

Review

Novel Roles of Kallistatin, a Specific Tissue Kallikrein Inhibitor, in Vascular Remodeling

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We have purified, cloned and characterized kallistatin, a tissue kallikrein-binding protein (KBP) in humans and rodents. Kallistatin is a unique serine proteinase inhibitor (serpin) with Phe-Phe residues at the P2 and P1 positions. Structural and functional analysis of kallistatin by site-directed mutagenesis and protein engineering indicate that wild-type kallistatin is selective for tissue kallikrein. Kallistatin is expressed and localized in endothelial and smooth muscle cells of blood vessels and has multiple roles in vascular function independent of the tissue kallikrein-kinin system. First, kallistatin induces vasorelaxation of isolated aortic rings and reduces renal perfusion pressure in isolated rat kidneys. Transgenic mice overexpressing rat kallistatin are hypotensive, and adenovirus-mediated gene delivery of human kallistatin attenuates blood pressure rise in spontaneously hypertensive rats. Second, kallistatin stimulates the proliferation and migration of vascular smooth muscle cells *in vitro* and neointima formation in balloon-injured rat arteries. Third, kallistatin inhibits the proliferation, migration and adhesion of endothelial cells *in vitro* and angiogenesis in the rat model of hindlimb ischemia. These results demonstrate novel roles of kallistatin in blood pressure regulation and vascular remodeling.

Key words: Angiogenesis/Blood pressure reduction/Kallistatin/Neointima hyperplasia/Tissue kallikrein-binding protein/Vascular cell growth.

Introduction

Tissue kallikreins belong to a subset of closely related serine proteinase that exhibit a narrow range of substrate specificity (Bhoola *et al.*, 1992). Members of the tissue kallikrein family are involved in the processing of biologically active peptide hormones and growth factors. The

expression of tissue kallikrein is regulated by a number of hormones and transcriptional factors (Clement, 1989, 1994). The activity and metabolism of kallikreins are modulated post-translationally by endogenous tissue kallikrein inhibitors. In 1928, a tissue kallikrein-binding protein that rapidly inhibits kallikrein's activity was first observed in human plasma (Frey and Kraut, 1928). However, the identity of this endogenous tissue kallikrein inhibitor remained unclear. During the early 1980's, we separated human serum samples on a denaturing SDS-polyacrylamide gel using radiolabeled human tissue kallikrein as a marker. Unexpectedly, we found that the labeled kallikrein that was added to the serum as an internal marker always migrated much slower than the enzyme running alone. This labeled high molecular weight kallikrein entity was resistant to treatment with SDS and boiling, suggesting a covalent linkage with a serum protein. For more than 10 years, we have continued to investigate the identity of this rapid tissue kallikrein-binding protein (KBP). We first purified, characterized and cloned the gene for rat tissue kallikrein-binding protein (Chao *et al.*, 1986, 1990; Chen *et al.*, 1990; Chai *et al.*, 1991a, b). However, we encountered difficulties in purifying human KBP from plasma due to contamination of α_1 -antitrypsin, which is present at a high concentration in the circulation. After many trials and errors, we finally succeeded in completely removing α_1 -antitrypsin from human KBP *via* a heparin-affinity column (Zhou *et al.*, 1992). Molecular cloning and sequence analysis of the human KBP gene revealed that KBP was a new member of the serine proteinase inhibitor (serpin) superfamily and thus it was designated as kallistatin (Chai *et al.*, 1993, 1994). Independent of its binding to tissue kallikrein, our recent studies showed that kallistatin had direct biological effects in vasodilatation, stimulation of vascular smooth muscle cell growth and inhibition of endothelial cell growth.

Biochemical and Molecular Properties of Kallistatin

Plasma kallistatins are acidic glycoproteins with molecular masses of 58–60 kDa and isoelectric points of 4.6 to 5.2 (Chao *et al.*, 1990; Zhou *et al.*, 1992). No immunological cross-reactivity was found by Western blot analysis between human kallistatin and other serpins using either polyclonal or monoclonal antibodies (Zhou *et al.*, 1992). Kallistatin is a heparin-binding protein. For most serpins,

