

Adenovirus-mediated kallikrein gene transfer inhibits neointima formation via increased production of nitric oxide in rat artery

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Abstract

Tissue kallikrein cleaves kininogen substrate to produce vasoactive kinin peptides that have been implicated to play a role in the proliferation of vascular smooth muscle cells (VSMC). In order to explore potential roles of the kallikrein–kinin system in vascular biology, we evaluated the effects of adenovirus-mediated kallikrein gene delivery on neointima formation in balloon-injured rat artery. Infection of isolated rat aortic segments with adenovirus containing the human tissue kallikrein gene resulted in a time-dependent secretion of recombinant human tissue kallikrein, and significant increases in nitric oxide (NO_x) and guanosine 3',5'-cyclic monophosphate (cGMP) levels post gene transfer. Human tissue kallikrein gene was delivered locally via adenoviral vectors into left common carotid artery after balloon angioplasty. Two weeks following gene transfer, we observed a 39% reduction in intima/media ratio at the injured vessel as compared to that of rats receiving control virus ($n = 8$, $P < .01$). Delivery of *N*^ω-nitro-L-arginine methyl ester (L-NAME), a NO_x synthase inhibitor via minipump for 2 weeks, blocked the protective effect and reversed the intima/media ratio to that of control rats ($n = 5$, $P < .01$). These results indicated that human tissue kallikrein gene delivery inhibits neointima formation via NO-cGMP signaling pathway. This study provides new insights into the role of the vascular kallikrein–kinin system and may have significant implications for gene therapy in treating occlusive vascular diseases. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Balloon angioplasty; Kallikrein; Gene transfer; Nitric oxide; Rat artery

Abbreviations: ACE, angiotensin-converting enzyme; Ad.CMV-CHK, adenovirus harboring the human tissue kallikrein cDNA under the control of the cytomegalovirus enhancer/promoter; Ad.CMV-LacZ, adenovirus harboring the *LacZ* gene under the control of the cytomegalovirus enhancer/promoter; AngII, angiotensin II; cGMP, guanosine 3',5'-cyclic monophosphate; L-NAME, *N*^ω-nitro-L-arginine methyl ester; NO, nitric oxide; pfu, plaque forming unit; VSMC, vascular smooth muscle cell

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1. Introduction

Endothelial injury by balloon catheter is known to promote vascular smooth muscle cell (VSMC) proliferation and growth, which leads to intimal hyperplasia and contributes to the pathogenesis of several cardiovascular disorders, including atherosclerosis and restenosis (Clowes et al., 1983). The process of neointima formation appears to involve the activation, proliferation and migration of VSMC from the media to the intima in response to mitogens and growth factors (Pauletto et al., 1994). The cellular

mechanisms of restenosis are not fully understood. Recent evidence indicates that angiotensin II (AngII) plays a pivotal role in the development of atherosclerosis by promoting VSMC growth and proliferation (Huckle and Earp, 1994). Angiotensin-converting enzyme (ACE) inhibition is effective in attenuating neointima formation after endothelial injury in rat carotid artery (Powell et al., 1989). Specific AngII receptor antagonists can also partially inhibit neointima formation, which suggests that the inhibition of neointima formation by ACE inhibitors is due, in part, to a decreased level of AngII (Kauffman et al., 1991). Inhibition of ACE activity not only prevents formation of AngII but also increases kinin levels by preventing kinin degradation. Increased local kinin accumulation may also contribute to inhibition of neointima formation since icatibant (Hoe 140), a bradykinin B₂ receptor antagonist, can partially block the protective effect of ACE inhibitors (Farhy et al., 1993). Therefore, the beneficial effects of ACE inhibition may also be attributed to increased kinin levels.

