Kallikrein Gene Delivery Inhibits Vascular Smooth Muscle Cell Growth and Neointima Formation in the Rat Artery After Balloon Angioplasty

Hideyuki Murakami, Katsutoshi Yayama, Robert Q. Miao, Cindy Wang, Lee Chao, Julie Chao

Abstract—Tissue kallikrein cleaves kininogen substrate to produce vasoactive kinin peptides that have been implicated in the proliferation of vascular smooth muscle cells (VSMCs). To explore potential roles of the kallikrein-kinin system in vascular biology, we evaluated the effects of adenovirus-mediated human kallikrein gene delivery on the growth of primary cultured VSMCs and in balloon-injured rat artery in vivo. Kallikrein gene transfer into cultured rat VSMCs resulted in time-dependent secretion of recombinant human tissue kallikrein and inhibition of cell proliferation. Balloon angioplasty reduced endogenous rat tissue kallikrein mRNA and protein levels at the injured site. In rats that received adenovirus-mediated human kallikrein gene delivery, we observed a 39% reduction in intima/media ratio at the injured vessel after delivery compared with that of rats that received control virus (n=8, P<0.01). Icatibant, a specific bradykinin B2 receptor antagonist, blocked the protective effect and reversed the intima/media ratio to that of the control rats (n=5, P<0.01). After gene delivery, human kallikrein mRNA was identified at the injured vessel and a 3-fold increase occurred in kininogenase activity. cAMP and cGMP levels in balloon-injured aorta increased significantly at 4, 7, and 14 days after kallikrein gene delivery, but icatibant abolished the increase. These results provide new insights into the role of the vascular kallikrein-kinin system and have significant implications for gene therapy to treat restenosis or atherosclerosis. (Hypertension. 1999;34:164-170.)

Key Words: angioplasty, balloon genes kallikrein arteries, rat

Injury to the arterial wall induces the synthesis of gene products that stimulate smooth muscle cell migration and proliferation, which leads to intimal hyperplasia.1 This process contributes to the pathogenesis of several cardiovascular disorders, including atherosclerosis and restenosis after angioplasty. Although the cellular mechanisms of restenosis are not fully understood, the process appears to involve proliferation and migration of smooth muscle cells from the media to the intima in response to mitogens and growth factors.2 A number of growth factors, including fibroblast growth factor, insulin-like growth factor, transforming growth factor, platelet-derived growth factor, and angiotensin II (Ang II), are released at the site of vascular injury, which indicates the significance of these mitogenic and chemotactic stimuli in vascular proliferation.3 Ang II exhibits mitogenic activity in vascular smooth muscle cell (VSMC) growth and proliferation.4 Angiotensin-converting enzyme (ACE) inhibitor suppresses neointima formation after endothelial injury in rat carotid artery and abdominal aorta.5 Specific Ang II receptor antagonists can also partially inhibit neointima formation, which suggests that the inhibition of neointima formation by ACE inhibitors is, in part, because of a decreased level of Ang II.6 Inhibition of ACE activity not only prevents formation of Ang II but also increases kinin levels by preventing kinin degradation. The increase in local kinin accumulation may also be involved in the inhibition of neointima formation because icatibant, a bradykinin B2 receptor antagonist, can partially block the protective effect of ACE inhibitors.7 Therefore, the beneficial effects of ACE inhibition may also be attributed to increased kinin levels. These studies suggest a protective role of the vascular kallikrein-kinin system in arterial thickening after vascular injury.

Abnormality of the tissue kallikrein-kinin system has been implicated in the pathogenesis of hypertension and cardiovascular and renal disorders.8,9 Tissue kallikrein cleaves low-molecular-weight kininogen to produce vasoactive kinin peptides. Intact kinin binds to bradykinin B2 receptor in target tissues and exerts a broad spectrum of biological effects including vasodilation, blood pressure (BP) reduction, smooth muscle relaxation and contraction, pain induction, and inflammation.10 We have recently shown that systemic delivery of the tissue kallikrein gene attenuates the development of hypertension and cardiac hypertrophy and enhances renal function in several hypertensive animal models.11–13 Cellular localization studies have identified tissue kallikrein expression in endothelial and smooth muscle cells of human
blood vessels. However, the functional role of the tissue kallikrein-kinin system in vascular physiology has not been fully established. To understand the role of tissue kallikrein in vascular biology, we analyzed the effects of kallikrein gene transfer on the growth of VSMCs in vitro and the expression of endogenous tissue kallikrein-kinin system components in balloon-injured rat vessels. To explore further the potential beneficial effects of tissue kallikrein on neointima formation after vascular injury, adenovirus carrying the human tissue kallikrein gene was delivered into rat blood vessels after balloon angioplasty. We showed that both local kallikrein and adenovirus were infused into the distal injured segment and incubated for 15 minutes at room temperature. After incubation, the cannula was removed and blood flow to the common carotid artery was restored. To investigate the potential kinin-mediated effect after kallikrein gene delivery, icatibant (Hoe 140), a specific antagonist for B2 receptor, was infused intraperitoneally at a rate of 70 μg · kg⁻¹ · d⁻¹ by use of osmotic minipumps (Alzet 2 ML2, Alza Corpor) immediately after the balloon angioplasty and Ad.CMV-cHK infusion. At 4, 7, and 14 days after gene delivery, rats were anesthetized and perfused with saline during the ascending aorta. Arterial and other tissues were isolated for RNA extraction or morphometric analysis.