heparin enhances their association with target serine proteinases (Griffith, 1982; Tollefsen *et al.*, 1983; Pratt and Church, 1992; Pratt *et al.*, 1992). However, heparin suppressed the binding of kallistatin to tissue kallikrein. Kallistatin inhibits both kininogenase and amidolytic activities of tissue kallikrein. Inhibition was accompanied by the formation of an equimolar heat- and SDS-stable complex between kallikrein and kallistatin, and by the generation of a small carboxyl-terminal fragment from the latter (Zhou *et al.*, 1992; Chao and Chao, 1995). The amino acid sequence derived from human kallistatin cDNA shares 44–46% homology with other human serpins such as α 1-antichymotrypsin, protein C inhibitor and α 1-antitrypsin (Chai *et al.*, 1993). The full-length human kallistatin cDNA encodes 427 amino acid residues, including a 26 residue signal peptide and a 401 residue mature protein (Chao *et al.*, 1990a; Chai *et al.*, 1991b, 1993). The structure and organization of kallistatin genes in human, rat and mouse, consisting of five exons and four introns, are similar to those of other serpin genes. The human kallistatin gene is located on human chromosome 14q31-32.1 within a serpin gene cluster (Chai *et al.*, 1994). Moreover, kallistatin is a negative-acute phase protein and its expression level is markedly reduced with endotoxin challenge (Chen *et al.*, 1997a). In transgenic mice expressing rat kallistatin under the control of the mouse metallothionein gene metal-response element (MRE) promoter, kallistatin levels were increased following endotoxin shock, since the MRE promoter was activated by cytokines. As a result, these transgenic mice have a significantly higher survival rate than control mice when subjected to endotoxin challenge. These results suggest that kallistatin may play a protective role in inflammation and septic shocks.

Structural Elements of Kallistatin Required for Interaction with Tissue Kallikrein

Figure 1 shows the three-dimensional structure of human kallistatin created by molecular modeling. Similar to other serpins, kallistatin consists of three β -sheets and eight helices (Carrell *et al.*, 1987). Functionally critical structural elements of kallistatin have been identified in the hinge region of the reactive center loop, reactive site residues P3 to P1, and two putative heparin-binding regions, F helix and the loop between the H helix and C2 sheet (Chen *et al.*, 2000a–d). Figure 2 shows the binding specificity between kallistatin (a serpin) and tissue kallikrein (a target serine proteinase). The P1Phe in kallistatin determines the binding and cleavage specificity with the S1 site in the catalytic center of tissue kallikrein. Similarly, the P2Phe residue binds to the S2 site and P3Lys residue of kallistatin binds to the S3 site of tissue kallikrein. We have analyzed the structural and functional relationship of kallistatin and tissue kallikrein inhibition by site-directed mutagenesis and protein engineering. Table 1 summarizes the structural elements of kallistatin crucial for tis-

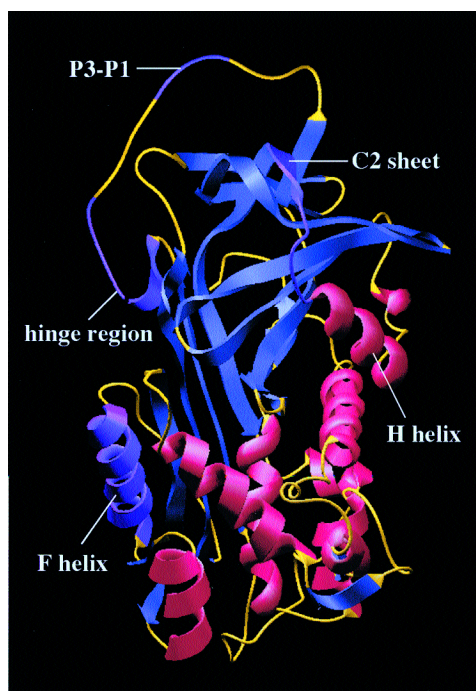


Fig. 1 The Three-Dimensional Structure of Kallistatin Created by Homology Modeling.

The P1–P3 residues and the hinge region of the reactive center loop, two putative heparin-binding sites, the F helix and the loop between the C2 sheet and the H helix are indicated in purple.

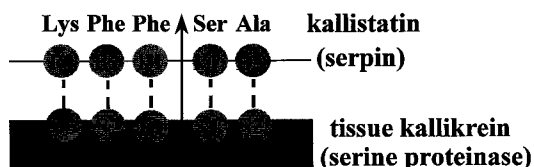


Fig. 2 Specificity Determinants of Kallistatin (Serpine) with Tissue Kallikrein (Target Serine Proteinase).

Cleavage or binding specificity of a serpin is primarily determined by the P1 residue. Other reactive residues, such as P2 and P3 or P1' and P2', may also interact with the subsites of the target enzyme, and determine the inhibitory specificity.

