The inhibitory effects of recombinant plasminogen kringle 1-3 on the neovascularization of rabbit cornea induced by angiogenin, bFGF, and VEGF

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Abstract

Angiostatin is a potent angiogenesis inhibitor that is composed of the first four kringles of plasminogen fragment. Angiostatin with one less kringle molecule (kringle 1 to 3) was recently demonstrated to be an effective angiogenic inhibitor. To determine whether recombinant plasminogen kringle 1-3 (rPK1-3) can inhibit the corneal neovascularization induced by potent angiogenic factors; angiogenin, bFGF, or VEGF, hydron polymer discs each containing 2.0 μg of angiogenin, 500 ng of bFGF, or 500 ng of VEGF respectively were implanted into the corneal stroma of 138 rabbit eyes, and then discs each containing 10 μg, 12.5 μg, 20 μg or 30 μg of rPK1-3 were implanted randomly. Discs containing phosphate buffered saline were also implanted as a control. The angiogenesis score on number and length of newly formed vessels on the each of the rabbit's cornea were recorded daily by two observers (blinded). The treated corneas were also examined histologically. Recombinant PK1-3 treated corneas showed less neovascularization induced by all angiogenic factors ($p < 0.05$). and the extent of inhibition of neovascularization was proportional to the concentration of rPK1-3 ($p < 0.05$). Histologic examination showed leukocyte infiltration into the corneal stroma on the PBS treated eyes whereas rPK1-3 treated eyes showed only traces of leukocytes. These results of the effective rPK1-3 inhibition of corneal neovascularization induced by angiogenin, bFGF, or VEGF suggest that this angiostatin related fragment, rPK1-3, may be useful in the treatment of various neovascular diseases.

Keywords: angiogenin, bFGF, corneal neovascularization, recombinant kringle 1-3 of plasminogen, VEGF

Introduction

Angiogenesis is essential in tissue reproduction, development, and wound repair. However, angiogenesis is also associated with pathogenesis of numerous eye diseases; loss of visual acuity, neovascular glaucoma, diabetic retinopathy, chemical burns and viral infections of the cornea (Epstein *et al.*, 1987). Corneal neovascularization is also thought to predispose to the rejection of corneal allograft by facilitating the exposure of antigens to the host immune system (Hill and Maske, 1988). The mechanism of corneal neovascularization has been extensively investigated, in lieu of various mediators such as basic fibroblastic growth factor (bFGF) (Adamis *et al.*, 1991), vascular endothelial growth factor (VEGF) (Amano *et al.*, 1998), prostaglandins (Ziche *et al.*, 1982), interleukin 2 and 8 (Koch *et al.*, 1992; Lipman *et al.*, 1992), and platelet derived endothelial cell growth factor (PD-ECGF) (Risau, 1990). Recently, a number of anti-angiogenic molecules in the physiological system have been identified (Folkman, 1995). Angiostatin purified from serum and urine of mice bearing a murine Lewis lung carcinoma has been demonstrated to be one of the potent angiogenesis inhibitors specially that targets proliferating endothelial cells (O'Reilly *et al.*, 1994; O'Reilly *et al.*, 1996). Angiostatin contains the first four out of five repeating loop of plasminogen molecule known as the kringle domain. Each kringle is composed of 80 amino acids polypeptide chain linked by disulfide bonds at an end and at midregion forming two, an inner and an outer loop structures. *In vitro*, it specifically inhibits endothelial cell growth, but not proliferation of other cell types, including tumor cells. *In vivo*, angiostatin suppresses neovascularization in the chick chorioallantoic membrane assay (CAM) and the mouse corneal assay (O'Reilly *et al.*, 1994; OReilly *et al.*, 1996). Small fragments of angiostatin have been demonstrated to display differential

effects on suppression of a growth of endothelial cell. The anti-proliferative activity of angiostatin on endothelial cell is shared by kringle 1 (K1), kringle 2 (K2), and kringle 3 (K3), but probably not by kringle 4 (K4). Slightly enhanced inhibition of BCE cell proliferation was reported when K4 was removed from angiostatin (Cao *et al.*, 1996). The purpose of this study is to assess whether human rPK1-3 (Figure 1 diagram) affects the growth of new vessels in the rabbit cornea induced by angiogenin, bFGF, and VEGF.