**Systemic Gene Delivery**

Male Sprague-Dawley rats (weight, 250 to 300 g) were anesthetized with sodium pentobarbital (50 mg/kg IP), and a 2F embolectomy balloon catheter was passed into the aorta by way of the femoral artery and placed distal to the renal artery. The balloon was inflated with sufficient saline and withdrawn slowly to the aortic bifurcation. This procedure was repeated 3 times. After the surgery, rats were injected with $2 \times 10^{10}$ pfu of Ad.CMV-cHK or control virus Ad.CMV-LacZ through the tail vein. Icatibant was infused intraperitoneally at a rate of 70 μg · kg⁻¹ · d⁻¹ by osmotic minipumps (Alzet 2002, Alza Corp) immediately after balloon angioplasty and gene delivery. At 4, 7, and 14 days after gene delivery, rats were anesthetized, and BP and heart rate (HR) were measured directly by cannulating the right carotid artery. Rats were then perfused and the aorta and other tissues were isolated for protein measurements, kininogenase assay, and RNA extraction.

**Enzymatic Assays Toward Low-Molecular-Weight Kininogen Substrate**

Canine low-molecular-weight kininogen was isolated according to the method described by Johnson et al. Kinin-releasing activities were measured by incubating rat aortic extracts (10 μg) with canine low-molecular-weight kininogen (3 μg) in 0.1 mol/L sodium phosphate (pH 8.5) in a total volume of 500 μL at 37°C for 30 minutes. The reactions were stopped by boiling for 20 minutes. Released kinin was assayed by a kinin radioimmunoassay, and total protein concentration was measured by the method of Lowry. Kininogenase activity is expressed as nanograms kinin released per milligram protein per 30 minutes.

**Reverse Transcription-Polymerase Chain Reaction Southern Blot Analysis**

Total RNA was extracted with Trizol reagent according to the protocol recommended by the manufacturer (BRL). Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) Southern blot analyses were used to determine the abundance of B2 receptors, rat tissue kallikrein, high- and low-molecular-weight kininogens, and T-kininogen mRNAs in nontreated, sham-operated, and injured carotid artery or abdominal aorta of rats after angioplasty to determine the expression of human tissue kallikrein in rat tissues after adenovirus-mediated gene delivery. Table 1 shows specific 5' primers and 3' primers used for RT-PCR and specific internal oligonucleotide probes for Southern blot analyses. Signals were detected by autoradiography at ~ 80°C and scanned into Adobe Photoshop 2.5 with the Hewlett Packard Scan Jet IICX/T.

**Assays for cAMP and cGMP Levels**

At 4, 7, and 14 days after angioplasty and gene delivery, rats were anesthetized and the abdominal aorta was dissected, homogenized (Polytron; Brinkmann Instruments) in 10 vol of 0.1N HCl at 4°C, and centrifuged at 15 000g for 30 minutes. cAMP and cGMP levels were measured in the supernatants by radioimmunoassay, and protein concentrations were determined by the method of Lowry as previously described.

**Morphometric Analysis**

Two weeks after gene delivery, rats were anesthetized and the left and right carotid artery were removed and embedded in paraffin. Each artery was divided into 3 segments that were separately...
embedded in paraffin. Cross-sectional rings (4 μm) were cut from each segment and stained with hematoxylin and eosin. The slides were photographed with a microscope at a magnification of ×100. The lumen, neointima, media areas were measured by use of the NIH Image 1.60 software package.

Statistical Analysis

Group data are expressed as mean±SEM. Data were compared between experimental groups by 1-way ANOVA. Differences between kallikrein and control groups were further evaluated by Fisher’s protected least-squares differences. Differences were considered significant at a value of *P*<0.05.