Table 1 Structural and Functional Relationships of Kallistatin and Tissue Kallikrein Probed by Site-Directed Mutagenesis and Protein Engineering.

Structural elements	Function
P10Ala, P12Ala	Hinge region, essential for inhibitory conformation
P1Phe	Cleavage site, specific for tissue kallikrein
P2Phe, P3Lys	Crucial for inhibition specificity
H helix and C2 sheet	A major heparin-binding site, acting as a secondary binding site to tissue kallikrein

sue kallikrein inhibition (Chen *et al.*, 2000a–d). Kallistatin mutants in the hinge region at the P10 and P12 positions, Ala379Pro and Ala377Thr, serve as a substrate instead of an inhibitor for tissue kallikrein (Chen *et al.*, 2000a). These results indicate that compact residues P10Ala and P12Ala at the hinge region are required for assuming an inhibitory conformation by kallistatin. Phenylalanine at the P1 position exhibits the highest selectivity toward tissue kallikrein. Substitutions of P1Phe with each of the other 19 amino acids revealed that several P1 mutants, P1Arg, P1Lys, P1Tyr, P1Met and P1Leu, displayed significant binding activity toward tissue kallikrein in addition to wild-type P1Phe. Wild-type P1Phe kallistatin only inhibits tissue kallikrein and has no effect on other serine proteinases. However, the P1Arg and P1Lys variants have a broad inhibitory spectrum toward other serine proteinases such as human plasma kallikrein, thrombin, activated protein C, plasmin and trypsin. These results indicate that P1Phe in wild-type recombinant kallistatin or plasma kallistatin is selective for human tissue kallikrein (Chen *et al.*, 2000a). Previous studies showed that human α -1-antitrypsin and protein C inhibitor were also capable of binding to human tissue kallikrein (Geiger *et al.*, 1981a, b; Ecker *et al.*, 1992). However, α -1-antitrypsin and protein C inhibitor also inhibit a broad spectrum of other serine proteinases. Thus, these results indicate that native kallistatin with a unique Phe residue at the P1 position is a specific tissue kallikrein inhibitor. In addition, P2Phe and P3 Lys of kallistatin are also crucial for its inhibitory specificity toward tissue kallikrein. Recombinant kallistatins with hydrophobic and bulky amino acids (Phe) at the P2 position and basic amino acids (Arg/Lys) at the P3 position display higher binding activity toward tissue kallikrein than other variants. The interaction of kallistatin with tissue kallikrein was probed by molecular modeling. The reactive center loop of kallistatin bound into the kallikrein's reactive crevice suggests that P2 residue required an extended and bulky hydrophobic side chain to reach and fill the hydrophobic S2 cleft generated by Tyr99 and Trp219 of tissue kallikrein. Basic amino acids at the P3 position could generate an electrostatic interaction with Asp98J and a hydrogen bond with Gln174 of tissue kallikrein to stabilize the complex formation (Bode *et al.*, 1989; Katz *et al.*, 1998).

We have identified two structural domains, the F helix and the region between the H helix and C2 sheet of kallistatin, containing clusters of positively charged residues, as putative heparin-binding regions by computational analysis, molecular modeling, and competition assay using synthetic peptides derived from both regions (Figure 1). Site-directed mutagenesis of the basic residues within these two domains indicates that the region between the H helix and C2 sheet of kallistatin is the major heparin-binding site responsible for its heparin-suppressed tissue kallikrein binding (Chen *et al.*, 2000c). Furthermore, we showed that the heparin-binding region acts as a secondary binding site for tissue kallikrein as identified by domain substitution of kallistatin with α -1-antitrypsin

(Chen *et al.*, 2000d). α -1-Antitrypsin was chosen as a scaffold since it is a non-heparin-binding serpin and a slow tissue kallikrein inhibitor. An α -1-antitrypsin chimera, incorporating the heparin-binding site from kallistatin, markedly increases the inhibitory activity of α -1-antitrypsin toward tissue kallikrein. These results confirm that the region between the H helix and C2 sheet is a heparin-responsive site and it acts a secondary binding site to accelerate the association between kallistatin and tissue kallikrein. Structural and functional analyses revealed structural elements of kallistatin crucial for the interaction with tissue kallikrein. These recombinant kallistatin variants are valuable tools for future studies in deciphering the structural elements essential for its roles in vascular biology.