Abnormality of the tissue kallikrein–kinin system has been implicated in the pathogenesis of hypertension, cardiovascular and renal disorders (Sharma et al., 1996). 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 reduction, smooth muscle relaxation and contraction, pain induction, and inflammation (Bhoola et al., 1992). However, the functional role of the tissue kallikrein–kinin system in vascular physiology has not been fully established. Gene transfer to blood vessels offers a new tool to study the role of tissue kallikrein–kinin system in blood pressure regulation and vascular biology. We have recently shown that systemic delivery of the human tissue kallikrein gene attenuated the development of hypertension, cardiac hypertrophy and enhanced renal function in hypertensive and/or normotensive rats (Jin et al., 1997; Chao et al., 1998; Murakami et al., 1998; Yayama et al., 1998). In order to understand the potential beneficial effects of the tissue kallikrein–kinin system on neointima formation following vascular injury, adenovirus carrying the human tissue kallikrein gene was delivered into rat carotid artery after balloon angio-

plasty. In this study, we showed that delivery of the kallikrein gene at the injured site suppressed neointima formation in rat artery and the effect was mediated via NO-cGMP signaling pathway. The present study provides new insights into the role of the tissue kallikrein–kinin system in vascular cell growth and in restenosis.

2. Materials and methods

2.1. Local gene delivery

Male Sprague–Dawley rats (weight, 350–400 g) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and a 2F embolectomy balloon catheter (Baxter Healthcare) was introduced into the left common carotid artery via the external carotid artery. The balloon was inflated with sufficient saline to distend the common carotid and was then pulled back to the external carotid artery. This procedure was repeated three times and the catheter was then removed. After balloon injury of the left common carotid artery, the injured distal segment was isolated by temporary ligatures. The adenoviral particles of Ad.CMV-cHK or Ad.CMV-*LacZ* (2×10^9 plaque forming unit (pfu) in 20 μ l) were infused into the distal injured segment and incubated for 15 min 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 following kallikrein gene delivery, *N*^ω-nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor was infused intraperitoneally at a rate of 1.44 mg/kg per day via osmotic minipumps (Alzet 2ML2, Alza) immediately post balloon angioplasty and Ad.CMV-cHK infusion.

2.2. Preparation of adenovirus carrying the human tissue kallikrein gene

Adenovirus containing the human tissue kallikrein gene, Ad.CMV-cHK, was generated as previously described (Chao et al., 1998). Large quantities of high-titered adenoviruses, Ad.CMV-cHK and Ad.CMV-*LacZ*, were prepared and purified for gene delivery (Becker et al., 1994).

2.3. Gene transfer to rat aorta *ex vivo*

Male Sprague–Dawley rats (200–250 g) were anesthetized with sodium pentobarbital (50 mg/kg, *i.p.*). The aortic segments were gently removed and placed into a dish containing the cold, oxygenated Eagle's MEM medium (Life Technologies). The loose fat and connective tissue were gently dissected away, and the vessels were cut into 3–5 mm segments. The aortic segments were then exposed to 150 μ l of vehicle, Ad.CMV-cHK (10^{11} pfu/ml) or control virus, Ad.CMV-*LacZ* (10^{11} pfu/ml) in Eagle's MEM for 3 h. Following viral exposure, the vessels were rinsed with $1 \times$ phosphate-buffered saline to remove non-adherent virus particles and then incubated in 200 μ l Eagle's MEM media supplemented with 5% fetal bovine serum (Life Technologies), 100 units/ml penicillin and 100 μ g/ml streptomycin sulfate at 37°C with 5% CO₂. The cultured media were collected every 24 h for measuring human tissue kallikrein and nitric oxide (NO) level. Aortic segments were extracted with 0.1 N HCl for cGMP assay.

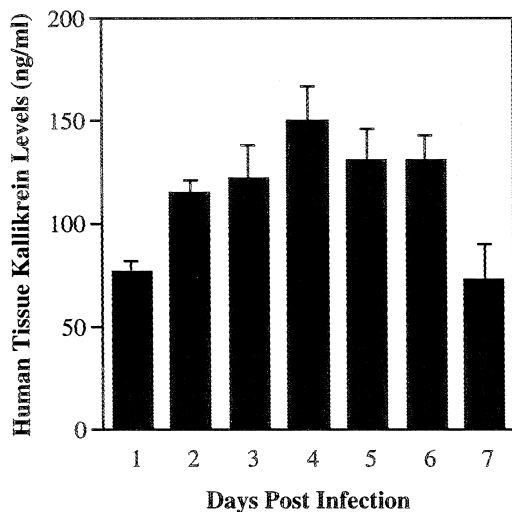


Fig. 1. Time-dependent expression of human tissue kallikrein in isolated rat aorta. Immunoreactive human tissue kallikrein levels in the cultured media of aortic segments following transfection of Ad.CMV-cHK were measured by ELISA. Data are expressed as mean \pm SEM.

