

Adenovirus-Mediated Kallikrein Gene Delivery Reduces Aortic Thickening and Stroke-Induced Death Rate in Dahl Salt-Sensitive Rats

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Background and Purpose—Kallikrein gene delivery has been shown to attenuate hypertension, cardiac hypertrophy, and renal injury in hypertensive animal models. The aim of this study was to investigate the potential protective effects of kallikrein gene delivery in salt-induced stroke and cerebrovascular disorders.

Methods—Adenovirus harboring the human tissue kallikrein gene (AdCMV-cHK) was delivered intravenously into Dahl salt-sensitive (DS) rats after 4 weeks of high salt loading, and blood pressure was monitored weekly for 9 weeks.

Results—A single injection of AdCMV-cHK caused a significant reduction of systolic blood pressure compared with that in control rats, with or without an injection of adenovirus carrying the LacZ (control) gene (AdCMV-LacZ). A maximal blood pressure reduction of 21 mm Hg was observed 2 weeks after gene delivery. The stroke mortality rate of DS rats (AdCMV-LacZ group versus the AdCMV-cHK group) was significantly decreased: 38% versus 9% at 3 weeks and 54% versus 27% at 5 weeks after gene delivery. Kallikrein gene delivery significantly attenuated salt-induced aortic hypertrophy, as evidenced by reduced thickness of the aortic wall. Recombinant human tissue kallikrein was detected in rat serum and urine after gene transfer. Kinin-releasing activities in the brain as well as urinary kinin and cGMP levels were significantly increased in rats receiving the kallikrein gene.

Conclusions—This is the first study to demonstrate the protective effect of kallikrein gene delivery in reducing salt-induced stroke mortality and vascular dysfunction. (*Stroke*. 1999;30:1925-1932.)

Key Words: gene transfer ■ hypertension ■ hypertrophy ■ kallikrein ■ mortality ■ stroke

Stroke is the third leading cause of death and long-term disability in the United States. Previous studies have shown that hypertension per se is critical in the development of stroke.¹⁻⁵ A high salt intake accelerates the development of malignant hypertension in stroke-prone, spontaneously hypertensive rats (SHRSP).⁶ In the brains of SHRSP, fibrinoid necrosis and associated thrombosis primarily affect cerebral arterioles, leading to their obstruction and infarction, whereas cerebral hemorrhage is caused by rupture of microaneurysms.⁷ In addition, a lethal form of hypertension has been shown to develop in Dahl salt-sensitive (DS) rats fed a high-salt diet at an early age.⁸ Pathological changes in the brains of DS rats affected by stroke include hemorrhage, edema, and infarction.

Abnormality of the tissue kallikrein-kinin system has been implicated in the pathogenesis of hypertension and renal diseases.^{9,10} Tissue kallikrein is capable of processing low-molecular-weight kininogen to release vasoactive kinin peptides.^{11,12} Kinin has been reported to be a powerful dilator of the cerebral arterial vessels and to act in part through the release of endothelium-derived relaxing factor/nitric oxide.¹³ Recent evidence has revealed that after nitric oxide synthase

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inhibition, an increase in blood pressure and the onset of stroke occur more rapidly.¹⁴ Moreover, kinin exerts a concentration-dependent relaxation of isolated cerebral vessels in a wide variety of species, such as humans, rabbits, and cats.¹⁵⁻¹⁷ Kinin-generating activity has been detected in brain homogenates from rabbits and rats.^{18,19} Our recent study showed that intracerebroventricular delivery of the human tissue kallikrein gene (cHK) in the form of naked DNA or an adenovirus (Ad) vector reduced blood pressure in SHR.²⁰ Antisense inhibition of the bradykinin B₂ receptor in the brain reduces blood pressure in SHR, whereas antisense inhibition of the B₁ receptor induces blood pressure elevation in SHR.^{21,22} These results suggest that the tissue kallikrein-kinin system may function in control of blood pressure homeostasis in brain.

Our recent studies showed that cHK gene delivery not only attenuated hypertension, cardiac hypertrophy, and renal injury but could also partially reverse salt-induced cardiac and renal lesions in DS rats on a high-salt diet.^{23,24} In this study, we further explored the potential effects of kallikrein gene

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delivery on salt-induced stroke in DS rats. We showed that a single injection of Ad carrying the cHK gene significantly reduced salt-induced hypertension, stroke-induced mortality rate, and aortic thickness in DS rats. These results suggest that successful application of this technology may have potential value in treating individuals at high risk for stroke and cerebrovascular dysfunction.

