Down-regulation of Vascular Endothelial Growth Factor and Up-regulation of Pigment Epithelium-derived Factor

A POSSIBLE MECHANISM FOR THE ANTI-ANGIOGENIC ACTIVITY OF PLASMINOGEN KRINGLE 5*

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We have previously shown that intravitreal injection of plasminogen kringle 5 (K5), a potent angiogenic inhibitor, inhibits ischemia-induced retinal neovascularization in a rat model. Here we report that K5 down-regulates an endogenous angiogenic stimulator, vascular endothelial growth factor (VEGF) and up-regulates an angiogenic inhibitor, pigment epithelium-derived factor (PEDF) in a dose-dependent manner in vascular cells and in the retina. The regulation of VEGF and PEDF by K5 in the retina correlates with its anti-angiogenic effect in a rat model of ischemia-induced retinopathy. Retinal RNA levels of VEGF and PEDF are also changed by K5. K5 inhibits the p42/p44 MAP kinase activation and nuclear translocation of hypoxia-inducible factor-1α, which may be responsible for the down-regulation of VEGF. Down-regulation of endogenous angiogenic stimulators and up-regulation of endogenous angiogenic inhibitors, thus leading toward restoration of the balance in angiogenic control, may represent a mechanism for the anti-angiogenic activity of K5.

Retinal neovascularization, abnormal formation of new vessels from pre-existing capillaries, is a common complication of many ocular diseases, such as advanced diabetic retinopathy, neovascular glaucoma, some forms of age-related macular degeneration, and retinopathy of prematurity (1, 2). Neovascularization leads to fibrosis and eventual damage to retinal tissues. It is a major cause of blindness in the industrialized countries and affects millions of people from infants to the elderly (1, 3, 4).

Angiogenesis is tightly controlled by two counter-balancing systems: angiogenic stimulators such as vascular endothelial growth factor (VEGF)1 and angiogenic inhibitors such as angiostatin and pigment epithelium-derived factor (PEDF) (5–7). Endogenous angiogenic inhibitors are essential for keeping the vitreous avascular (8). In some pathological conditions, such as diabetic retinopathy and retinopathy of prematurity, regions in the retina become hypoxic. Local hypoxia increases the production of angiogenic stimulators and decreases the production of angiogenic inhibitors, breaking the balance between the positive and negative regulators of angiogenesis. As a result, capillary endothelial cells over proliferate, leading to neovascularization (1, 6).

A number of endogenous angiogenic inhibitors have been shown to be fragments or cryptic domains of large protein molecules (9–11). For example, proteolysis of plasminogen releases a group of angiogenic inhibitors. Plasminogen contains 5 kringle, with each consisting of 80 amino acids (12). Angiostatin (kringle 1–4), kringle 1–5, kringle 1–3, and kringle 5 (K5) are all angiogenic inhibitors (9, 11). Among them, K5 displays the most potent inhibitory activity to endothelial cell proliferation (13). K5 induces apoptosis and causes cell cycle arrest in proliferating endothelial cells (14). K5 also inhibits endothelial cell migration (14, 15). Recently, we have shown that intravitreal injection of recombinant K5 prevents the development and arrests the progression of ischemia-induced retinal neovascularization in a rat model (16). In contrast to its potential therapeutic significance, little is known about the mechanism underlying the anti-angiogenic activity of K5 and other fragments of plasminogen. The present study reports that K5 down-regulates endogenous VEGF while up-regulating PEDF in cultured retinal vascular cells and in the retina. The results herein support the idea that the regulation of endogenous angiogenic factors may be responsible for the anti-angiogenic activity of K5.

**Experimental Procedures**

Animals—Brown Norway rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Care, use, and treatment of all animals in this study were in strict agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, as well as the guidelines set forth in the Care and Use of Laboratory Animals by the Medical University of South Carolina.

Isolation and Culture of Human Retinal Capillary Endothelial Cells (HRCEC) and Pericytes—HRCEC and pericytes were isolated from donor eyes obtained through the South Carolina Lion’s Eye Bank Association, as described by Grant and Guay (17) with some modifications. At passage 3 or 4, the purity of the cells in culture was determined. The identity of HRCEC was confirmed by a characteristic cobblestone morphology and the incorporation of acetylated low-density lipoprotein labeled with a fluorescent probe, Dil (1,1’-dioctadecyl-3,3,3’-tetramethylindocarbocyanine perchlorate) (Biomedical Technologies Inc., Stoughton, MA). Purity of the pericyte culture was determined by immunostaining using an fluorescein isothiocyanate-conjugated antibody specific to α-smooth muscle actin (Sigma).