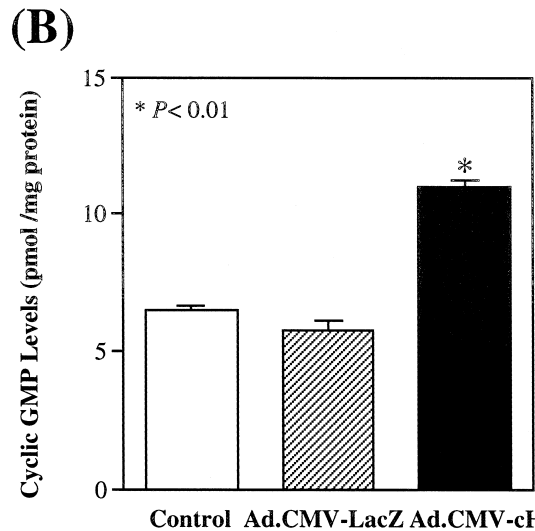
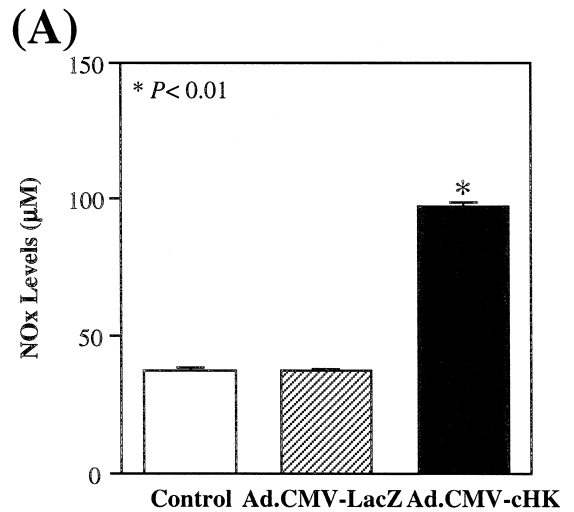


Fig. 2. NOx (A) and cGMP (B) levels after adenovirus-mediated kallikrein gene delivery in isolated rat aorta. Control, incubated with regular growth medium; Ad.CMV-*LacZ*, aortic segments transfected with adenovirus carrying the *LacZ* gene; Ad.CMV-cHK, aorta segments transfected with adenovirus carrying the human tissue kallikrein gene. Data are expressed as mean \pm SEM.

2.4. Immunoassays for human tissue kallikrein, NOx and cGMP levels

Immunoreactive human tissue kallikrein levels in the media of rat aortic segments were measured by enzyme-linked immunosorbent assay (ELISA) as previously described (Wang et al., 1994). NO levels

in cultured media were measured by modified fluorometric assay for quantification of nitrite/nitrate (Misko et al., 1993). cGMP levels in aortic extracts were measured by a radioimmunoassay (Jin et al., 1997) and protein concentration was determined by Lowry's method (Lowry et al., 1951).

2.5. Morphometric analysis

Two weeks after gene delivery, rats were anesthetized and a catheter was placed in the ascending aorta and right atrium was cut open. The vascular system was perfused with saline and then perfusion-fixed with 4% formaldehyde at a perfusion pressure of 100 mmHg. The left and right carotid artery were removed and embedded in paraffin. Each artery was divided into three segments that were separately embedded in paraffin. Cross-section 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 $100\times$. The lumen, neointima, media areas were traced and measured by using NIH Image 1.60 software package.