Materials and Methods

Manufacturing of recombinant kringle 1-3 of human plasminogen

Construction, expression and purification of the rPK1-3 were carried out as follows; rPK1-3 (amino acid residues from 82 to 335 of human plasminogen) was amplified by polymerase chain reaction (PCR) using the following synthetic oligonucleotide primers on a template consisting of the cDNA of human plasminogen: (1) 5'primer: 5'-CGGGATCCCATATGTCAG-AGTGCAAGACTGGGA-3', (2) 3'-primer: 3'-CATTCTATGGCAGGACACTGAGGAT-CA-TTCCTAGGGC-5'. A *Nde*I site was included in the 5'-PCR primer. *Bam* HI site and stop codon (ATC, ATT) were incorporated into the 3-PCR primer. The cDNA encoding the rK1-3 was ligated into *Nde* I/*Bam* HI site of the *Escherichia coli (E. coli)* expression vector, pET 11a, resulted in expression plasmids pMETK1. *E.coli* BL21 clone containing the highest number of the pMETK1 plasmid was selected and rK1-3 expression was induced by 1 mM isopropyl thio-β-D-galactopyranoside. Cells were finally harvested by centrifugation for 30 minutes at $4{,}000 \times g$. The large-scale production of rK1-3, refolding and purification were carried out as follows: (1) The cells were disrupted by microfludizer. The insoluble body (IB) was collected by centrifugation and washed with lipophilic buffers until IB was 95% pure; (2) 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; (3) The refolded rPK1-3 was purified to homogenicity by utilizing SP-Sepharose and Lysine-Sepharose columns (Pharmacia, NJ).

The analysis of its amino acid composition, N-terminal sequence, Circular Dichroism, and affinity toward 6-amino hexanoic acid-ligand of the purified rK1-3 showed that they were identical to those of the glycosylated native kringle 1-3 purified from the elastase digest of plasminogen. Furthermore, the immuno-reactivity against the monoclonal antibody to native K1-4 of plasminogen and biological properties measured by CAM assay, and bovine capillary endothelial cell proliferation assay *in vitro* showed comparable activities with those of the native PK1-3.

The purified rPK1-3 was stable during storage in lyophilized state at 4°C for more than a year.

Chorioallantoic membrane assay

Fertilized 3 day-old eggs were incubated at 37°C, 90% humidity. After 4.5 days of incubation, thermonox cover slips with bFGF + rK1-3 or bFGF + PBS (control) were applied on the CAM of each embryo. After 7 days of incubation, embryos and CAMs were analyzed for neovasculization with stereomicroscope.

Preparation of pellet

A rabbit corneal micropocket assay described by Hu *et al.* (1994) in detail was used. Briefly, 1 μg/ml of suspension of sterile phosphated buffered saline (Sigma, St. Louis, Mo) containing bovine angiogenin, recombinant bFGF (Scios Nova, Mountainview, CA), and human recombinant VEGF (Sigma, St. Louis, MO) respectively was added to 24% Hydron (polyhydroxyethyl-methacrylate [polyHEMA], Sigma, St. Louis, MO) in ethanol. A 4 μl aliquot of this mixture was then pipetted onto teflon pegs and allowed to dry under a laminar flow hood at room temperature. All procedures were performed under sterile condition. By this methods, each uniform pellet containing 2 μg of bovine angiogenin, 500 ng of bFGF, or 500 ng of VEGF respectively was prepared. Also, hydron pellets containing 10, 12.5, 20, or 30 μg of rPK1-3 or PBS were prepared as described previously.