Treatment of Cells with K5—Human K5 was expressed, purified, and
analyzed as described previously (16). HRCEC were grown to ~80% confluence in 100-mm cell culture dishes and shifted to an endothelial cell serum-free medium containing bFGF, EGF, and human plasma fibronectin (Invitrogen, Gaithersburg, MD) plus different concentrations of K5. Pericytes were grown to 80% confluence and shifted to Dulbecco's modified Eagle's medium plus 2% fetal bovine serum and different concentrations of K5. The cells were then incubated at 37°C for 24 or 48 h in normoxia or hypoxia (cells kept in a chamber that was perfused with a mixture of 95% N₂ + 5% CO₂ for 15 min) and harvested for Western or Northern blot analysis.

For Western blot analysis of MAP kinase activity, cells were grown to confluence in 100-mm cell culture dishes, and the culture medium was replaced by the endothelial serum-free medium without bFGF, EGF, and human plasma fibronectin for 24 h to suppress baseline MAP kinase activation. The medium was then replaced with the endothelial cell serum-free medium containing bFGF, EGF, and human plasma fibronectin in the presence or absence of 160 nM K5. After 5 min of incubation, the cells were lysed for analysis of phosphorylation of p42/p44 mitogen-activated protein (MAP) kinase.

Ischemia-induced Retinopathy and Intravitreal Injection of K5—Induction of retinal neovascularization was performed as described by Smith et al. (18) with some modifications. Pigmented Brown Norway rats were used for this study as they are more susceptible to hypoxia-induced retinal neovascularization (16). K5 was injected intravitreally, and retinal vasculature analyzed by fluorescein angiography as described previously (16).

Western Blot Analysis—A polyclonal anti-PEDF antibody was raised, affinity purified with a PEDF epitope column, and characterized as described (19). VEGF and PEDF levels were analyzed by Western blot analysis using the anti-VEGF (2.4 μg/ml) antibody and an anti-PEDF antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Phosphorylation of p42/p44 was measured using a phosphorylation-specific antibody (1:1,000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Measurement of Nuclear Levels of Hypoxia-inducible Factor-1α (HIF-1α)—The retina was dissected 24 h after intravitreal injection of K5 or PBS (control). Nuclear proteins were prepared as described (20). The same amount of nuclear proteins was applied to Western blot analysis using an anti-HIF-1α antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:500 dilution.

Northern Blot Analysis—Total RNA was isolated using Trizol reagents (Invitrogen, Gaithersburg, MD) according to the protocol recommended by the manufacturer. Total RNA was hybridized with a cDNA probe of PEDF in the UltraHyb Solution (Ambion, Inc., Austin, TX) following the manufacturer's protocol. The RNA blot was stripped and re-probed with the rat VEGF cDNA and then probed with a labeled oligonucleotide specific for 18 S RNA. The RNA levels were semiquantified by densitometry and normalized by 18 S RNA levels. Each analysis was repeated at least twice, and results averaged.

RESULTS

Down-regulation of VEGF Expression by K5—The purity of primary HRCEC was demonstrated by the uptake of low density lipoprotein, and the nuclei were counterstained with 4,6-diamidino-2-phenylindole. More than 95% of the cells in the HRCEC culture were low density lipoprotein receptor positive, indicating their identity as endothelial cells (Fig. 1A). Similarly, the purity of the pericyte cultures was demonstrated by immunostaining with an fluorescein isothiocyanate-conjugated anti-α-smooth muscle actin antibody (Fig. 1B).

In primary HRCEC, K5 decreased VEGF level in a concentration-dependent manner from 40 to 640 nM (Fig. 2). At 640 nM, K5 showed a maximal inhibition of VEGF expression by 3.5-fold after normalization with β-actin levels (p < 0.01, n = 3) (Fig. 2A).