2.6. Statistical analysis

Group data are expressed as mean \pm SEM. Data were compared between experimental groups by one-way analysis of variance (ANOVA). Differences between kallikrein and control groups suggested by the ANOVA were further evaluated by Fisher's protected least-squares differences. Differences were considered significant at a value of $P < .05$.

3. Results

3.1. Time-dependent secretion of recombinant human tissue kallikrein in rat aorta *ex vivo*

Following infection of adenovirus carrying the human tissue kallikrein gene into isolated rat aortic

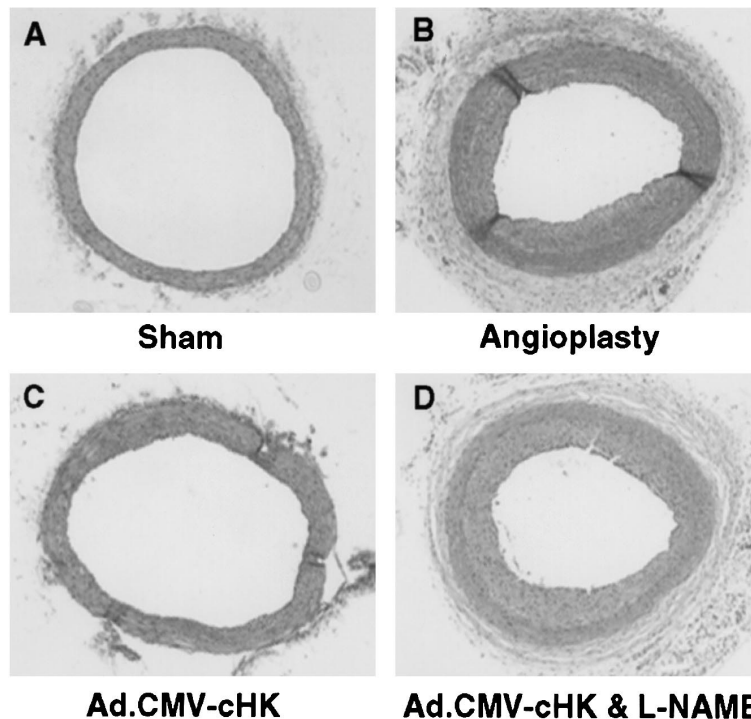


Fig. 3. Inhibition of neointima formation in rat carotid artery after adenovirus-mediated kallikrein gene delivery. (A) through (D) shows representative histological sections. (A) normal rat carotid artery; (B) rat aorta at 2 weeks after balloon angioplasty; (C) balloon angioplasty plus delivery with adenovirus, Ad.CMV-cHK carrying the human tissue kallikrein gene; (D) balloon angioplasty plus delivery with Ad.CMV-cHK and infusion of L-NAME via minipump for 2 weeks. Original magnification $100\times$.

segments, cultured media were collected from day 1 to day 7 and immunoreactive human tissue kallikrein levels were measured by an ELISA. Recombinant human tissue kallikrein was detected in the first day post infection and the highest level was 149 ng/ml at 4 days after gene transfer (Fig. 1).

3.2. Human tissue kallikrein gene transfer increased NO and cGMP levels in isolated rat aorta

In order to evaluate the effects of kallikrein gene delivery on production of potential mediators in rat aorta, we measured NO and cGMP production in aorta tissues. Fig. 2A shows NOx levels in the media at 5 days post infection. Adenovirus-mediated kallikrein gene delivery significantly increased NO levels as compared to controls with or without infection of adenovirus carrying the *LacZ* gene (Fig. 2A). Fig. 2B shows cGMP levels in aortic extracts at 5 days after the infection. Aortic tissues transfected with adenovirus containing the human tissue kallikrein gene had significantly higher levels of cGMP than that of rats with or without infection of control virus. In both assays there were no significant differences between the control groups with or without infection with Ad.CMV-*LacZ*.