Pellet implantation

Each angiogenic factor-impregnated pellets (including angiogenin, bFGF, or VEGF) were implanted in 69 of New Zealand male white rabbits, weighing between 2.5 kg-3.0 kg. All animals were handled and maintained in accordance with the ARVO Resolution on the Use of Animals in Ophthalmic and Vision Research. The animals were anesthetized by intramuscular injection of ketamine hydrochloride (5 mg/kg) and xylazine (2 mg/kg). The eyes were topically anesthetized with 0.5% propacaine (Ophthetic®, Alcon, TX), and the corneas were proptosed with a speculum. A intrastromal linear keratotomy (approximately 1.5 mm length) was performed under an operating microscope (Zeiss, Oberkochen®, Germany) with a surgical blade (Bard-Parker no. 15; Becton Dickinson®, Fraklin Lakes, NY) and lamellar micropocket was dissected with a 1.5 mm wide pliable iris spatula at 1.2 mm apart from the superior limbus. A hydrogel pellet containing 2 μg of angiogenin was implanted in the micropocket located in the transparent corneal stroma of each 34 New Zealand white rabbit eyes. By the same manner, each hydrogel pellet containing 500 ng of bFGF or 500 ng of VEGF was implanted in the corneal stroma of the rabbit eyes (*bFGF model*; 40 eyes, *VEGF model*; 64 eyes). A second hydrogel pellet was placed in each eye adjacent to the first one, and then randomized to

contain either rK1-3 or PBS (*angiogenin model*; pellets containing 12.5 mg of rK1-3 in 20 eyes and PBS in 14 eyes, *bFGF model*; pellets containing 10 μg of rK1-3 in 10 eye and 30 μg of rK1-3 in 10 eyes and PBS in 20 eyes, *VEGF model*; pellets containing 10 μg of rK1-3 in 10 eye, 20 μg of rK1-3 in 12 eye and 30 μg of rK1-3 in 10 eyes and PBS in 32 eyes). During the surgery, the cornea was irrigated with 0.5% chloramphenicol eye drops. On completion of the implantation, antibiotic eye ointment (Tobrex®, Alcon, TX) was applied to the operated eye, not only to prevent infection but also to lubricate an irregular ocular surface.

Biomicroscopic examination and score of angiogenesis

The eyes were examined daily under slit lamp biomicroscopy by two blind observers. After rabbits were anesthetized with ketamine hydrochloride and xylazine, the eyes were proptosed. Pictures of corneal neovascularization were taken with zoom photographic slit lamp (model SM-50F; Takagi®, Nakano, Japan). Corneal neovascularization was measured directly from slides using an image analyzer consisting of CCD camera (SONY® CCD TR-900, Japan) coupled with the digital analyzer system (Optima® version 5.1.1) on IBM compatible computer. Angiogenic activity was recorded and calculated by number of newly developed vessels multiplied by length of vessels from limbus on postoperative day 3 and 6 post pellet implantation. Length value of less than 0.3 mm was assigned to score 0; 1, from 0.3 mm to 0.6 mm; 2, from 0.7 mm to 0.9 mm; and 3, for more than 1.0 mm. In the case of a vessels branch into several vessels, a longest vessel was selected as a representative score. The scores of two blind-observer were summed and mean was used as final score. After recording the findings, the operated eyes were treated daily with 0.5% chloramphenicol eyedrops and Tobrex® ointment.

Histological examination

On post operation day 7, experimental animals were put into euthanasia by an air embolization after sedation with an intramuscular injection of ketamine hydrochloride. All of the eyes were enucleated immediately after euthanasia. Eyes enucleated on the 7th day of implantation were immersed in 4% paraformaldehyde solution, and then dissected under the operating microscope to make tissue blocks of 5×5 mm in size. Each tissue block contained neovascularized cornea and upper limbus in full thickness. Slides were made with hematoxylin and eosin (H&E) staining.