The effect of K5 on VEGF expression was also examined in HRCEC under hypoxia as VEGF expression is known to be induced by hypoxia, and this induction plays a key role in the development of retinal neovascularization (21, 22). Consistent with previous observations, Western blot analysis showed that hypoxia increased the cellular VEGF level by ~2.3-fold in HRCEC (Fig. 2B). Under hypoxic conditions, K5 showed a concentration-dependent inhibition of VEGF expression with a maximal inhibition of 13-fold at 640 nM after normalization with β-actin levels, compared with the VEGF level under hypoxia without K5 (p < 0.01, n = 3). VEGF was undetectable in pericytes by Western blot analysis under hypoxia or normoxia (data not shown), indicating a low-expression level of VEGF in pericytes from human retinal capillaries.

Up-regulation of PEDF Expression by K5 in Both HRCEC and Pericytes—Under normoxia, K5 (40–320 nM for 24 h) did not significantly change PEDF at the protein level. At 640 nM, K5 only elevated PEDF by 20% (Fig. 3A). Hypoxia down-regulated PEDF expression by 60% in HRCEC (Fig. 3B). Under hypoxia, K5 increased PEDF protein levels in a concentration-dependent manner from 40 to 160 nM in HRCEC (Fig. 3B). At 160 nM, K5 treatment produced a 10-fold increase in PEDF levels over the hypoxic control (under hypoxia, no K5) (p < 0.01, n = 3).

In pericytes cultured under normoxia, K5 treatment for 24 h increased PEDF levels only at high concentrations, with 32 and 55% increases at 160 and 320 nM K5, respectively (Fig. 3C). Similar to that in HRCEC, hypoxia down-regulated PEDF expression by 55% in pericytes (Fig. 3D). Under hypoxia, K5 treatment caused significant increases in PEDF expression in a concentration-dependent manner within the range of 40–160 nM (Fig. 3D). Densitometry analysis showed that K5 at 160 nM up-regulated PEDF by 14-fold over the hypoxic control, at 160 nM after normalization by β-actin (p < 0.01, n = 3).

Effect of K5 on Retinal Neovascularization and Expression of VEGF and PEDF in the Retina—A single-dose intravitreal injection of K5 has been shown to inhibit ischemia-induced retinal neovascularization in rats (16). To determine whether this effect correlates with the regulation of VEGF and PEDF expression, retinal VEGF and PEDF levels were measured after K5 injection. K5 was injected intravitreally into the right eye of retinopathy rats at P13 (1 day after the animals were returned from hypoxia to normoxia), and to age-matched normal rats. The left eye of each animal received the same volume of PBS as a control. Retinal VEGF and PEDF were measured at the RNA and protein levels at 48 or 72 h after K5 injection, respectively.

Consistent with our previous observations (16), K5 injection substantially reduced neovascularization (Fig. 4, A–C). Correlated with the anti-angiogenic effect, K5 injection resulted in a 15-fold decrease in retinal VEGF levels (p < 0.01, n = 3) and a
2.5-fold increase in PEDF levels, when compared with the control retinas injected with PBS (p < 0.01, n = 3) (Fig. 4E). In age-matched normal controls (kept in constant normoxia), the K5 injection resulted in only a 2-fold decrease in retinal VEGF levels and a 20% increase in PEDF levels (Fig. 4, D and E).

Decrease of VEGF and PEDF mRNA Levels by K5—Northern blot analysis demonstrated that K5 decreased the VEGF mRNA level by 2-fold in the retinas with retinopathy (p < 0.01, n = 4), but not in the retinas of age-matched normal animals (Fig. 5A). Similar to the changes at the protein level, the PEDF mRNA was increased slightly in normal retina, but increased by 1.5-fold in the retina with retinopathy after K5 injection (p < 0.05, n = 4) (Fig. 5B). The changes at the RNA levels of VEGF and PEDF occurred at 48 h after the injection, 24 h prior to the protein changes.

Reduced Activation of MAP Kinase Pathway by K5—Serum-deprived HRCEC were stimulated with bFGF, and phosphorylation of p42/p44 was measured. A 5-min challenge with bFGF resulted in a 2-fold increase in p42/p44 phosphorylation when compared with those in the unchallenged cells (Fig. 6A). The difference between challenged and unchallenged cells was considered to reflect the activation of p42/p44 by bFGF, as reported previously (23). K5 decreased the bFGF-induced p42/p44 phosphorylation by 2-fold in HRCEC (Fig. 6A).