3.3. Adenovirus-mediated kallikrein gene transfer inhibited neointima formation and the protective effect was abolished by NOx synthase inhibitor

In order to assess the effects of kallikrein gene delivery on vascular cell proliferation *in vivo*, we employed balloon angioplasty to induce vascular injury. In this model, a consistent neointima formed within the first 2 weeks after angioplasty. Adenovirus containing the human tissue kallikrein gene (Ad.CMV-cHK) or control virus containing the *LacZ* gene (Ad.CMV-*LacZ*), were delivered into left common carotid artery at the time of balloon injury and the vessels were harvested 2 weeks after local gene delivery. Fig. 3 shows histology of rat artery stained with hematoxylin and eosin. Carotid artery of the sham-operated rat showed normal morphology (Fig. 3A). Rats receiving angioplastic surgery showed severe neointima thickening (Fig. 3B) and similar morphological change was observed in rats receiving control virus, Ad.CMV-*LacZ* (data not shown). Lo-

cal delivery of the tissue kallikrein gene attenuated the formation of neointima (Fig. 3C) and L-NAME infusion via minipump for 2 weeks abolished the protective effect of kallikrein on injured vessels (D). Fig. 4 shows morphometric analyses of the intima area and intima/media ratio in carotid artery after angioplasty. Adenovirus-mediated kallikrein gene delivery significantly suppressed intima area in rat carotid artery when compared to rats following angioplasty with or without control adenovirus,

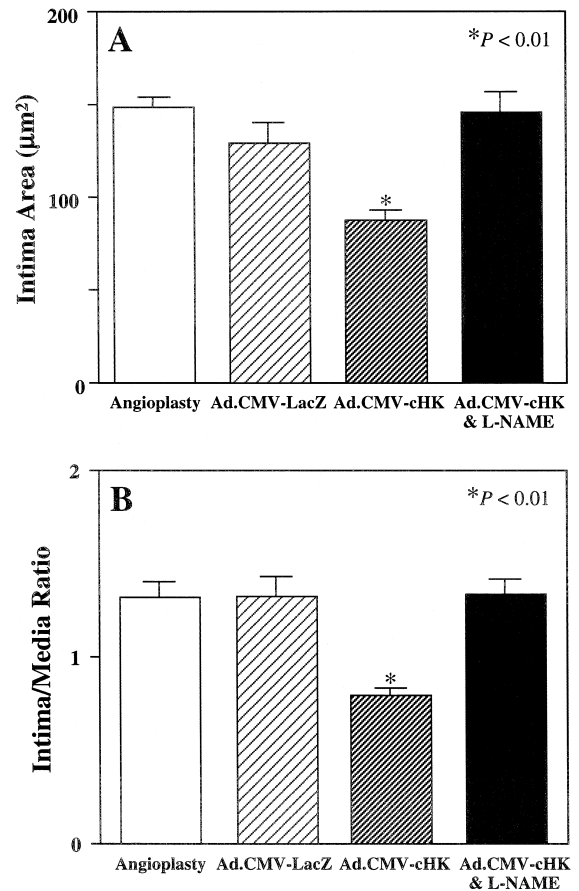


Fig. 4. Morphometric analyses of the intima area (A) and intima/media area ratio (B) in rat carotid artery after balloon angioplasty. Intima/media area ratios were measured in histological sections of vessels at 2 weeks after vascular injury. Angioplasty, balloon injury; Ad.CMV-*LacZ*, balloon-injured rats receiving adenovirus carrying the *LacZ* gene; Ad.CMV-cHK, balloon-injured rats receiving adenovirus carrying the human tissue kallikrein gene; Ad.CMV-cHK and L-NAME, balloon-injured rats receiving adenovirus carrying the human tissue kallikrein gene and L-NAME infusion via minipump for 2 weeks.

