

# Kallistatin Stimulates Vascular Smooth Muscle Cell Proliferation and Migration In Vitro and Neointima Formation in Balloon-Injured Rat Artery

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**Abstract**—Kallistatin, a serine proteinase inhibitor (serpin), is expressed in the endothelial and smooth muscle cells of blood vessels. The potential function of kallistatin in vascular biology was investigated by studying its role in the proliferation and migration of cultured primary aortic vascular smooth muscle cells (VSMCs) in vitro and in neointima formation in rat artery after balloon angioplasty in vivo. Exogenous kallistatin induced a >2-fold increase of VSMC proliferation and cell growth as measured by [<sup>3</sup>H]thymidine incorporation and cell counts and a 2.3-fold increase of cell migration in modified Boyden chambers. In balloon-injured vessels, endogenous kallistatin mRNA and protein levels increased up to 10-fold as determined by competitive polymerase chain reaction and by ELISA. Intense staining of kallistatin mRNA was identified in the proliferating VSMCs of balloon-injured arteries during cell migration from media to neointima by in situ hybridization histochemistry and immunohistochemistry. We observed an induction of kallistatin expression by platelet-derived growth factor (PDGF) and upregulation of p42/44 mitogen-activated protein kinase (MAPK) activity by kallistatin in cultured VSMCs. Conversely, adenovirus-mediated transfer of kallistatin antisense cDNA into cultured VSMCs inhibited PDGF-induced p42/44 MAPK activity and cell proliferation. Furthermore, local delivery of adenovirus carrying kallistatin antisense cDNA significantly downregulated kallistatin mRNA levels and attenuated neointima formation in balloon-injured rat arteries in vivo. These results indicate that kallistatin may play an important role in mediating PDGF-induced MAPK pathway on VSMC proliferation and in neointima formation after balloon angioplasty. (*Circ Res.* 2000;86:418-424.)

**Key Words:** kallistatin ■ vascular smooth muscle cell ■ neointima ■ proliferation ■ migration

Neointimal hyperplasia and restenosis are the major problems limiting the long-term efficacy of percutaneous transluminal coronary angioplasty.<sup>1,2</sup> The common characteristics of vascular responses to balloon injury are proliferation and migration of vascular smooth muscle cells (VSMCs) and neointima formation in the injured vessels. The latter is an important initial step in the progression of atherosclerotic lesions and restenosis.<sup>2,3</sup> Although the mechanisms responsible for the proliferation and migration of VSMCs are not fully understood, several factors produced in response to vascular injury have been implicated in this process. These include proto-oncogenes (*c-fos*, *c-jun*, and *c-myc*), mitogens such as basic fibroblast growth factor (bFGF) and interleukin-1, and growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor- $\beta_1$ .<sup>2,3</sup> PDGF is both mitogenic and chemotactic for medial VSMCs.<sup>2,4</sup> Denudation of endothelial cells after balloon angioplasty results in release of PDGF, and PDGF or other growth factors stimulate VSMC proliferation and migration into the intima resulting in intimal hyperplasia.<sup>2</sup>

Kallistatin is a serine proteinase inhibitor (serpin), which was first discovered as a tissue kallikrein binding protein

(KBP) and was capable of inhibiting the enzymatic activity of kallikrein.<sup>5</sup> The structure and organization of kallistatin gene are similar to those of other serpins, with typically 5 exons and 4 introns.<sup>6</sup> Two putative activator protein-1 (AP-1) binding sites and hormone response elements were identified in its 5'-flanking region.<sup>7</sup> The expression of kallistatin in rats was upregulated in the liver by estrogen, progesterone, growth hormone, and thyroxin<sup>7-9</sup> and was induced in crushed muscle tissues after injury.<sup>10</sup> In addition to its function as a proteinase inhibitor, kallistatin also has a potent vasodilatory effect on rat vasculature.<sup>11</sup> Kallistatin reduced mean arterial blood pressure in anesthetized rats and renal perfusion pressure in isolated rat kidneys and induced vasorelaxation in rat aortic rings.<sup>11</sup> Specific kallistatin-binding sites in the aortic membrane proteins were identified by a kallistatin-ligand binding assay.<sup>11</sup> Furthermore, kallistatin was localized in the endothelial and smooth muscle cells of human blood vessels of various sizes.<sup>12</sup> These findings suggest that kallistatin may play a role in the regulation of vascular function in autocrine and/or paracrine mechanisms. To explore the potential roles of kallistatin in vascular biology, we evaluated the effects of