Statistical analysis

The data are reported as mean ± standard deviation of experiments. Statistical analysis was performed by analysis of variance followed by *Mann-Whitney U* test to determine specific differences between two groups. *P* value of <0.05 was considered statistically significant.

Results

Chorioallantoic membrane assay

After 3 days of the application of thermonox coverslips with bFGF and with or without rPK1-3 (Figure 1), embryos and CAMs were observed with stereomicroscope. An-giogenin and VEGF had also similar effects on the chorioallantoic membrane study. There was less visible vascularization in the areas covered with thermonox coverslips in the CAMs treated with rPK1-3. Whereas the control group showed prominent vascularization in those areas.

Rabbit corneal study

Biomicroscopic examination: Two pellets containing angiogenic factors and either angiostatin or PBS were implanted in rabbit corneas. Biomicroscopic observation revealed the healing process of the corneal epithelium and the stroma with mild edema within 24 h after surgery.

Angiogenin model: Two pellets containing bovine angiogenin and either rPK1-3 ($n = 20$) or PBS ($n = 14$) have been implanted in both groups. Limbal vessels began to develop toward the implant within a previously avascular cornea on postoperative day 2. And definite congestions of limbal blood vessel accompanied by mild angiogenic activity were observed in both groups. On the third day of post-implantation of angiogenin, the eyes treated with rPK1-3 had less angiogenesis (mean score 4.9 ± 10.0) than the eyes treated with PBS (mean
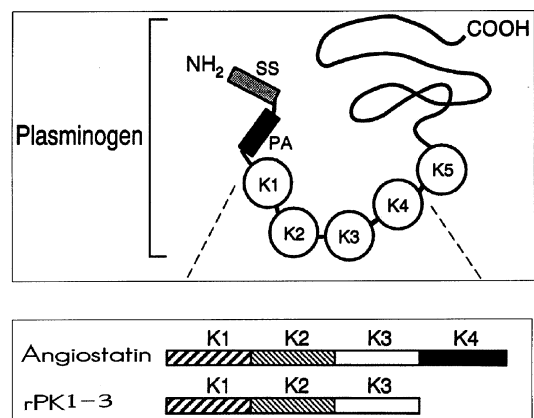

Figure 1. Schematic diagram of plasminogen (top) and angiostatin and rPK1-3 (bottom). Top graph shows a diagram of plasminogen where circles denote kringle 1-5 (k1, k2, K3, K5 & K5) and the bottom horizontal bar graph shows angiostatin which is made up of serially linked kringles 1-4 and rPK1-3 with kringles 1-3.
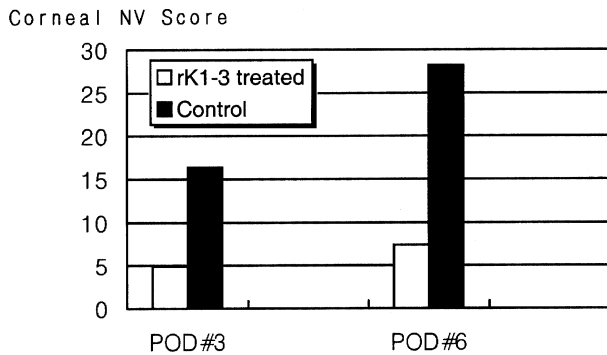
Corneal NV Score

Figure 2. Inhibition of angiogenin-induced corneal neovascularization by recombinant kringle 1-3 (rPK1-3) (*n*=20 eyes). Control animals received PBS (*n*=14 eyes). Histogram shows mean angiogenesis score ± standard deviation on post operative days 3 and 6

score 16.4 ± 20.0) (*p* < 0.05, Figure 2). On the sixth day, the eyes treated with rPK1-3 also had less angiogenesis (mean score 7.4 ± 10.1) than the eyes treated with PBS (mean score 28.2 ± 42.7) (*p* < 0.05, Figure 2). As the vessels increased in length during this period of time, the extent of circumferential neovascularization stabilized by fifth to sixth day and decreased on the seventh day.