Materials and Methods

Animals

Thirty-two DS rats (male, 4 weeks old) from Sprague-Dawley Harlan (Indianapolis, Ind) were used in this study. Rats were divided into 2 groups. The first group was fed with standard rat chow (0.4% NaCl), and the other group was fed with a high-salt diet (4% NaCl; Harlan Teklad, Madison, Wis). All rats had free access to water. All procedures complied with the standards for care and use of animal subjects as stated in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Resources, National Academy of Sciences, Bethesda, Md).

Preparation of Replication-Deficient Ad Vector AdCMV-cHK

Plasmid cytomegalovirus carrying the cHK gene (CMV-cHK) was constructed as previously described.²³ In this construct, expression of human tissue kallikrein cDNA was under the control of the CMV enhancer/promoter and was followed by a bovine growth hormone polyadenylation signal sequence. The purified DNA was sent to the Institute for Human Gene Therapy, Wistar Institute, Philadelphia, Pa, for generation of the Ad, AdCMV-cHK, harboring the CMV-cHK-polyadenylation transcription unit. Ad harboring the LacZ gene under control of the CMV enhancer/promoter (AdCMV-LacZ) was purchased from the Institute for Human Gene Therapy.

Intravenous Delivery of Ad Vectors

DS rats in the group fed with the high-salt diet containing 4% NaCl for 4 weeks were randomly divided into 3 groups and were intravenously injected with either AdCMV-cHK (n=13) or AdCMV-LacZ (n=6) at a dosage of 2.4×10^{10} plaque-forming units per rat through the tail vein. The remaining 6 DS rats on the 4% NaCl diet did not receive any Ad injection.

Blood Pressure Measurement

The systolic blood pressure of the rats was measured with a manometer tachometer (Nastume KN-210; Nastume Seisakusho Co, Ltd, Tokyo, Japan) by using the tail-cuff method.²⁵ An average of 10 readings was taken for each animal after they became acclimated to the environment.

Urine Collection and Tissue Preparation

Twenty-four-hour urine volume was collected from the rats in metabolic cages at 5 and 11 days after gene delivery. To eliminate contamination of urine samples, animals received only water during the 24-hour collection period. Urine was collected and centrifuged at 1000g to remove particles. The volume of the supernatant was measured and the samples were used for further analysis. At the end of the experiment, the rats were anesthetized intraperitoneally with pentobarbital at a dose of 50 mg/kg body weight. The brain was removed and homogenized in phosphate-buffered saline (pH 7.0) to measure kinin-releasing activity. Total protein in the supernatant was determined by the method of Lowry et al.²⁶

Immunoassays for Human Tissue Kallikrein, Kinin, and cGMP

Human tissue kallikrein levels in rat urine and sera were determined by an ELISA specific for human tissue kallikrein as previously described.²⁵ Urinary kinin levels were determined in samples at 5 days after gene delivery by a kinin radioimmunoassay.²⁷ Urinary

cGMP levels were measured in samples at 11 days after gene delivery by a cGMP radioimmunoassay.²⁸

Enzymatic Assays Toward Low-Molecular-Weight Kininogen Substrates

Canine low-molecular-weight kininogen was isolated according to the method of Johnson et al.²⁹ Kinin-releasing activities were measured by incubating rat brain extracts (10 μ g) with kininogen (1.5 μ g) in 0.1 mol/L sodium phosphate (pH 8.5) in a total volume of 500 μ L at 37°C for 30 minutes. The reactions were stopped by boiling for 20 minutes. Released kinin was assayed by a kinin radioimmunoassay.²⁷ Kallikrein activity is expressed as nanograms of kinin released per milligram protein per 30 minutes.

Monitoring Stroke Development

The rats were monitored daily for the occurrence of stroke. The symptoms associated with stroke development have been previously described in SHRSP but not in DS rats.^{30,31} Initially, rats develop convulsive, repetitive forearm movement followed by inappropriate posture. In this study, stroke was often associated with lethargy and poor grooming. There is no fixed period between onset of the first behavioral symptoms of stroke and death. Some animals died abruptly after the first behavioral symptoms of stroke. Some animals were killed later, at a point when death was judged likely to occur within a day.