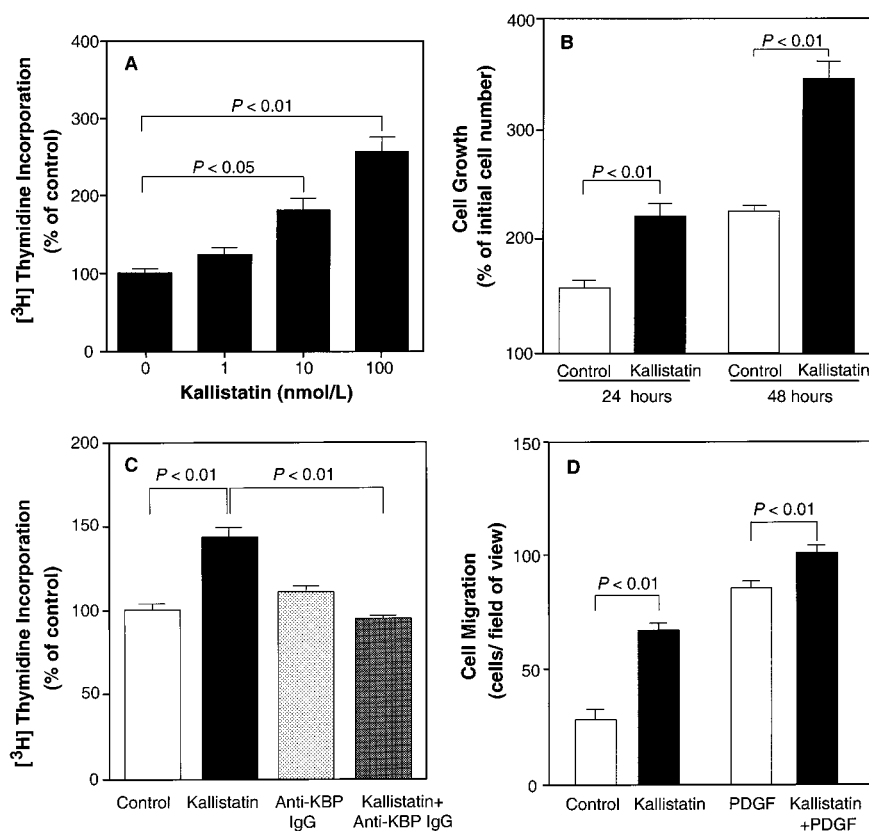
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**Figure 1.** A, Effects of rat kallistatin on [ $^3\text{H}$ ]thymidine incorporation in rat VSMCs. Cells were incubated with the indicated concentrations of kallistatin for 24 hours. DNA synthesis was measured as [ $^3\text{H}$ ]thymidine incorporation. Results are expressed relative to controls incubated in the absence of kallistatin. Values are mean  $\pm$  SEM (n=4). B, Effects of kallistatin on rat VSMC growth. Cells were incubated with 100 nmol/L kallistatin. Cell number was counted with a hemacytometer at different time points. The results are expressed relative to initial cell number before the treatment of kallistatin. Each value represents mean  $\pm$  SEM (n=4). C, Effects of rat kallistatin on [ $^3\text{H}$ ]thymidine incorporation in rat intimal smooth muscle cells. Rat intimal smooth muscle cells were isolated from the neointima induced by balloon angioplasty. Cells were incubated with kallistatin (100 nmol/L) alone or in combination with antibody against kallistatin (anti-KBP IgG; 11  $\mu\text{g}/\text{mL}$ ) for 24 hours. DNA synthesis was measured as [ $^3\text{H}$ ]thymidine incorporation. Results are expressed relative to controls incubated in the absence of kallistatin. Values are mean  $\pm$  SEM (n=4). D, Kallistatin stimulates rat VSMC migration in the absence or presence of PDGF-BB using modified Boyden chambers. Kallistatin, 1  $\mu\text{mol}/\text{L}$ ; PDGF-BB, 10 ng/mL. Average number of cells from 4 randomly chosen high-power ( $\times 400$ ) fields on the lower surface of the filter was counted. Each experiment was performed in triplicate, and 2 independent experiments were performed.

exogenous kallistatin on VSMC proliferation and migration in vitro as well as the expression and localization of endogenous kallistatin in balloon-injured rat arteries in vivo. The results of the present study provide new insights into the biological function of kallistatin in vascular cell growth and migration.

## Materials and Methods

### Animal Treatment

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) (300 to 400 g body weight) were used as previously described,<sup>13</sup> and all protocols conformed to institutional guidelines. For local gene delivery, the injured distal segment was temporally ligated after local balloon injury of the left common carotid artery. The adenovirus (Ad.CMV-AS.KBP) containing rat kallistatin antisense (AS) cDNA under control of cytomegalovirus (CMV) promoter/enhancer, or control virus (Ad.CMV-GFP) containing the green fluorescent protein (GFP) gene under control of CMV promoter/enhancer ( $4 \times 10^9$  plaque-forming units in 20  $\mu\text{L}$ ), was infused into the distal injured segment of the left common carotid artery and incubated for 15 minutes at room temperature. The cannula was then removed, and blood flow to the common carotid artery was restored. At the designated time, rats were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg). Left and right common carotid arteries or abdominal aortas were removed for RNA isolation, protein extraction, or morphometric analysis. For morphometric analysis, cross-section rings (4  $\mu\text{m}$ ) were cut from each paraffin segment and stained with hematoxylin and eosin. The slides were photographed at 100 $\times$  magnification with an Olympus microscope. The lumen, neointima, and media areas were traced and measured by using the NIH Image 1.61 software package.