Since VEGF also regulates MAP kinase activity, we determined whether or not K5 inhibition of MAP kinase activity occurred prior to the VEGF change by measuring the time course of the K5 effect on VEGF. As shown in Fig. 6B, the earliest VEGF decrease was detected at 3 h after the K5 treatment, while decreased p42/p44 phosphorylation appeared 5 min after the K5 addition (Fig. 6A), suggesting that the MAP kinase changes are upstream of the VEGF decrease.

The retinas from rats with retinopathy and age-matched normal rats were dissected and pooled for MAP kinase activity assay 48 h after the K5 injection. Consistent with the results in HRCEC, K5 decreased p42/p44 phosphorylation by 2.5-fold in the retina of the retinopathy model, but not in the age-matched normal control (Fig. 6C).

Inhibition of Nuclear Translocation of HIF-1α by K5—Nuclear HIF-1α levels were measured in the retinas with neovascularization and in the retinas of age-matched normal rats, after PBS or K5 injection. Nuclear HIF-1α levels were 4 times higher in the retina with neovascularization than that in the normal retina. Intravitreal injection of K5 significantly decreased nuclear HIF-1α level in the retinas with neovascularization (p < 0.05, n = 3) but not in the age-matched normal rats (Fig. 7).

DISCUSSION

K5 is a potent angiogenic inhibitor and is believed to have therapeutic potential in the treatment of solid tumors (13). Recently, we have shown that intravitreal injection of K5 prevents the development and stops the progression of ischemia-induced retinal neovascularization in rats (16). Toward the understanding of its mechanism, the present study demonstrates that K5 down-regulates the expression of an endogenous angiogenic stimulator, VEGF, through inhibiting HIF-1 and p42/p44 MAP kinase activation, while enhancing the expression of an angiogenic inhibitor, PEDF. This regulation leads to the restoration of a normal balance between angiogenic...
stimulators and inhibitors. These results reveal a link between angiogenic inhibitors and angiogenic stimulators.

It is evident that there exists a delicate balance between angiogenic stimulators and angiogenic inhibitors, and this balance plays a key role in maintaining the homeostasis of angiogenesis (6, 7, 19). Under certain hypoxic conditions in the retina as found in proliferative diabetic retinopathy and retinopathy of prematurity, the angiogenic stimulators are overproduced while the angiogenic inhibitors are decreased (7, 19). The consequent disruption in the balance between these factors results in retinal neovascularization. VEGF is a major angiogenic stimulator in the retina, and increased VEGF levels have been shown to be a common pathologic factor in neovascularizing ocular diseases of humans, as well as in the animal model of ischemia-induced retinopathy (21, 24–26). PEDF has been identified as a major angiogenic inhibitor in the vitreous (6). Decreased PEDF levels have been associated with ischemia-induced retinal neovascularization and proliferative diabetic retinopathy in patients (19, 27). Recently, we have shown that the ratio between angiogenic stimulators and inhibitors is crucial for the control of angiogenesis in the retina. Elevated retinal angiogenic stimulators such as VEGF and decreased angiogenic inhibitors such as PEDF, resulting in an increased ratio of angiogenic stimulators to angiogenic inhibitors, contribute to retinal neovascularization in the ischemia-induced retinopathy rat model (19). The down-regulation of VEGF and the up-regulation of PEDF by K5 can decrease the VEGF/PEDF ratio to near normal levels, restoring the balance be-
Regulation of VEGF and PEDF by K5

Regulation of endogenous angiogenic stimulators and inhibitors may be responsible for the anti-angiogenic effect of K5.

Multiple angiogenic stimulators and inhibitors are expressed in the retina and vascular cells. Insulin-like growth factor-1 has been shown to regulate the expression of VEGF in RPE cells, suggesting that regulatory interactions exist among angiogenic stimulators. However, the regulatory interactions between the two counter-balancing systems, angiogenic stimulators and inhibitors, have not been reported previously. The present study demonstrates for the first time that an angiogenic inhibitor can suppress the expression of angiogenic stimulators while enhancing the expression of other endogenous angiogenic inhibitors. These regulatory interactions accelerate the restoration of the balance between angiogenic stimulators and inhibitors and thus, may represent a mechanism of angiogenic control.

HIF-1 is a major positive regulator of VEGF expression under hypoxia. Nuclear translocation of HIF-1α is a critical step in the induction of VEGF expression. The present study demonstrated that the nuclear HIF-1α level was elevated significantly in the retina with neovascularization, correlating with increased VEGF expression. K5 injection significantly reduced the nuclear HIF-1α levels in the retina of the retinopathy model, suggesting a decreased HIF-1α nuclear translocation. These results suggest that inhibiting HIF-1α activation is responsible, at least partially, for the decreased VEGF expression by K5.

