF | bFGF | VEGF | bFGF | VEGF | |
| Angiostatin (PK1-4) | | 100 | | | | MacDonald *et al.*, 1999 |
| PK1-3 | | 180 | | | | |
| Angiostatin (PK1-4) | 50 | | | | | Ji *et al.*, 1998b |
| PK1 | >1000 | | | | | |
| PK2 | 1000 | | | | | |
| PK3 | 1000 | | | | | |
| PK4 | 500 | | | | | |
| PK1-3 | >1000 | | | | | |
| PK2-3 | 100 | | | | | |
| PK5 | 500 | | | | | Ji *et al.*, 1998a |
| UK1 | | | 1 | | | Kim *et al.*, 2003d |
| LK6-8 | | 230 | | | | Kim *et al.*, 2003c |
| HGF/NK4 | | | | 30 (HDMEC)[c] | 30 (HDMEC)[c] | Kuba *et al.*, 2000 |

[a]Bovine capillary endothelial cell, [b]human umbilical vein endothelial cell, and [c]human adult dermal microvascular endothelial cell.

**Table III.** Summary of inhibition of *in vivo* angiogenesis and tumor growth by kringle proteins

| Kringle | CAM assay | Anti-tumor effect | References |
|---|---|---|---|
| Angiostatin (PK1-4) | Dose dependent inhibition began at 20 ug and reached saturation at approximately 100 ug/ embryo | Mediates suppression of metastasis by a Lewis Lung Carcinoma | ① |
| PK1-3 | | Suppression of B16-BL6 (Lung cancer) | ② |
| PK1-3 | | Suppresson of U-87 (Human malignant glioma) | ③ |
| PK1-3 | 5 ug/embryo Marked inhibition | | ④ |
| PK1-5 | 5~25 ug/embryo Potent inhibition | Suppression of primary tumor growth (T241 fibrosarcoma) and tumor Neovascularization | ⑤ |
| UK1 | 20 ug/embryo Marked Effect | | ⑥ |
| TK1-2 | 20 ug/embryo Potent inhibition | | ⑦ |
| HGF/NK4 | 60 ug/embryo Marked Effect | Suppression of LLC (Lewis lung carcimona) and Jyg-MC (mammary carcimoma) | ⑧ |
| HGF/NK4 | | Suppression of PC3 (Human prostate cancer) | ⑨ |
| LK6-8 | 10 ug/embryo Marked Effect | Suppression of A549 (lung cancer) and HCT119 (colon cancer) | ⑩ |
| PTBK-2 | 20 ug/embryo Potent inhibition | | ⑪ |

① O'Reilly *et al.*, 1994, ② MacDonald *et al.*, 1999, ③ Joe *et al.*, 1999, ④ Lee *et al.*, 2000, ⑤ Cao *et al.*, 1999, ⑥ Kim *et al.*, 2003d, ⑦ Kim *et al.*, 2003b, ⑧ Kuba *et al.*, 2000, ⑨ Davies *et al.*, 2003, ⑩ Kim *et al.*, 2003c, and ⑪ Lee *et al.*, 1998.

activities of individual kringles employing the same endothelial cells and chemotactic factors, are necessary for the characterization of structure-function relationships.

Finally, the effects of individual kringles on *in vivo* angiogenesis and anti-tumor effects were summarized in Table III. Various kringles including angiostatin show inhibition of *in vivo* angiogenesis in chick chorioallantoic membrane. All the kringles tested show marked inhibition at doses of 5-20 μg. Although *in vitro* data showed the more potent inhibitory activity for PK5 (Cao *et al.*, 2002), it has been found to less active than angiostatin in suppression of chick chorioallantoic membrane assay and the mouse corneal angiogenesis model. Insufficient suppression of *in vivo* angiogenesis by PK5 was explained by its relatively short half-life *in vivo*. Therefore, it delivers us an important notion that antiangiogenic effect of a given compound must be tested *in vivo* angiogenesis models in addition to *in vitro* endothelial cultures.

Anti-tumor effects of kringle domains were observed in the treatment of angiostatin, PK1-3, PK1-5, NK1-4, and LK6-8 (Cao *et al.*, 1999; Davies *et al.*, 2003; Kim *et al.*, 2003c; Kuba *et al.*, 2000; O'Reilly *et al.*, 1994). All these kringles have been found to inhibit *in vivo* angiogenesis as well as endothelial cell proliferation and migration. TK1-2 also suppressed the growth of several primary tumors including human lung carcinoma and human colon carcinoma in xenograft animal models (in submission). Interestingly, all the treatments of kringle proteins showed the identical immunohistochemical data pattern of tumor tissues that apoptosis index (TUNEL staining) is increased upon the treatment, whereas proliferation index (PCNA staining) is not changed. At the present, the relative potency in antitumor activity of these kringle proteins could not be compared. Other kringles are also needed for testing their antitumor activity.

## Mechanisms

The molecular mechanisms by which angiostatin affects endothelial cells are not fully understood. Two proteins, ATP synthase and angiomotin, were independently found to bind to angiostatin (Moser *et al.*, 2001; Troyanovsky *et al.*, 2001). Cell cycle arrest and apoptosis resulting from angiostatin treatment may occur by additional independent mechanisms (Claesson-Welsh *et al.*, 1998; Griscelli *et al.*, 1998; Lucas *et al.*, 1998). PK5 also causes cell cycle arrest and apoptosis (Lu *et al.*, 1999). Recently, specific interactions of angiostatin and integrin $\alpha_v\beta_3$ were reported (Tarui *et al.*, 2001). Angiostatin as well as NK1-4 was found to inhibit HGF-induced signaling by acting as antagonists against HGF receptor, c-met (Date *et al.*, 1997; Wajih and Sane, 2002). For now, other kringles have been poorly studied for their action mechanism for anti-angiogenesis. Therefore, it remains as an important question whether the molecular targets of other kringles for anti-angiogenesis are identical to that of angiostatin. After all, it should be resolved whether all the kringle domains undergo single general mechanism or different mechanism for their antiangiogenic and antitumor activity.

## Prospect of this field

During the last decade, the angiogenesis field was exponen-

tially expanded as considered by the number of papers published in a year. Various angiogenesis inhibitors with different mechanisms have been developed for the clinical trials. Pharmaceutical companies are particularly interested in this area with strong hopes to develop effective anticancer drugs. Therefore, the search for novel angiogenesis inhibitors with therapeutic potentials has been competitive. In this regard, the unique structure of angiostatin has offered a possibility of searching for other structural homologues with perhaps more potent antiangiogenic activity. Thus, the kringle structure provides the example of discovery of novel angiogenesis inhibitors based on structural similarities. Besides, the information of structure-function relationship will help to define the functional epitope of kringle domains, and might provide possible clues for explanation of mechanisms underlying their anti-angiogenic activity.

Since biochemical characteristics of angiogenesis inhibitors differ from conventional cytotoxic chemotherapy, there are several challenges that face the application of anti-angiogenic therapy to the clinic. These include the need for surrogate markers for therapeutic efficacy and the requirement for long-term therapy (Kerbel and Folkman, 2002). Using angiogenesis inhibitors in combination with other therapeutic approaches have also been suggested for the effective approach to controlling tumor angiogenesis. As different angiogenesis inhibitors are constantly being developed and become more widely available, the number of these reagents that enter clinical trials will increase. Thus, in relation to angiogenesis, further research is required to uncover the mechanisms of action of angiogenesis inhibitors, which will give us clues for efficient therapy with angiogenesis inhibitors.

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