### Preparation of Adenovirus Carrying Rat Kallistatin Antisense cDNA

Rat kallistatin cDNA<sup>7</sup> was cloned in antisense orientation into the adenoviral shuttle vector pAdTrack-CMV.<sup>14</sup> The resultant adenoviral plasmid carrying rat kallistatin antisense cDNA or GFP under control of CMV promoter/enhancer along with all Ad5 sequences except for the E1 and E3 genes was transfected into human embryonic kidney 293 packaging cells (Quantum, Quebec, Canada). Large quantities of high-titer adenoviruses were produced in human embryonic kidney 293 cells and purified by CsCl banding as previously described.<sup>15</sup>

### Primary Aortic Smooth Muscle Cell Culture

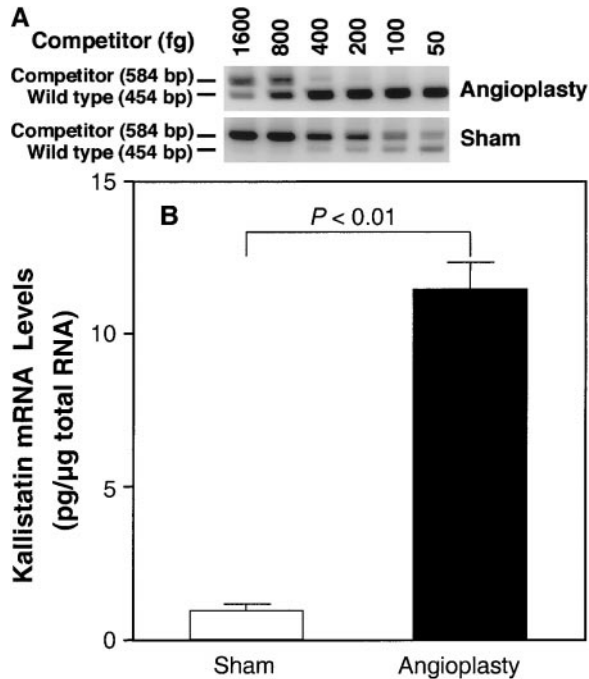
Rat primary VSMCs were isolated from the normal aorta of male Sprague-Dawley rats (200 to 250 g) by the combined collagenase and elastase digestion method.<sup>16</sup> Cells were serially passaged and used between passages 3 and 10. Intimal smooth muscle cells were isolated from neointima in injured aorta at 2 weeks after balloon angioplasty as described.<sup>17</sup>

### [ $^3\text{H}$ ]Thymidine Incorporation

Quiescent VSMCs in 24-well plates were treated with different concentrations of kallistatin in serum-free medium for 18 hours and then pulse-labeled with 1  $\mu\text{Ci}/\text{mL}$  of [ $^3\text{H}$ ]thymidine (DuPont NEN) for another 6 hours. Cells were then washed 3 times with PBS, precipitated with 10% trichloroacetic acid at 4 $^{\circ}\text{C}$  for 30 minutes, washed twice with 95% ethanol, solubilized with 0.25 mol/L NaOH plus 0.1% SDS, and neutralized with 1 mol/L acetic acid. Radioactivity was determined using a liquid scintillation counter (Packard).

### Cell Migration Assays

VSMC migration was assessed using modified Boyden chambers (Corning Inc),<sup>18,19</sup> which were coated with a solution of 5  $\mu\text{g}/\text{mL}$  fibronectin and 100  $\mu\text{g}/\text{mL}$  type I collagen (Sigma). VSMCs ( $2 \times 10^5$



**Figure 2.** Expression of kallistatin mRNA in sham and balloon-injured rat arteries. Total RNA was isolated from the abdominal aortas of 4 rats at 1 week after balloon angioplasty. Two of the rats had received angioplasty surgery, and 2 had been sham-operated. An aliquot of the reverse transcription product was coamplified with a serial dilution of the competitors for quantification of mRNA by competitive PCR. Amount of loading samples was normalized by using the  $\beta$ -actin housekeeping gene. Two independent experiments were performed. A, Representative competitive PCR image. B, Kallistatin mRNA levels calculated from the linear regression plot of the ratio plotted logarithmically against the initial input of competitor DNA. Values are mean  $\pm$  SEM.

cells) suspended in DMEM containing 0.1% BSA were added to the upper chamber, and tested samples were placed in the bottom chamber. After 4 hours of incubation at 37°C, cells were fixed and stained with hematoxylin and eosin. The average number of cells from 4 randomly chosen high-power ( $\times 400$ ) fields on the lower surface of the filter was counted.