The p42/p44 MAP kinase pathway has been suggested to play a role in the regulation of VEGF expression. Our results show that K5 inhibits the activation of p42/p44. As VEGF itself is an activator of this pathway through its interactions with VEGF receptors, we have also determined if the decreased MAP kinase activation by K5 is a cause, or a consequence, of decreased VEGF levels. In endothelial cells, K5 displayed a fast inhibition of p42/p44 activation, and this effect occurred as early as 5 min after the addition of K5, while the earliest change in VEGF levels occurred at 3 h after the addition of K5, suggesting that the MAP kinase inhibition occurs prior to the down-regulation of VEGF and is unlikely a consequence of the decreased VEGF levels. Interestingly, angioatin has recently been shown to reduce the activation of MAP kinase ERK-1/ERK-2 (p42/p44) in human dermal microvascular endothelial cells. Therefore, angioatin and K5 may have similar anti-angiogenic mechanisms.

The MAP kinase pathway is a well studied intracellular signal transduction pathway mediating biological effects of many activated receptors. The finding that K5 specifically inhibits the activation of p42/p44 raises the question of how K5 interacts with this intracellular pathway. Recently, we have performed a receptor-binding assay using 125I-labeled K5 and cultured endothelial cells. No specific binding of K5 with endothelial cells was detected (data not shown), suggesting that K5 does not have a specific receptor on endothelial cells. As VEGF can also activate the MAP kinase pathway through its receptor, blocking the VEGF receptor may also result in the inhibition of MAP kinase pathway. Therefore, we have also measured the effect of K5 on VEGF binding with VEGF receptor, and the results showed that K5 does not interfere with VEGF binding to its receptor (data not shown). These results indicate that the inhibitory effect of K5 on MAP kinase is neither through binding to a specific receptor on the endothelial cells nor through blocking the VEGF binding. It is possible that K5 may block the binding of other factors to their receptors and subsequently inhibit certain signal transduction pathways. It is also possible that the K5 effect is mediated by molecules in the extracellular matrix such as integrin that is essential for the sustained activation of MAP kinase by angiogenic stimuliators (41, 42). It remains a future challenge to determine how K5 inhibits the MAP kinase pathway.

In contrast to VEGF, little is known about the regulation of PEDF expression. Dawson et al. (6) showed that PEDF is regulated by oxygen concentration in a retinoblastoma cell line only at the protein level. However, Coljee et al. (43) demonstrated that the mRNA level of PEDF (EPC-1/PEDF) is regulated by serum stimulation in fibroblasts. Their studies demonstrated that the decreased PEDF mRNA level by serum stimulation results from a reduced RNA stability and changed hnRNA processing. Recently, we have shown that the PEDF mRNA is decreased in the retina of the rat model of ischemia-induced retinal neovascularization (19). The discrepancy between these observations suggests that PEDF expression may be regulated at different levels depending on cell types and regulators. Regulation of PEDF expression by other growth factors and transcription factors has not been reported previously. Herein we show that K5 up-regulates PEDF expression in vascular cells and in the retina. As PEDF has been shown to induce apoptosis, the up-regulation of PEDF expression by K5 may be responsible for K5’s effect on the induction of apoptosis in endothelial cells.

The present study demonstrates that K5 has more significant regulation of VEGF and PEDF expression under hypoxia than under normoxia. This may be explained by the fact that the basal level of VEGF is elevated, while that of PEDF is decreased by hypoxia in the absence of K5. In newborn rats, exposure to hyperoxia for 5 days followed by exposure to normoxia results in local hypoxia in the retina. The retinal hypoxia elevates VEGF, but reduces PEDF levels. Consistent with the findings in cultured vascular cells, the K5 regulation on VEGF and PEDF is more significant in the retinopathy animal model than in normal retinas. As increased retinal VEGF expression and decreased PEDF are believed to be the cause of retinal neovascularization (19), the more significant regulation of...
VEGF and PEDF by K5 in the retinopathy model than in normal controls may explain our previous observation that K5 has anti-angiogenic activity only in the retina with neovascularization, but not in the normal retina (16).

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