The Reactivity Center Loop of Kallistatin Is Not Essential for Its Biological Effects on Vascular Smooth Muscle Cells

We previously showed that kallistatin bound to active kallikrein but not to the active-site blocked kallikrein or latent (inactive) kallikrein (Chao *et al.*, 1986; Chao and Chao, 1988). These results indicate that the interaction between kallistatin and tissue kallikrein requires binding of the reactive center loop of kallistatin to the active site of tissue kallikrein. Tissue kallikrein activity is abolished by aprotinin, a potent tissue kallikrein inhibitor, or by affinity-purified antibody to tissue kallikrein. Our recent studies showed that purified tissue kallikrein inhibited the proliferation of vascular smooth muscle cells and the effect was abolished by either bradykinin B₁, or B₂ receptor antagonist (Agata *et al.*, 2000), while kallistatin simulates the proliferation of vascular smooth muscle cells (Miao *et al.*, 2000a). In order to decipher whether the effects of kallistatin on the proliferation of vascular smooth muscle cells is dependent on the binding of its reactive center loop to active kallikrein, we examined the effect of aprotinin and kallikrein antibody on vascular smooth muscle cell proliferation. We found that neither of these two kallikrein inhibitors mimicked the stimulatory effect of kallistatin on human aortic smooth muscle cell proliferation. These results indicate that the biological effects of kallistatin on vascular cell growth are independent of the interaction of its reactive center loop with active kallikrein. This notion is further supported by structural and functional analysis of kallistatin using site-directed mutagenesis and protein engineering. A substitution of the P12Ala residue at the hinge region with Thr (P12Thr) results in a complete loss of its inhibitory activity toward tissue kallikrein. However, the AlaP12Thr kallistatin variant still exerts the same stimulatory effect as wild-type kallistatin on vascular smooth muscle cell proliferation. These results indicate that the reactive center loop of kallistatin is not essential for its biological effects on vascular smooth muscle cell growth. Another serpin, pigment epithelial-derived factor (PEDF), has been shown to function as a growth factor by promot-

ing neurite growth (Becerra *et al.*, 1995). Similar to kallistatin, the effects of PEDF's growth-promoting activity does not require the serpin's reactive center loop as recombinant PEDF polypeptide fragments with truncations from the C-terminal end still showed neurotrophic activity. These studies showed that serpins could affect cell growth and migration independent of its interaction with serine proteinases.

Expression and Localization of Human Kallistatin

Kallistatin and tissue kallikrein are expressed in a wide variety of human tissues (Chen *et al.*, 1994, 1995; Chao *et al.*, 1995; Ma *et al.*, 1997; Wolf *et al.*, 1998, 1999). Table 2 shows the expression and localization results of kallistatin and kallikrein mRNA in human tissues obtained by *in situ* hybridization histochemistry. Kallistatin and tissue kallikrein mRNA were identified and localized to endothelial and smooth muscle cells of large, medium and small normal blood vessels by *in situ* hybridization histochemistry and RT-PCR followed by Southern blot analysis (Wolf *et al.*, 1999). Immunoreactive kallistatin and tissue kallikrein were also localized in the corresponding blood vessels by immunohistochemistry and immunoassays using polyclonal or monoclonal antibodies (Wolf *et al.*, 1999). The major difference in the site of synthesis between kallistatin and tissue kallikrein is that kallistatin is synthesized in the liver while tissue kallikrein is not expressed in this organ (Chao and Chao, 1995). Kallistatin transcripts were localized in many cells and tissues (Table 2). Kallistatin is also expressed in primary cultured renal proximal tubular cells, umbilical vein endothelial cells and in platelets, lymphocytes, monocytes as well as in the

colon carcinoma cell line T84 (Chao *et al.*, 1995). Consistent with the results of kallistatin mRNA distribution, immunoreactive kallistatin was also identified in these tissues, blood cells and bodily fluids. Unlike other serpins, human kallistatin is not only present in the circulation but also in urine, saliva, seminal fluids, amniotic fluid, milk, sweat and tears (Chao *et al.*, 1995). The wide distribution of kallistatin in tissues and body fluids suggests that kallistatin may play important roles in complex physiological and pathophysiological functions.