Results

Inhibition of Growth of Primary Cultured VSMCs by Adenovirus-Mediated Human Kallikrein Gene Transfer

Expression of recombinant human tissue kallikrein in primary cultured VSMCs was measured from 1 to 6 days after infection with Ad.CMV-chK. Recombinant human tissue kallikrein achieved the highest level, 310±39 ng/mL (n=3), at 2 days after gene transfer and decreased to 135±8 ng/mL (n=3) at 6 days after gene delivery (Figure 1A). Human tissue kallikrein was not detected in the culture media of VSMCs after infection with Ad.CMV-LacZ (data not shown). Expression of the human tissue kallikrein transgene in VSMCs caused a significant inhibition in cell proliferation as measured by [3H] thymidine incorporation into DNA at 4 days after gene transfer (Figure 1B). The growth rate of VSMCs transfected with Ad.CMV-chK (2568±198 cpm per well) was reduced to 59% of that of control cells with or without transfection with Ad.CMV-LacZ (4745±329 or 4343±120 cpm per well, respectively, n=4, *P*<0.01).

Differential Expression of Vascular Tissue Kallikrein-Kinin System Components After Balloon Angioplasty

The expression of endogenous tissue kallikrein-kinin system components in rat blood vessels was analyzed with RT-PCR followed by Southern blot analysis using 3 gene-specific oligonucleotides for each transcript (Table 1). Figure 2 shows the transcripts of endogenous tissue kallikrein, B2 receptors, and kininogens in rat carotid artery (Figure 2A) and abdominal aorta (Figure 2B) after balloon angioplasty. At 1 and 2 weeks after angioplasty by way of the common carotid artery, the relative level of rat tissue kallikrein mRNA was markedly reduced versus control sham-operated rats. No changes in high- or low-molecular-weight kininogens, T-kininogen, and bradykinin B2 receptor mRNA expression were observed in the injured carotid artery. Similar levels of β-actin were detected in both the sham-operated and angioplasty groups, which indicates that the RNA quality of these samples is internally consistent (Figure 2A). A similar pattern in the differential expression of rat tissue kallikrein, B2 receptors, and kininogens was observed in rat abdominal aorta after balloon angioplasty through the femoral artery (Figure 2B).

<table>
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<tr>
<th>TABLE 1. Gene-Specific Primers and Probes for Rat Kallikrein-Kinin Systems and Human Tissue Kallikrein in RT-PCR Southern Blot Analysis</th>
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<td>Human tissue kallikrein</td>
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Figure 1. A. Time-dependent expression of recombinant human tissue kallikrein in primary cultured VSMCs after adenovirus-mediated gene transfer. B. Effect of adenovirus-mediated kallikrein gene transfer on [3H] thymidine incorporation into DNA in VSMCs. The results are expressed as mean±SEM (n=4).

Figure 2. Differential expression of rat tissue kallikrein, B2 receptors, and kininogens in rat carotid artery (A) and abdominal aorta (B) after balloon angioplasty.
(218.05±18.87 versus 372.46±39.41 ng kinin released per milligram protein per 30 minutes, mean±SEM, n=8, P<0.01, Figure 3). Decreased kininogenase activity is consistent with reduction of endogenous rat tissue kallikrein mRNA at the injured vessels (Figure 2). Rats that received adenovirus-mediated kallikrein gene delivery had a 3-fold greater increase in kinin-releasing activity in aorta after balloon angioplasty than control rats that received Ad.CMV-LacZ (717.00±83.98 versus 249.64±31.01 ng kinin released per milligram protein per 30 minutes, mean±SEM, n=5, P<0.01) (Figure 3).

Expression of Human Tissue Kallikrein mRNA in Balloon-Injured Rat Artery After Gene Delivery

At 4 days after local administration of Ad.CMV-cHK into the balloon-injured left common carotid artery, human tissue kallikrein mRNA was detected only in the injured left carotid artery but not in the control right carotid artery, aorta, heart, liver, or kidney (Figure 4A). At 4 days after systemic delivery of Ad.CMV-cHK by way of the tail vein, human tissue kallikrein mRNA was detected in the aorta, and in the liver, kidney, and heart (Figure 4B). Human tissue kallikrein mRNA was not detected in rats receiving Ad.CMV-LacZ (Figure 4). Similar levels of β-actin were detected in both experimental and control groups, which indicates the integrity of RNA in these samples (Figure 4).