Confirmation of Brain Infarction Area due to Stroke

Serial, coronal brain sections were stained with 2,3,5-triphenyltetrazolium chloride (TTC,³² Sigma Chemical Co). Brain slices were immersed in normal saline containing 2% TTC at 37°C for 30 minutes. TTC, a colorless salt, is reduced to form an insoluble red formazan product in the presence of a functioning mitochondrial electron transport chain. Noninfarcted area, which contains dehydrogenase, was stained brick red by reacting with TTC, whereas infarcted tissues remained unstained because of the lack of dehydrogenase. Each slice was photographed and the image was then transferred to the computer. The infarct areas were measured by using the National Institutes of Health IMAGE software.

Morphological and Histological Investigations

Tissue sections were preserved in 4% buffered formaldehyde solution and embedded in paraffin. Five-micron-thick sections were cut, stained with hematoxylin-eosin, and analyzed microscopically and morphometrically. Ten measurements of thickness of the aortic wall taken from different positions of each aorta were averaged. In addition, 6 measurements of the number of elastic fiber and smooth muscle cell layers in the media of aorta from each rat were taken from different positions, and aortic sections from 4 rats in each group were measured and averaged. All sections were evaluated by independent personnel with no prior knowledge of the group from which the rats were obtained.

Statistical Analysis

Data were analyzed using standard statistical methods. Repeated blood pressure measurements were taken after gene delivery for comparison between control and experimental groups at each time point with the use of ANOVA and Fisher's protected least significant difference test. Group data were expressed as mean \pm SEM. Survival curves were constructed using Kaplan-Meier analysis. Statistical significance of these data was measured by ANOVA and χ^2 by using the SAS software package. Values of blood pressure and other parameters were considered significantly different at a value of $P < 0.05$.

Results

Blood Pressure Reduction After Intravenous Injection of AdCMV-cHK

DS rats (4 weeks old) were fed either a high-salt (4% NaCl) diet or normal rat chow (0.4% NaCl) for 4 weeks until blood pressure

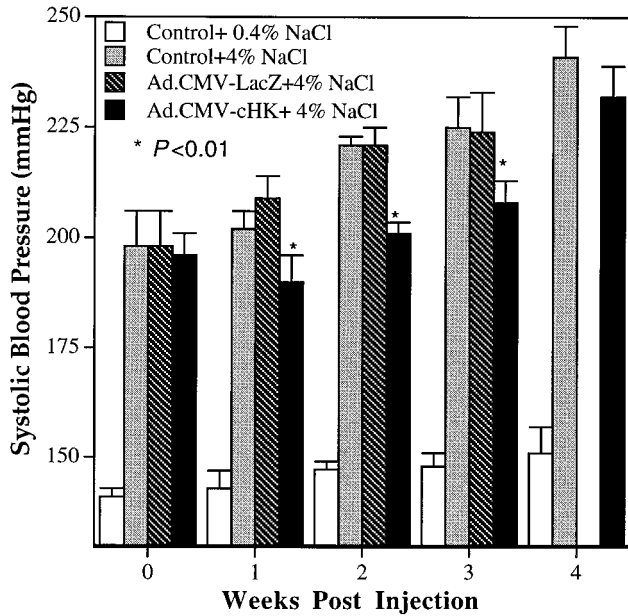


Figure 1. Systolic blood pressure of Dahl salt-sensitive rats after intravenous injection of adenovirus (Ad) carrying the cytomegalovirus enhancer/promoter (CMV) and either human tissue kallikrein (cHK) or the control LacZ (AdCMV-LacZ). Blood pressure values are expressed as mean \pm SEM (n=7). $P<0.01$ AdCMV-cHK group vs high-salt and AdCMV-LacZ groups.

differences between these 2 groups exceeded 50 mm Hg. Rats on the high-salt diet were divided into 3 groups: 2 groups were injected with either AdCMV-cHK or AdCMV-LacZ through the tail vein; the third group was injected with saline. Blood pressure was monitored weekly after gene delivery. Figure 1 shows systolic blood pressures of DS rats fed a normal-salt diet (0.4% NaCl) or a high-salt diet (4% NaCl) from 1 to 4 weeks after gene delivery. Systemic delivery of AdCMV-cHK caused a significant reduction in blood pressure within 1 week after injection. A maximal blood pressure reduction of 21 mm Hg was observed 14 days after gene delivery (200 \pm 3 versus 221 \pm 8 mm Hg, mean \pm SEM; n=7, $P<0.01$). In contrast, the blood pressures of control rats on a normal-salt diet (0.4% NaCl) remained at 135 to 155 mm Hg throughout the experimental period (Figure 1).