Ad.CMV-*LacZ* (cross-sectional area: 85.9 ± 7.1 vs. 148.9 ± 4.6 , $129.5 \pm 10.4 \mu\text{m}^2$, mean \pm SEM, $n = 8$, $P < .01$). Suppression of neointima formation after kallikrein gene delivery was significantly abolished by L-NAME (85.9 ± 7.1 vs. $146.3 \pm 11.2 \mu\text{m}^2$, mean \pm SEM, $n = 5$, $P < .01$). In contrast, media area was not significantly different among all the groups (angioplasty, 115.1 ± 4.1 ; Ad.CMV-*LacZ*, 98.3 ± 3.5 ; Ad.CMV-CHK, 110.7 ± 4.6 ; Ad.CMV-CHK and L-NAME, $111.2 \pm 4.5 \mu\text{m}^2$, mean \pm SEM). There is a 39% reduction in intima/media ratio in rats receiving kallikrein gene delivery as compared to rats receiving control virus (0.80 ± 0.06 vs. 1.32 ± 0.10 , mean \pm SEM, $n = 8$, $P < .01$). On the other hand, L-NAME reversed its effect to the control levels (1.34 ± 0.08 , mean \pm SEM, $n = 5$). No statistical difference was found between control groups with or without receiving Ad.CMV-*LacZ*, and the group receiving Ad.CMV-CHK with L-NAME infusion.

4. Discussion

This study demonstrated that a continuous supply of tissue kallikrein via gene transfer suppressed neointima formation in balloon-injured artery and the effect is, in part, mediated by increased production of NO. Delivery of the tissue kallikrein gene at the injured site significantly reduced intima/media ratio and thus neointima formation. L-NAME, a NO synthase inhibitor, blocked the protective effect of tissue kallikrein on arterial thickening. Expression of recombinant human tissue kallikrein in aortic tissues post gene transfer can be identified by specific ELISA and was accompanied by increased NO and cGMP levels. These results indicated that adenovirus-mediated kallikrein gene delivery attenuated neointima formation in injured artery and the effect was mediated by activation of second messengers such as NO and cGMP in blood vessels. Suppression of neointima formation after kallikrein gene delivery may be mediated by the antiproliferative activity of NO and cGMP. These findings provide important insights regarding the role of the vascular kallikrein–kinin system in occlusive vascular diseases such as atherosclerosis or restenosis.

ACE inhibition not only inhibits AngII formation but also prevents kinin degradation (Bhoola et al., 1992). A previous study showed that administration of ACE inhibitors could attenuate neointimal hyperplasia and the effects can be blocked by icatibant, Hoe 140, a bradykinin B₂ receptor antagonist (Farhy et al., 1992). These results indicated a potential protective role of the kallikrein–kinin system in vascular injury. This notion was supported by the findings that the components of the kallikrein–kinin system are present in the vasculature (Oza et al., 1990; Wolf et al., 1999). In addition, bradykinin has been shown to inhibit both basal and serum-stimulated cultured primary smooth muscle cell proliferation (Dixon and Dennis, 1996). These findings suggest that enhanced activity of the vascular kallikrein–kinin system is responsible, at least in part, for inhibiting neointima formation.

In this study, we showed that the expression of recombinant human tissue kallikrein was accompanied by increased NO and cGMP levels in isolated rat aortic tissues following kallikrein gene delivery. Kallikrein gene delivery suppressed neointima formation in balloon-injured rat artery *in vivo* and the protective effect was abolished by L-NAME, a NO synthase inhibitor. Human tissue kallikrein is capable of cleaving rat kininogen to produce kinin (Wang et al., 1994). Expression of recombinant human tissue kallikrein at the injured site results in increased kinin formation. Our previous studies showed increases in kinin, NO and cGMP levels in the urine and/or kidney after systemic delivery of the kallikrein gene (Jin et al., 1997; Chao et al., 1998; Yayama et al., 1998). These results suggest that kallikrein–kinin may inhibit vascular cell growth via induction and/or activation of NO synthase. This notion is consistent with the finding that L-NAME could block the beneficial effect of ACE inhibition on neointima formation in balloon-injured rat carotid artery (Farhy et al., 1993). Furthermore, recent studies showed that overexpression of human endothelial NO synthase (Chen et al., 1998) or NO-generating vasodilators (Garg and Hassid, 1989) inhibited VSMC proliferation. These findings are consistent with our observations that kallikrein gene transfer inhibits the VSMC growth via increased NO production.

This study provides new insights into the role of the tissue kallikrein–kinin system in vascular biol-

ogy and may have significant implication in future therapeutic applications of treating restenosis with kallikrein gene therapy.

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