### Statistical Analysis

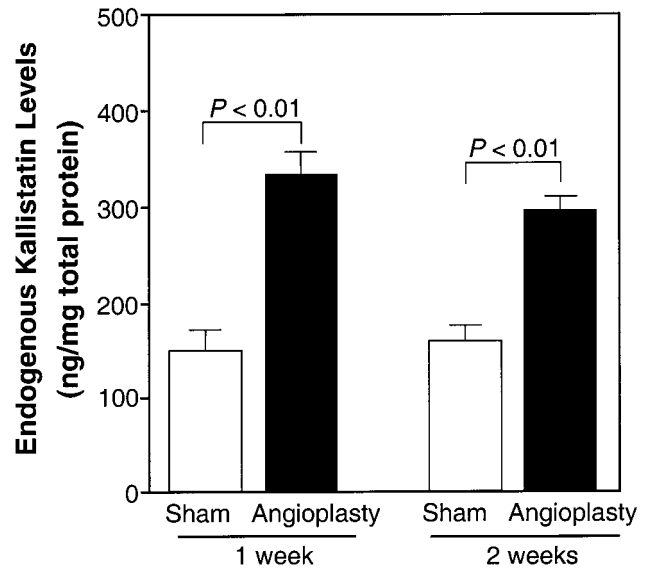
Statistical significance was determined by 1-way ANOVA with the Fisher multiple comparison test. All data are expressed as mean  $\pm$  SEM, and differences are considered significant at a value of  $P < 0.05$ .

An expanded Materials and Methods section is available online at <http://www.circresaha.org>.

## Results

### Effects of Kallistatin on the Proliferation and Migration of Cultured VSMCs

Figure 1 shows that purified kallistatin stimulated proliferation and growth of cultured primary rat VSMCs isolated from normal aortas, as assessed by [ $^3$ H]thymidine incorporation and cell counts. Kallistatin increased VSMC proliferation in a dose-dependent manner and at 100 nmol/L stimulated cell proliferation by 2.5-fold as compared with the control ( $n=4$ ,  $P < 0.01$ , Figure 1A). Kallistatin at 100 nmol/L increased cell growth by  $>2$ -fold in cell number as compared with the control ( $n=4$ ,  $P < 0.01$ , Figure 1B). Kallistatin at 100 nmol/L

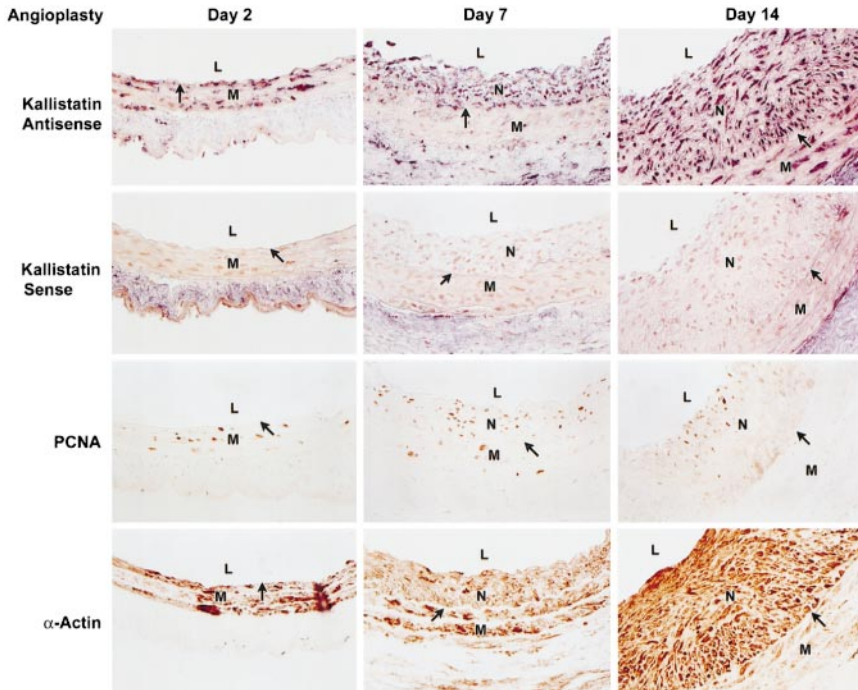


**Figure 3.** Kallistatin protein levels in sham and balloon-injured rat arteries. Total proteins were extracted from abdominal aortas of 8 rats at 1 or 2 weeks after balloon angioplasty. Five rats had received angioplasty surgery, and 3 had been sham-operated. Kallistatin protein levels were measured by an ELISA specific for rat kallistatin. Results are mean  $\pm$  SEM. Two independent experiments were performed.

also increased the proliferation of intimal smooth muscle cells by 1.4-fold ( $n=4$ ,  $P < 0.01$ , Figure 1C). The effect of kallistatin on intimal smooth muscle cell proliferation was blocked by a specific antibody against kallistatin, whereas the antibody alone had no effect on cell proliferation ( $n=4$ ,  $P < 0.01$ , Figure 1C). The effect of kallistatin on VSMC migration in the presence or absence of PDGF-BB was evaluated in modified Boyden chambers. In the absence of PDGF-BB, kallistatin at 1  $\mu$ mol/L stimulated VSMC migration by 2.3-fold ( $n=3$ ,  $P < 0.01$ , Figure 1D). Similarly, in the presence of PDGF-BB, kallistatin also significantly stimulated VSMC migration as compared with the control ( $n=3$ ,  $P < 0.01$ , Figure 1D).