bFGF model: Two pellets containing bFGF and either rPK1-3 (*n* = 20) or PBS (*n* = 20) have been implanted in both groups. Limbal vessels began to develop toward the implant within a previously avascular cornea. Limbal hyperemias accompanied by angiogenic activity were observed in both groups. On the third day of post-implantation of bFGF, the eyes treated with 10 μg and 30 μg of rPK1-3 had less neovascularization than the eyes treated with PBS (*p* < 0.05, Figure 3). On the sixth day, the eyes treated with rPK1-3 also had less angiogenesis than the eyes treated with PBS (*p* < 0.05, Figure 3). As the vessels increased in length during this period of time, the extent of circumferential neovascularization regressed on the seventh day after implantation.

VEGF model: Two pellets containing VEGF and either
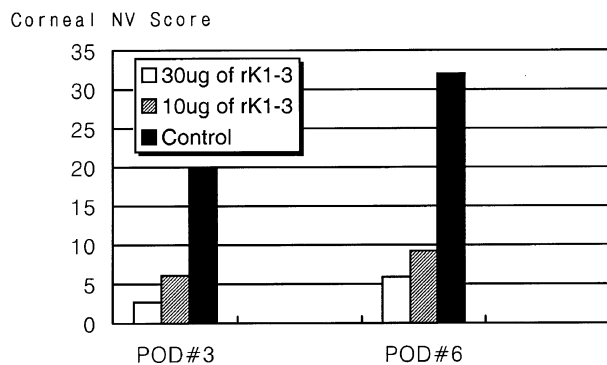
Corneal NV Score

Figure 3. Recombinant kringle 1-3 (rPK1-3) treated eyes have significantly less angiogenesis score than PBS treated eyes in bFGF induced corneal neovascularization. Inhibitory activity of rPK1-3 on corneal angiogenesis are proportional to its concentration.
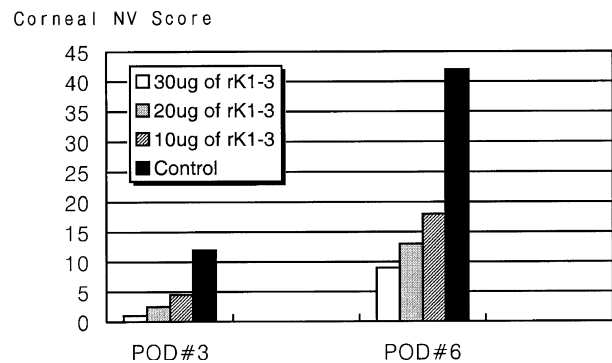
Corneal NV Score

Figure 4. Recombinant kringle 1-3 (rPK1-3) treated eyes have significantly less angiogenesis score than PBS treated eyes in VEGF induced corneal neovascularization. Inhibitory activity of rPK1-3 on corneal angiogenesis are proportional to its concentration.

rPK1-3 (*n* = 32) or PBS (*n* = 32) have been implanted in both groups. Prominent limbal hyperemias accompanied by angiogenic activity were observed in both groups. On the third day of post-implantation of VEGF, the eyes treated with rPK1-3 had less neovascularization than the eyes treated with PBS in all groups (*p* < 0.05, Figure 4). On the sixth day, the eyes treated with rPK1-3 also had less angiogenesis than eye treated with PBS (*p* < 0.05, Figure 4). As the vessels increased in length
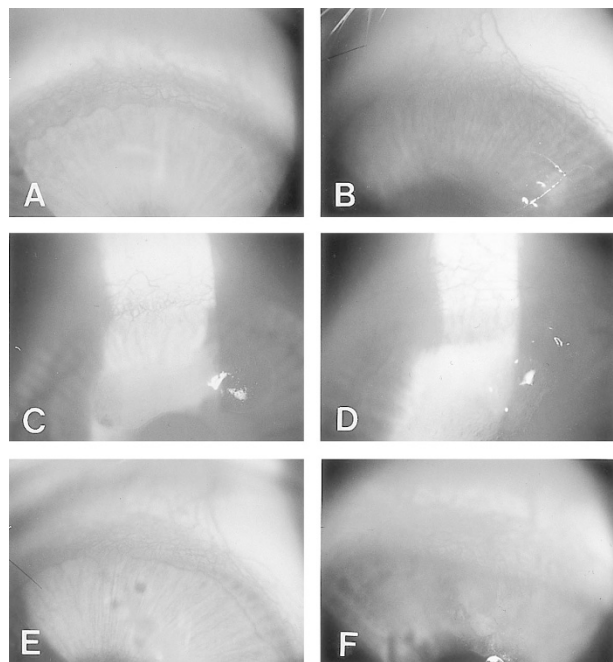