Adenovirus-Mediated Kallikrein Gene Transfer Inhibited Neointima Formation

Figure 5 shows typical morphology of artery segments 14 days after local angioplasty and tissue kallikrein gene delivery. Carotid artery of sham-operated rats showed normal morphology (Figure 5A), whereas angioplasty caused neointima formation and narrowing of the lumen area (Figure 5B). Kallikrein gene delivery significantly reduced thickening of the arterial wall (Figure 5C), whereas icatibant treatment abolished the protective effect of kallikrein (Figure 5D). Figure 6 shows morphometric analyses of the intima area and intima/media ratio in carotid artery after angioplasty. Rats that received adenovirus-mediated kallikrein gene delivery had significantly suppressed neointima formation in carotid artery versus control rats that received Ad.CMV-LacZ (cross-sectional area, 85.9±7.1 versus 129.5±10.4 μm², mean±SEM, n=8, P<0.01, Figure 6A). A 39% reduction in intima/media ratio was found in rats receiving kallikrein gene delivery versus rats receiving control virus (0.80±0.06 versus 1.32±0.10, mean±SEM, n=8, P<0.01, Figure 6B). Suppression of neointima formation and intima/media ratio after kallikrein gene delivery was significantly blocked by icatibant (85.9±7.1 versus 130.5±6.5 μm², n=5, P<0.01, Figure 6A; 0.80±0.06 versus 1.26±0.05, mean±SEM, n=5, P<0.01, Figure 6B). No statistical differences in neointima and intima/media ratio were found between the control groups that did or did not receive Ad.CMV-LacZ and the group that received Ad.CMV-cHK with icatibant infusion (Figure 6A and 6B). No significant difference in the media area was found among these groups (Figure 6C). Lumen area in the Ad.CMV-cHK group was significantly higher than in the other groups (Figure 6D). These results indicated that reduced neointima area was not attributable to remodeling by reduction of diameter of the vessel.

Similarly, kallikrein gene delivery also reduced neointima formation after systemic gene delivery. Morphometric analysis indicated that neointima formation in rat aorta was significantly reduced versus that in control rats (cross-sectional area=92.1±3.8 versus 135.0±5.5 μm² [mean±SEM], n=7, P<0.01). A 35% reduction in intima/
media ratio was noted in rats receiving kallikrein gene delivery versus rats receiving Ad.CMV-LacZ (0.88 ± 0.03 versus 1.34 ± 0.06, mean ± SEM, n = 8, *P* < 0.01). No statistical difference was found between injured abdominal aorta after angioplasty with or without infection with Ad.CMV-LacZ.

**Effects of Kallikrein Gene Delivery on cGMP and cAMP Levels in Aorta, BP and HR**

Table 2 summarizes the time course of cGMP and cAMP levels in rat aortic extracts and rat BP and HR after balloon angioplasty and gene delivery. Both cGMP and cAMP levels increased significantly in the group receiving the adenovirus containing the kallikrein gene versus the group injected with the control virus, but icatibant abolished the increase in cAMP and cGMP levels at 4, 7, and 14 days after gene delivery. No significant differences in BP and HR were found among all the groups throughout the study.

**Discussion**

The present study is the first to demonstrate that a continuous supply of tissue kallikrein by way of gene transfer suppresses neointima formation in balloon-injured artery. Balloon angioplasty resulted in decreased endogenous rat tissue kallikrein expression. Expression of recombinant human tissue kallikrein at the injured site results in significant reduction of intima/media ratio and neointima formation, and time-dependent increases of cAMP and cGMP levels. Icatibant, a bradykinin B₂ receptor antagonist, abolished both the protected effect of kallikrein on arterial thickening and the increases in cAMP and cGMP levels. Kallikrein gene transfer also suppresses the proliferation of VSMCs in vitro. These results suggest that inhibition of neointima formation in injured artery after kallikrein gene delivery is mediated by kinin with subsequent activation of second messengers such as cAMP and cGMP in blood vessels. These findings provide important insights into the role of the vascular kallikrein-kinin system in occlusive vascular diseases such as atherosclerosis or restenosis.

Our present study supports a role for the local vascular kallikrein-kinin system. Components of this system are present within the blood vessel wall. We showed differential expression of tissue kallikrein-kinin components in rat artery...
reached a peak level site (Figure 3). Proliferation and migration of VSMCs consistent with reduced kininogenase activity at the injured vascular kallikrein mRNA levels after balloon angioplasty are kallikrein gene expression (Figure 2). Moreover, decreased Southern blot analysis. These results showed that both local after local and systemic balloon angioplasty by RT-PCR.