### Induction of Endogenous Kallistatin Expression in Balloon-Injured Artery

The expression of endogenous kallistatin in rat aortas after balloon angioplasty was analyzed by competitive polymerase chain reaction (PCR) and ELISA specific for rat kallistatin. The representative competitive PCR image was shown in Figure 2A and kallistatin mRNA levels calculated from the linear regression plot of the ratio plotted logarithmically against the initial input of competitor DNA were shown in Figure 2B. At 1 week after balloon angioplasty, kallistatin mRNA levels in rat abdominal aortas increased up to 10-fold as compared with control sham-operated rats ( $11.43 \pm 1.05$  versus  $0.91 \pm 0.05$  pg/ $\mu$ g total RNA, Figure 2). Immunoreactive kallistatin levels in the aortas were increased by 2.3-fold and 1.9-fold, respectively, at 1 and 2 weeks after balloon angioplasty as compared with control sham-operated rats ( $n=5$  and 3,  $P < 0.01$ , Figure 3).



**Figure 4.** Localization of kallistatin mRNA and proliferating smooth muscle cell in balloon-injured rat arteries at different time points. Serial sections obtained from carotid arteries at 2, 7, and 14 days after balloon angioplasty were hybridized with kallistatin antisense or sense riboprobes (as negative control) and were immunolabeled with anti-PCNA or anti-smooth muscle  $\alpha$ -actin antibody ( $\alpha$ -actin). Positive in situ hybridization staining using nitroblue tetrazolium appears as purple. Positive PCNA and  $\alpha$ -actin immunostaining using 3,3'-diaminobenzidine appear as brownish-black. L indicates lumen; N, neointima; M, media; and arrow, internal elastic lamina. Original magnification,  $\times 100$ .

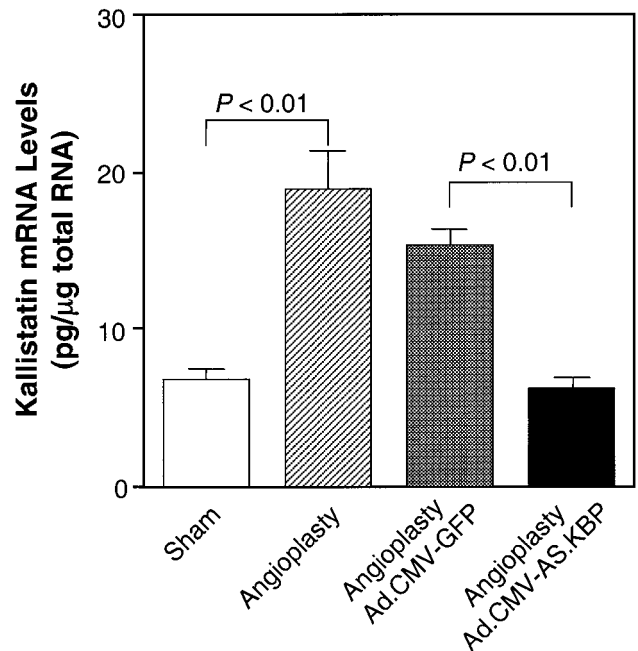
### Expression and Cellular Localization of Kallistatin in Rat Artery After Balloon Angioplasty

To further explore the role of rat kallistatin in neointima formation after balloon angioplasty, time-dependent expression and cellular localization of rat kallistatin mRNA in balloon-injured carotid arteries were identified by in situ hybridization using the kallistatin antisense riboprobe (Figure 4). Normal artery showed a very weak hybridization signal in the medial layer (data not shown). In the injured artery at 2 days after balloon angioplasty, a strong hybridization signal was noted in the media, particularly the inner layer. At 7 and 14 days after balloon angioplasty, intense staining of kallistatin mRNA was identified in the neointima with relatively low expression in the underlying media (Figure 4). Kallistatin mRNA was identified in the cytoplasm or around nuclei of the proliferating VSMCs. Only background levels of nonspecific hybridization signals were detected in the serial sections stained with the kallistatin sense riboprobe (Figure 4) or in the RNase A-pretreated sections stained with the kallistatin antisense riboprobe (data not shown). These negative results confirmed the specificity of in situ hybridization signals of these experiments. Proliferating cell nuclear antigen (PCNA) and smooth muscle  $\alpha$ -actin in the proliferating VSMCs of balloon-injured rat arteries were identified immunohistochemically using their respective antibodies (Figure 4). The results show that the site of kallistatin expression was spatially and temporally colocalized with PCNA and  $\alpha$ -actin in serial sections.