Figure 5. Slit lamp photographs of rabbit corneas 3 and 6 days after implantation show that recombinant kringle 1-3 (rPK1-3) treated eyes have less angiogenesis than PBS treated eyes. Angiogenin models; A: rPK1-3 treated eye, post operative day 6. B: phosphated buffered saline (PBS) treated eye (control), postoperative day 6, bFGF models; C: rPK1-3 treated eye, post operative day 6. D: PBS (control) treated eye, post operative day 6. VEGF models; E: rPK1-3 treated eye, post operative day 6. F: PBS (control) treated eye, post operative day 6.
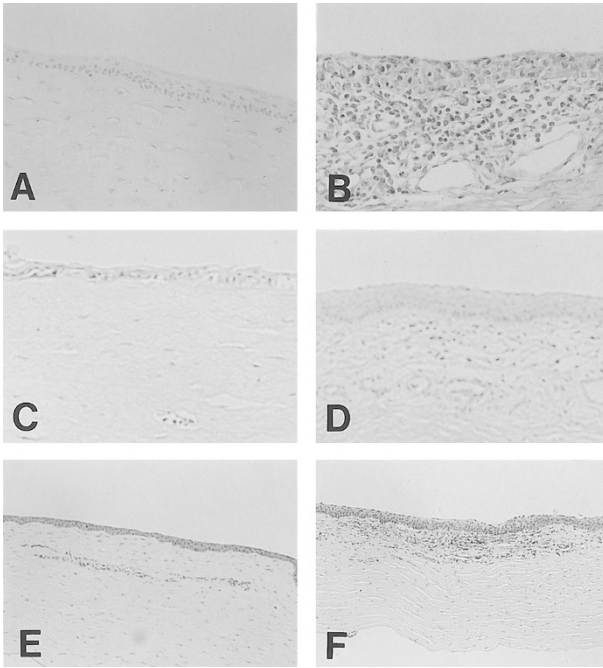
Figure 6. Histopathologic study reveals the control corneas treated with PBS have significantly more stromal leukocyte infiltration than rPK1-3 treated corneas at postopertive 6th day in all models (H&E stain, x40). A. rPK1-3 treated cornea in angiogenin model, B. PBS treated cornea in angiogenin model, C. rPK1-3 treated cornea in bFGF model, D. PBS treated cornea in bFGF model, E. rPK1-3 treated cornea in VEGF model, F. PBS treated cornea in VEGF model.

during this period of time, the extent of circumferential neovascularization regressed on the seventh day after implantation.

Histological examination: In the cornea of H&E stained section, there were significantly more leukocyte infiltration in PBS treated group. Preceding the burst of the new vessel in the group treated with PBS, extensive influx of cellular infiltrates composed mainly of polymorphonuclear cells, lymphocytes, and macrophages were observed (Figure 6).