A Direct Role of Kallistatin in Blood Pressure Regulation

Our previous study showed that infusion of purified kallistatin into cannulated rat jugular vein produced a dose-dependent reduction of blood pressure and the effect was not abolished by Icatibant (Hoe140), a bradykinin B₂-receptor antagonist (Chao *et al.*, 1977). Moreover, kallistatin induced relaxation of isolated aortic rings while neither kinin nor kallikrein had any effect on vascular relaxation of aortic rings. Kallistatin also caused vasodilatation of the renal vasculature in isolated, perfused rat kidneys. Icatibant has no effect on kallistatin-induced vasorelaxation. These results suggest that kallistatin is a potent vasodilator which may function directly through a vascular smooth muscle mechanism independent of bradykinin B₂ receptor (Chao *et al.*, 1997). In order to understand the role of kallistatin in blood pressure regulation, we developed transgenic mouse lines overexpressing rat kallistatin under the control of mouse metallothionein gene MRE promoter. Functional analysis showed that these mice have significantly lower blood pressure than control littermates (Chen *et al.*, 1996). Moreover, rat kallistatin levels in spon-

Table 2 Localization of Kallistatin and Tissue Kallikrein mRNA in Human Tissues by *in Situ* Hybridization Histochemistry.

Tissues	Kallistatin	Kallikrein	Specific cells stained
Blood vessels	+	+	Endothelial and vascular smooth muscle cells
Liver	+	+	Hepatic cells and bile ducts
Kidney	+	+	Distal tubules and collecting ducts
Pancreas	+	+	Acinar cells
Prostate	+	+	Glandular epithelial cells
Lung	+	+	Cartilage chondrocytes Tracheal glandular cells
Adrenal	+	+	Kallistatin: zona fasciculata cells Kallikrein: zona fasciculata cells zona reticularis medullar cells
Spleen	+	+	White pulps
Colon	+	+	Glandular epithelial cells
Hippocampus	+	+	Pyramidal cells and granule cells
Ventricle (left)	+	+	Myocardial cells
Stomach	+	+	Gastric glandular cells
Salivary	+	+	Serous alveoli and striated ducts

taneously hypertensive rats (SHR) are significantly reduced as compared to normotensive rats (Chao and Chao, 1988). Expression of recombinant kallistatin in SHR following adenovirus-mediated delivery of the human kallistatin gene caused a prolonged blood pressure reduction (Chen *et al.*, 1997b). Taken together, these results suggest that kallistatin may function as a vasodilator *in vivo* and that the blood pressure-lowering effect is independent of its binding to tissue kallikrein.

Kallistatin Stimulates Neointima Hyperplasia in Balloon-Injured Rat Arteries

Kallistatin is expressed in both endothelial and smooth muscle cells of blood vessels, implicating a potential role in vascular biology. To examine this possibility, we first evaluated the effect of kallistatin on the proliferation and growth of primary rat vascular smooth muscle cells by measuring [³H]-thymidine incorporation and cell counts (Miao *et al.*, 2000). We found that purified plasma kallistatin markedly increased the proliferation and growth of vascular smooth muscle cells in a dose- and time-dependent manner. Kallistatin also stimulates the migration of vascular smooth muscle cells as measured by modified Boyden chambers. To further investigate its potential role in vascular cell growth *in vivo*, we evaluated the expression of rat kallistatin in balloon-injured vessels. We found that balloon angioplasty resulted in marked increases of kallistatin mRNA and protein levels in the injured vessels as determined by competitive RT-PCR and ELISA. Consistent with this finding, intense staining of kallistatin mRNA was identified in the proliferating vascular smooth muscle cells of balloon-injured arteries during cell migration from media to neointima by *in situ* hybridization histochemistry using antisense riboprobes of rat kallistatin. A role of kallistatin in vascular cell growth *in vivo* was supported by the finding that local delivery of rat kallistatin antisense cDNA significantly attenuated kallistatin mRNA levels as well as neointima formation in rat artery after balloon angioplasty. These results indicate a role of kallistatin in vascular cell growth at the injured

sites. We further evaluated the potential mechanisms of kallistatin in neointima formation using cultured vascular smooth muscle cells. We found that kallistatin enhanced platelet-derived growth factor (PDGF)-induced p42/44 mitogen-activated protein kinase (MAPK) activity, while adenovirus-mediated transfer of rat kallistatin antisense cDNA into vascular smooth muscle cells inhibited both PDGF-induced p42/44 MAPK activity and proliferation. These results indicate that kallistatin stimulates vascular smooth muscle cell growth in injured vessels *via* mediating PDGF-induced MAPK signaling pathways and thus plays a role in the pathogenesis of neointima hyperplasia (Figure 3).