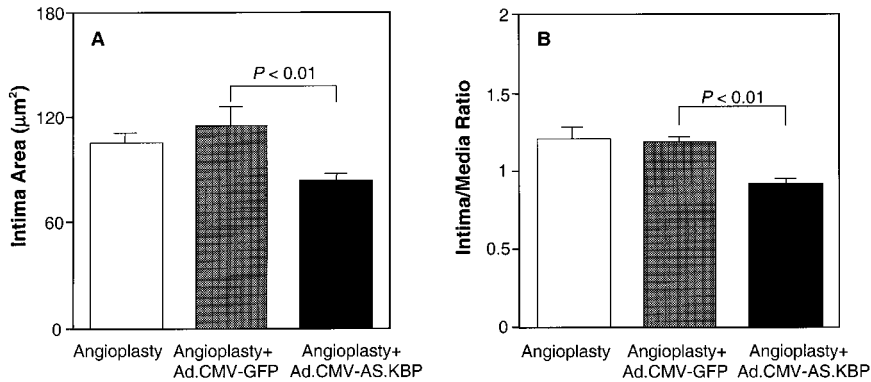
### Adenovirus-Mediated Kallistatin Antisense cDNA Delivery Inhibited Kallistatin mRNA Expression and Neointima Formation in the Balloon-Injured Artery

To further investigate the role of kallistatin in neointima formation in vivo, adenovirus Ad.CMV-AS.KBP or control

virus Ad.CMV-GFP was delivered locally into the balloon-injured rat carotid arteries. Competitive PCR showed that adenovirus-mediated delivery of kallistatin antisense cDNA significantly reduced kallistatin mRNA levels to those of sham rats ( $n=4$ ,  $P<0.01$ , Figure 5). Reduction of kallistatin expression by antisense inhibition was accompanied by significant suppression of neointima formation in balloon-



**Figure 5.** Effects of adenovirus-mediated kallistatin antisense cDNA delivery on expression of kallistatin mRNA in balloon-injured rat arteries. An aliquot of the reverse transcription product was coamplified with a serial dilution of the competitors for quantification of mRNA by competitive PCR. Amount of loading samples was normalized by using the  $\beta$ -actin housekeeping gene. Values are mean  $\pm$  SEM ( $n=4$ ).



**Figure 6.** Morphometric analyses of intima area (A) and intima/media area ratio (B) in rat carotid arteries after balloon angioplasty. Intima and media areas were measured in histological sections of vessels at 2 weeks after balloon angioplasty. Angioplasty, balloon-injured rats (n=6); Ad.CMV-GFP, balloon-injured rats receiving adenovirus carrying GFP gene (n=4); Ad.CMV-AS.KBP, balloon-injured rats receiving adenovirus carrying rat kallistatin antisense cDNA (n=7). Results are mean  $\pm$  SEM.

injured arteries when compared with that in those arteries infected with control virus (cross-sectional area:  $83 \pm 5$  [n=7] versus  $115 \pm 11 \mu\text{m}^2$  [n=4], mean  $\pm$  SEM,  $P < 0.01$ , Figure 6A). There was a 25% reduction in intima/media ratio in rats receiving kallistatin antisense cDNA delivery as compared with rats receiving or not receiving control virus at 2 weeks after balloon angioplasty ( $0.90 \pm 0.04$  [n=7] versus  $1.17 \pm 0.05$  [n=4] or  $1.20 \pm 0.08$  [n=6], mean  $\pm$  SEM,  $P < 0.01$ , Figure 6B). No statistical difference was found between injured rat carotid arteries after angioplasty with or without control virus infection.

#### PDGF-BB Increased Endogenous Kallistatin Synthesis and Kallistatin Antisense cDNA Inhibited PDGF-BB-Induced Cell Proliferation and p42/44 Mitogen-Activated Protein Kinase (MAPK) Activity in Cultured VSMCs

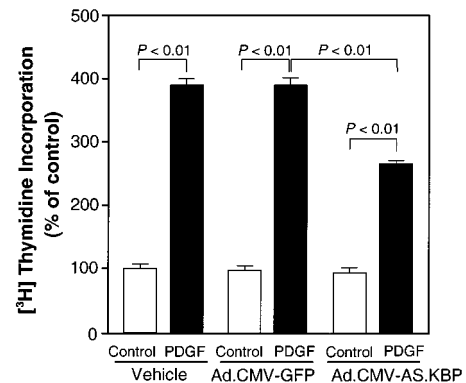
PDGF-BB markedly induced endogenous kallistatin synthesis in cultured VSMCs as compared with the control ( $304 \pm 23$  versus  $57 \pm 13 \text{ pg/mg}$  total protein [n=3],  $P < 0.01$ ). To confirm the possibility that kallistatin may be involved in the PDGF-induced MAPK pathway on the proliferation of VSMCs, cell proliferation and MAPK activity in VSMCs with or without infection of Ad.CMV-AS.KBP or Ad.CMV-GFP were examined after treatment of PDGF-BB or kallistatin. Figure 7 shows that PDGF-BB stimulated proliferation of VSMCs with or without control virus infection, whereas adenovirus-mediated transfer of kallistatin antisense cDNA attenuated PDGF-BB-induced VSMC proliferation as compared with the control (n=4,  $P < 0.01$ ). Furthermore, Figure 8 shows that kallistatin and PDGF-BB increased MAPK activity by 2- and 3-fold, respectively, compared with the control basal level, whereas kallistatin antisense cDNA inhibited 35% of PDGF-BB-induced MAPK activity (n=3,  $P < 0.01$ ). No significant downregulation of MAPK activity was observed in VSMCs treated with PDGF-BB and infected with control virus. Specific MAPK induced by kallistatin in VSMCs was further identified by Western blot analysis using phosphospecific MAPK antibodies. Activation of p42/44 MAPK, but neither p38 kinase nor stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), was induced in VSMCs treated with kallistatin. Kallistatin increased phosphorylation of p42/44 MAPK compared with the control, and kallistatin antisense cDNA inhibited PDGF-BB-induced phosphorylation of p42/44 MAPK (Figure 9A). The levels of

total p42/44 MAPK were identical in all of the samples (Figure 9B).