Discussion

Neovascularization affects several disease of the eye and results in loss of visual acuity. These diseases include neovascular glaucoma, diabetic retinopathy, chemical burns and viral infections of the cornea (Epstein *et al.*, 1987). Corneal neovascularization is also thought to predispose to the rejection of corneal allografts by facilitating the exposure of antigens in donor cornea to the immune system (Hill and Maske, 1988). A variety of angiogenic factors can stimulate angiogenesis *in vitro* and *vivo*. This includes angiogenin (Fett *et al.*, 1985; Bond and Vallee, 1988; Risau, 1990), basic fibroblastic growth factor (bFGF) (Adamis *et al.*, 1991), vascular endothelial growth factor (VEGF) (Amano *et al.*, 1998),

prostaglandins (Ziche *et al.*, 1982), interleukin 2 and 8 (Koch *et al.*, 1992; Lipman *et al.*, 1992), and platelet derived endothelial cell growth factor (PD-ECGF) (Risau, 1990). And also a number of antiangiogenic molecules have been identified (Folkman *et al.*, 1995). Angiogenin is unique among the established angiogenic proteins (Schott and Marrow, 1993) in that it is isolated based solely on its ability to induce neovascularization in chick embryo chorioallantoic membrane (CAM) (Loughnan *et al.*, 1996). It also induces blood vessel formation in the cornea and meniscus of the knee of the rabbit (King and Vallee, 1991). The activity of angiogenin, as other angiogenic proteins, can also be regulated by cellular mechanism (Badet *et al.*, 1989). *In vitro* experiment, it has shown to stimulate endothelial cells in order to produce most of the characteristics of new capillary formation, including activation of proteases that digest the basement membrane and extracellular matrix (Hu *et al.*, 1994), induction of cell adhesion (Soncin, 1992), and invasion (Hu *et al.*, 1994) as well as organization of cells into tubules (Jim *et al.*, 1995). Basic fibroblast growth factor is a multifunctional, single-chain peptide composed of 146 aminoacids (Esch *et al.*, 1985) that has important effects on cell morphology, transformation, proliferation, and differentiation *in vitro* (Gospodarowicz *et al.*, 1987). It stimulates chemotaxis in vascular endothelial cells and is a potent mitogen for mesoderm-derived cells *in vitro* (Terranova *et al.*, 1985). Moreover, it induces capillary endothelial cells to invade a three-dimensional collagen matrix, to organize into tubules that resemble blood capillaries, and to produce plasminogen activator and proteases *in vitro* (Montesano *et al.*, 1986). Because bFGF is capable of directly inducing series of steps required for angiogenesis, it is an appropriate choice to use in model system. Vascular endothelial growth factor (VEGF) is a heparin-binding angiogenic factor and is highly conserved, multifunctional cytokine expressed and secreted at high levels by activated macrophages and many tumor cells of animal and human origin (Senger *et al.*, 1993). Among the various angiogenic factors identified to date, VEGF is unique because it specifically acts on endothelial cells (Connolly, 1991) and its cell-surface receptors are exclusively expressed in endothelial cells (Plate *et al.*, 1993). The overexpression of VEGF and its receptors are associated with chronic inflammation, tumor growth and other angiogenic diseases. Thus, VEGF is a prime target for the development of specific angiosuppressive drugs recently. A number of angiogenesis inhibitors have been characterized as proteolytic fragments. Proteolytic processing plays critical dual roles in the control of angiogenesis. When angiogenesis 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, to control the over-

growth of blood vessels. Tumors not only overexpress angiogenic factors, but also retain their abilities of producing angiogenesis inhibitors. The switch to the angiogenic phenotype requires both up-regulation of angiogenic stimulators and down-regulation of angiogenesis inhibitors (Cao *et al.*, 1996). It is not yet clear how expression of these angiogenesis inhibitors is regulated. However some tumors produce proteases that subsequently cleave circulating plasminogen to generate angiostatin, others may use different strategies for generation of angiostatin.