![Figure 6](https://hyper.ahajournals.org/)

Figure 6. Morphometric analyses of A, intima area; B, intima/media area ratio; C, media area; and D, lumen area in carotid artery 2 weeks after balloon angioplasty. Angioplasty, (n = 8); Ad.CMV-LacZ (n = 8); Ad.CMV-cHK (n = 8); Ad.CMV-cHK and icatibant (n = 5). The results are expressed as mean ± SEM.

after local and systemic balloon angioplasty by RT-PCR. Southern blot analysis. These results showed that both local and systemic angioplasty resulted in suppression of tissue kallikrein gene expression (Figure 2). Moreover, decreased vascular kallikrein mRNA levels after balloon angioplasty are consistent with reduced kininogenase activity at the injured site (Figure 3). Proliferation and migration of VSMCs reached a peak level 1 week after angioplasty and completed neointima formation at 2 weeks. Decreased endogenous tissue kallikrein levels during this time period after angioplasty suggested that vascular kallikrein may in part be responsible for suppressing proliferation and migration of VSMCs. This idea is supported by a previous report that kinin attenuated the proliferation of cultured VSMCs and fibroblasts.23 In the present study, we also found that infection of cultured VSMCs with adenovirus containing the kallikrein gene resulted in expression of recombinant human tissue kallikrein and inhibition of vascular cell proliferation (Figure 1). Furthermore, our preliminary results showed that purified tissue kallikrein inhibited vascular cell growth and icatibant abolished the inhibitory effect of kallikrein (data not shown). Together, these findings indicate that inhibition of smooth muscle proliferation after kallikrein gene transfer is mediated by kinin.

In the present study, we showed that recombinant human tissue kallikrein can be processed in VSMCs. Adenovirus carrying the entire coding sequence of the human tissue kallikrein gene under the control of the cytomegalovirus enhancer/promoter was transduced into cultured VSMCs in vitro and in rat artery in vivo. We can measure time-dependent expression of recombinant human tissue kallikrein in the secreted media of primary cultured rat VSMCs by an ELISA specific for human kallikrein. The immunoreactive human tissue kallikrein detected in the media is in an active form, because our antiserum to human tissue kallikrein only recognizes active kallikrein, not latent kallikrein.24 Previous studies reported that 20% to 30% of the vascular kallikrein in the rat is present in the active form.25,26 These combined results indicated that VSMCs contain a kallikrein-activating mechanism. However, the ratio of active versus inactive forms of recombinant human tissue kallikrein expressed in rat aorta after gene transfer remains to be evaluated.

Our results indicate that the inhibitory effect of cell proliferation after kallikrein gene delivery is in part mediated by the kinin-cAMP/kinin-cGMP signal pathways. Kinin has been shown to increase cAMP levels as well as to inhibit cell proliferation in cultured aortic smooth muscle cells.27 Binding of kinin to bradykinin B1 receptor stimulates phospholipase A2 with increased prostacyclin formation. Prostacyclin activates adenylate cyclase, which results in increased cAMP.

<table>
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Data were analyzed by ANOVA. Values for each group were expressed as mean±SEM (n = 6 to 8).

*P<0.01, Ad.CMV-cHK vs Ad.CMV-LacZ and Ad.CMV-cHK and icatibant.

†P<0.05, Ad.CMV-cHK vs Ad.CMV-LacZ and Ad.CMV-cHK and icatibant.

‡P<0.01, Ad.CMV-cHK vs Ad.CMV-cHK and icatibant.
levels. Elevation of cAMP attenuates mitogen-activated protein kinase signaling induced by platelet-derived growth factor in VSMCs and results in G1 phase arrest of the cell cycle by stimulating p27kip1, an inhibitor of cyclin-dependent kinase 4. The mechanism by which kinin inhibits vascular growth may also be attributed to activation of vascular nitric oxide synthase (NOS). This notion is supported by a study that shows that the NOS inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester could block the beneficial effect of ACE inhibition on neointima formation in balloon-injured rat carotid artery. Furthermore, a recent study showed that bradykinin significantly increased nitrite release from isolated canine coronary microvessels and that this increased release of nitrite was dramatically reduced by NOS inhibitor or by bradykinin B\textsubscript{2} receptor antagonist. Increased levels of NO lead to increased production of cGMP, a potent inhibitor of VSMC growth. The effect of cGMP on growth inhibition may be mediated by one of the following potential pathways: (1) cGMP activation of tyrosine phosphatase to inhibit G\textsubscript{1}/G\textsubscript{2} transition or the early G\textsubscript{1} phase of the cell cycle, (2) cGMP-dependent protein kinase inhibition of growth factor-activated tyrosine kinase or Raf-1 protein in the early G\textsubscript{1} phase of the cell cycle, or (3) cGMP cross-activation of cAMP-dependent protein kinase and inhibition of cell proliferation. Considered together, these results suggest that suppression of neointima formation in balloon-injured vessels after kallikrein gene delivery is mediated by the antiproliferative activity of cAMP and cGMP.

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