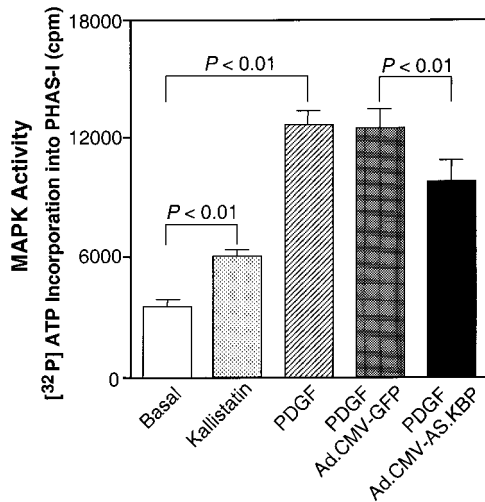
### Discussion

This is the first study to demonstrate that kallistatin plays a role in vascular injury and restenosis. The expression of endogenous kallistatin increased markedly in balloon-injured blood vessels. The site of expression was spatially and temporally colocalized with PCNA and  $\alpha$ -actin in proliferating VSMCs during cell migration from media to neointima. Inhibition of kallistatin expression by its antisense cDNA significantly suppressed neointima formation in the balloon-injured artery in vivo. The potential role of kallistatin as a growth factor or a mediator of growth factors in neointima formation was further confirmed by in vitro studies. Exogenous kallistatin significantly stimulated the proliferation and migration of cultured primary VSMCs, and antisense inhibition of kallistatin expression attenuated PDGF-induced p42/44 MAPK activity and proliferation in VSMCs. These results indicate that kallistatin in proliferative VSMCs may function as a mediator of growth factors in the pathogenesis of vascular injury.

In this study, we show that both mRNA and protein levels of endogenous kallistatin were markedly increased at the



**Figure 7.** Effects of adenovirus-mediated kallistatin antisense cDNA transfer on PDGF-induced VSMC proliferation. VSMCs were transiently infected with adenovirus (Ad.CMV-AS.KBP or control virus Ad.CMV-GFP) (20 plaque-forming units/cell) in DMEM for 6 hours. At 48 hours after infection, cells were incubated with or without PDGF-BB (15 ng/mL) for another 24 hours. DNA synthesis was measured as [ $^3\text{H}$ ]thymidine incorporation. Results are expressed relative to controls incubated in the absence of PDGF and without virus infection. Values are mean  $\pm$  SEM (n=4).



**Figure 8.** Effects of adenovirus-mediated kallistatin antisense cDNA transfer on MAPK activity in VSMCs. Cell lysate (40  $\mu$ g), prepared after incubating VSMCs for 10 minutes at 37°C with no addition (control) or with 1  $\mu$ mol/L kallistatin or 20 ng/mL PDGF-BB in the presence or absence of infection with Ad.CMV-AS.KBP or control virus Ad.CMV-GFP, was used for the phosphorylation of phosphorylated heat- and acid-stable protein regulated by insulin (PHAS-I; Stratagene, La Jolla, Calif) in vitro. Data represent mean  $\pm$  SEM (n=3).

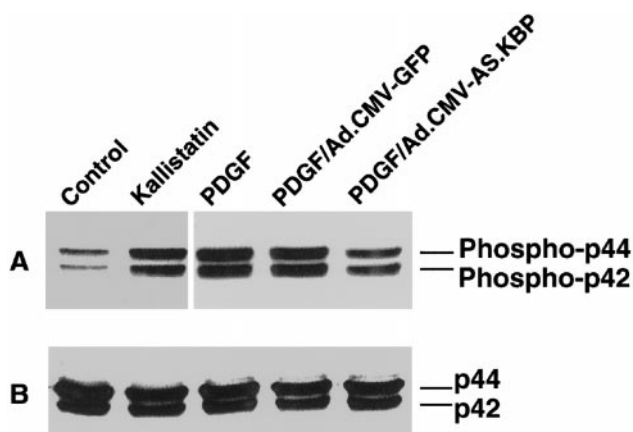
injured sites after balloon angioplasty. This indicates that kallistatin is upregulated at the transcriptional level in the injured vessels. The elevated expression of kallistatin mRNA in the proliferating VSMCs during the process of neointima formation was also identified by in situ hybridization histochemistry. In response to mitogens and growth factors stimulated by vascular injuries, medial VSMCs may enter into the growth cycle between 2 and 3 days after balloon angioplasty. The majority of medial cells would complete their proliferation and migration within 7 days after angioplasty, whereas