Angiostatin is a circulating angiogenesis inhibitor that has been purified from serum and urine of mice bearing a murine Lewis lung carcinoma (O'Reilly *et al.*, 1994; O'Reilly *et al.*, 1996). Angiostatin has been characterized as a potent inhibitor of angiogenesis which specially targets proliferating endothelial cells (Cao *et al.*, 1996). Angiostatin is a 38 kD internal fragment of plasminogen containing the first four kringle structures, which is known as the most effective inhibitor of tumor growth, as well as endogenous and synthetic angiogenesis inhibitors (Cao *et al.*, 1996). Recombinant kringle 1 and kringle 3 show a potent inhibitory activity in the endothelial cell proliferation assay *in vitro*. Also recombinant kringle 2 displays a with lesser degree of inhibition. In contrast, kringle 4 is an ineffective inhibitor of bFGF-stimulated endothelial cell proliferation. Among the tandem kringle arrays, the most potent inhibitory activity is seen in the kringle 1-3 fragment compared to others (Cao *et al.*, 1996). According to a recent report, kringle 5 of plasminogen also showed an antiproliferative activity on the endothelial cell (Cao *et al.*, 1997) and inhibits endothelial cell migration (Ji *et al.*, 1998).

The molecular mechanism underlying how angiostatin and its related kringle fragment specifically inhibit endothelial cell growth is unclear. Knowledge of this mechanism by which angiostatin and its related kringle fragments inhibit neovascularization is crucial to an understanding of its function in *vivo* and angiogenesis in general. Whether these inhibitors are angiostatin or angiostatin-related molecule remains to be investigated. Recent studies demonstrated that angiostatin induces apoptosis in endothelial cells (Claesson-Welsh *et al.*, 1998; Lucas *et al.*, 1998) and also inhibits migration and tube formation of endothelial cells (Luo et *al.*, 1998). Angiostatin selectively upregulates E-selectin in proliferating endothelial cells *in vitro*. This selectivity may provide insights into the mechanism by which angiostatin inhibits angiogenesis *in vivo* without apparent effects on quiescent endothelium (Redlitz *et al.*, 1999). Ji *et al.* (1998) reported that kringle domains of angiostatin may contribute to overall anti-angiogenic function by their distinct anti-migratory activities. Redlitz *et al.* (1999) demonstrated that angiostatin inhibits primarily the invasion of endothelial cells and exerts minimal effects on their proliferation.

Previous studies have shown that an inflammatory process played a major role in the neovascular response with bFGF (Loughnan *et al.*, 1996). In this study, histolopathogic findings demonstrated that there was an intrastromal vascularization with many inflammatory cell infiltration on cornea implanted with pellets containing angiogenic factors and PBS. Less inflammatory cells were observed in rabbits implanted with pellets containing angiogenic factors and recombinant kringle 1-3 (rPK1-3). Thus, these results suggest that an inflammatory process plays an important role in the development of neovascularization by angiogenin, bFGF, and VEGF. These also suggest that angiostatin and its kringle structures may suppress inflammatory reaction in the angiogenesis process.

In this study, the eyes treated with recombinant kringle 1-3 showed less induction of corneal neovascularization than PBS treated control eyes in all angiogenic factor models (p < 0.05). And the extent of inhibition of neovascularization was proportional to the concentration of recombinant kringle 1-3 (p < 0.05). These findings provide a direct evidence that neovascularization induced by angiogenin, bFGF, and VEGF is inhibited by rPK1-3 i*n vivo*. Based on our *in vivo* studies of the rabbit cornea micropocket assay, it is apparent that intrastromally implanted rPK1-3 is an inhibitor of angiogenesis induced by angiogenin, bFGF, or VEGF. Leukocyte infiltration has an important role in neovascularization induced by angiogenin, bFGF, and VEGF and angiostatin may suppress these processes. This study provides the direct evidence that neovascularization by angiogenin is inhibited by rPK1-3 *in vivo*. There were no noticable physiological ill effect during the course of this study. Therapeutic use of endogenous inhibitors is less likely to cause side effects such as suppression of hematopoiesis and gastrointestinal symptoms (Folkman J. 1995). Thus, rPK1-3, endothelial cell-specific inhibitor such as angiostatin related fragment, may be useful for long term therapy in suppression of angiogenesis.

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