Kallistatin Inhibits Angiogenesis in the Rat Model of Hindlimb Ischemia

Kallistatin acts as a growth stimulator in vascular smooth muscle cells, while it acts as a growth inhibitor in endothelial cells (Miao *et al.*, 2000). We found that purified kallistatin significantly inhibits the proliferation, migration and adhesion of cultured endothelial cells induced by vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) (Miao *et al.*, data not shown). To further investigate the role of kallistatin in endothelial cell growth *in vivo*, we prepared an adenovirus carrying the human kallistatin gene under the control of the cytomegalovirus promoter/enhancer. The effect of adenovirus-mediated kallistatin gene delivery on angiogenesis was evaluated in a rat model of hindlimb ischemia. Histological and morphometric analysis showed significantly reduced capillary density in the ischemic muscle as compared to that of control rats injected with adenovirus containing the green fluorescence protein gene. The anti-angiogenic effect of kallistatin was associated with reduced regional blood flow in the ischemic hindlimb as measured by the microsphere assay. Expression of recombinant human kallistatin was identified in the injected muscle and circulation of the rats following kallistatin gene delivery. These results demonstrate a novel role of kallistatin in the inhibition of angiogenesis, and perhaps in the retardation of tumor growth. Similarly, other serpins such as maspin, PEDF, and the cleaved form of antithrombin III have also been shown to have anti-angiogenesis and anti-tumor growth activities (Dawson *et al.*, 1999; O'Reilly *et al.*, 1999; Zhang *et al.*, 2000). The potential mechanisms as well as the structural elements of kallistatin required for its inhibition on endothelial cell growth/angiogenesis remain to be investigated.

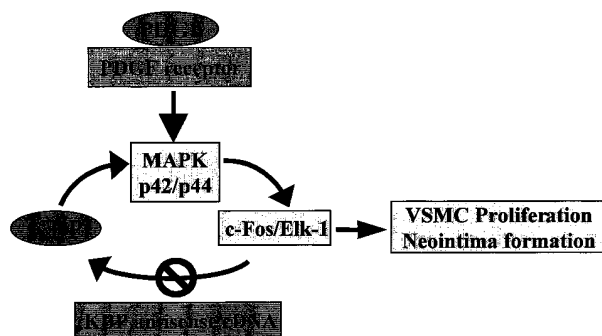


Fig. 3 Scheme for Potential Mechanisms of Kallistatin Action on the Proliferation of Vascular Smooth Muscle Cells (VSMC).

Concluding Remarks

We have identified, purified and cloned tissue kallikrein-binding proteins from human, rat and mouse, and demonstrated that kallistatin is a new member of the serpin superfamily. Structural and functional analyses of

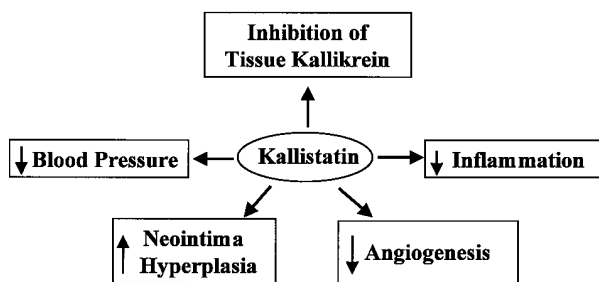


Fig. 4 Multiple Roles of Kallistatin in Vascular Function.

Kallistatin has multiple roles in carciofascular function including reduction of blood pressure and inflammation, neointima hyperplasia and anti-angiogenesis in addition to inhibition of tissue kallikrein.

kallistatin by molecular modeling, site-directed mutagenesis and protein engineering shows that native or wild-type kallistatin is selective for tissue kallikrein but not for other serine proteinases. Kallistatin also functions as a vasodilator and a regulator of vascular cell growth, independent of its interaction with tissue kallikrein. Kallistatin regulates cultured vascular cell growth by stimulating the proliferation and migration of vascular smooth muscle cells, while it inhibits the growth, migration and adhesion of endothelial cells. Consistent with the *in vitro* studies, kallistatin reduces blood pressure in hypertensive rats, stimulates neointima hyperplasia in blood vessels after balloon angioplasty and inhibits angiogenesis in the rat model of hindlimb ischemia. These results indicate that kallistatin has multiple roles in the regulation of biological activities including inhibition of tissue kallikrein, vasodilation, protection of inflammatory reaction, stimulation of vascular smooth muscle growth and migration and inhibition of endothelial cell growth (Figure 4). These findings are crucial for the future design of therapeutic agents in treating cardiovascular diseases.

Acknowledgement

This work was supported by the National Institutes of Health, grants HL 44083 and HL 29397.

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