the neointima area would show a dramatic increase from 7 to 14 days. No significant growth of neointima occurs beyond 14 days after balloon angioplasty in rats.<sup>2,20</sup> The intimal cell proliferation contributes considerably to the subsequent accumulation of neointima mass.<sup>20</sup> Our results show that the sites of kallistatin expression were localized in the media at 2 days after angioplasty and in neointima at 7 and 14 days after angioplasty during the migration of proliferative VSMCs from media to neointima. The time and spatial coordination between kallistatin expression and cellular proliferation suggests that kallistatin may play the important role of autocrine growth factors in mediating VSMC proliferation and migration and in neointima formation after balloon angioplasty.

Unlike other mitogens, such as PDGF, bFGF, and epidermal growth factor,<sup>17</sup> kallistatin not only stimulated medial VSMC proliferation but also had mitogenic effects on intimal smooth muscle cell proliferation. A previous study showed that  $\approx$ 50% of medial VSMCs activated by balloon injury migrated and underwent division. These cells constituted eight ninths of the final neointimal cell population, whereas the other 50% migrated without further proliferation and made up one ninth of the neointimal cell population.<sup>20</sup> These results suggest that balloon injury stimulates a proportion of the medial VSMCs to enter the growth cycle, and proliferation of intimal smooth muscle cells accounts for most of intimal accumulation of VSMCs. We isolated intimal smooth muscle cells from neointima at 2 weeks after balloon angioplasty and showed that kallistatin stimulated the proliferation of these cells. The stimulatory activity of kallistatin on cell proliferation is specific because it was neutralized by its specific antibody. Taken together, these results suggest that kallistatin could also play an important role in intimal smooth muscle cell proliferation and accumulation.

Our studies show that PDGF stimulated the expression of endogenous kallistatin in cultured VSMCs. PDGF is one of the crucial growth factors induced by vascular injury, and it has both mitogenic and chemotactic activities on medial VSMCs during intimal hyperplasia.<sup>2,4</sup> However, PDGF alone can not optimally stimulate cell proliferation. It requires a second group of growth factors, termed "progression factors," to initiate DNA synthesis and cell division.<sup>4,21,22</sup> A number of progression factors, such as bFGF,<sup>23</sup> epidermal growth factor,<sup>24</sup> and osteopontin,<sup>25</sup> have been identified for intimal hyperplasia. Our results indicate that the expression of kallistatin is spatially and temporally colocalized with proliferating VSMCs of balloon-injured arteries in vivo, and kallistatin can stimulate the proliferation and migration of VSMCs independent of PDGF in vitro. These results suggest that kallistatin may function as an autocrine progression growth factor in response to vascular injury.

Using kallistatin antisense strategy, we demonstrate a potential role of kallistatin in mediating the PDGF-induced MAPK pathway resulting in proliferation of VSMCs. Our results show that PDGF induced kallistatin expression in cultured VSMCs. Moreover, kallistatin significantly increased p42/44 MAPK activity and stimulated VSMC proliferation in the absence of PDGF. MAPK-mediated cell proliferation is one of the major pathways for the regulation of PDGF-induced VSMC proliferation and growth.<sup>4</sup> Activation



**Figure 9.** Effects of adenovirus-mediated kallistatin antisense cDNA transfer on the phosphorylation of p42/44 MAPK in VSMCs. Cell lysate (20  $\mu$ g), prepared after incubating VSMCs for 10 minutes at 37°C with no addition (control) or with 1  $\mu$ mol/L kallistatin or 20 ng/mL PDGF-BB in the presence or absence of infection with Ad.CMV-AS.KBP or control virus Ad.CMV-GFP, was used in Western blot analysis. A, Western blot analysis of phosphorylated p42/44 MAPK (exposure times: left panel, 90 seconds; right panel, 30 seconds). B, Western blot analysis of total p42/44 MAPK (exposure time: 30 seconds).

of MAPK induces expression of proto-oncogenes *c-fos* and *c-jun*, which form transcription factors such as AP-1. Functional AP-1 further initiates the transcription of cyclins cell cycle-regulatory genes, and cyclins stimulate cell proliferation.<sup>26,27</sup> We observed that adenovirus-mediated kallistatin antisense cDNA delivery inhibited PDGF-induced p42/44 MAPK activity and cell proliferation in cultured VSMCs in vitro. Moreover, kallistatin antisense cDNA also suppressed neointima formation in balloon-injured arteries in vivo. Collectively, these results indicate that kallistatin may participate in mediating PDGF-induced p42/44 MAPK pathway. The detailed mechanisms by which kallistatin mediates PDGF-induced p42/44 MAPK pathway remain to be elucidated.

In summary, our results indicate that inhibition of endogenous kallistatin expression in injured blood vessels may have protective effects on neointima formation by inhibiting VSMC proliferation and migration. This study suggests that kallistatin may serve as a new potential therapeutic target for neointimal hyperplasia and restenosis after angioplasty.

### Acknowledgments

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