Time-Dependent Expression of Human Tissue Kallikrein After Gene Delivery

After intravenous injection of AdCMV-cHK, immunoreactive human tissue kallikrein levels in rat sera were measured by ELISA (Figure 2). The highest level of recombinant human tissue kallikrein (967 \pm 4 ng/mL, mean \pm SEM; n=4) was observed on the fourth day after gene delivery. Human tissue kallikrein levels were highest at 4 to 5 days after injection and then decreased gradually. Human tissue kallikrein levels in rat urine were 22.7 \pm 1.3 and 6.1 \pm 0.9 μ g \cdot 100 g body weight $^{-1}$ \cdot d $^{-1}$ (n=5, mean \pm SEM) at 5 and 11 days after kallikrein gene delivery. Human tissue kallikrein was not detected in control rats injected with AdCMV-LacZ.

Kallikrein Activities in Brain Extracts After Gene Delivery

Figure 3 shows kallikrein activity in rat brain extracts after gene delivery. Kallikrein activity increased 2.8-fold in rat

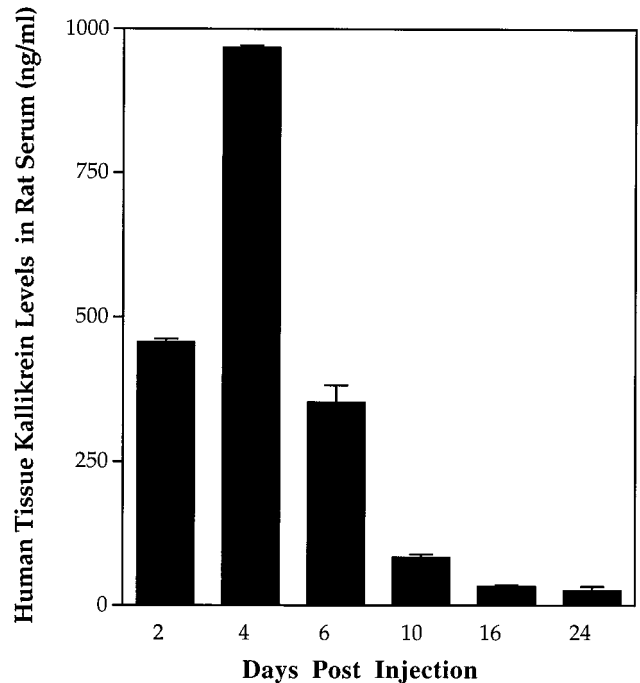


Figure 2. Time-dependent expression of human tissue kallikrein in rat serum. Human tissue kallikrein was measured by ELISA. Data are expressed as mean \pm SEM (n=4).

brain extracts after kallikrein gene delivery compared with control rats receiving AdCMV-LacZ (3.6 \pm 0.2 versus 1.3 \pm 0.1 ng kinin released per mg protein per 30 minutes, mean \pm SEM; n=4, $P<0.05$). Kallikrein activities of DS rats on a 4% NaCl diet (1.3 \pm 0.1 ng kinin released per mg protein per 30 minutes) were similar to those of rats on a 0.4% NaCl diet (1.4 \pm 0.2 ng kinin released per mg protein per 30 minutes, n=4).

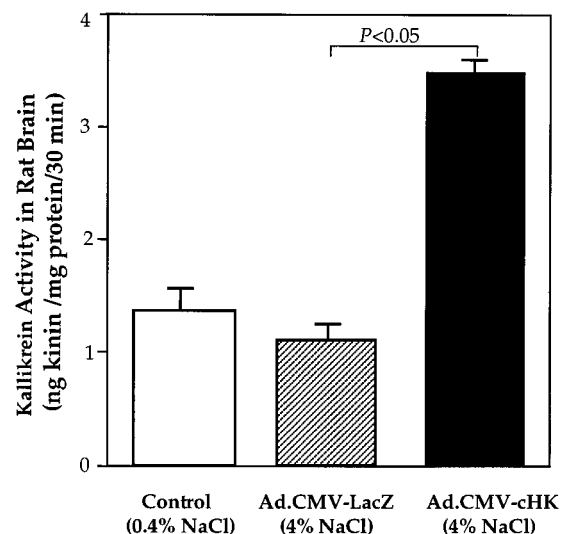


Figure 3. Kallikrein activities of rat brain 34 days after adenovirus-mediated kallikrein gene delivery. Kallikrein activity is expressed as ng kinin released per mg protein per 30 minutes. Values for each group are reported as mean \pm SEM (n=4). $P<0.05$ indicates a significant difference between the groups indicated. See the legend to Figure 1 for explanation of abbreviations.

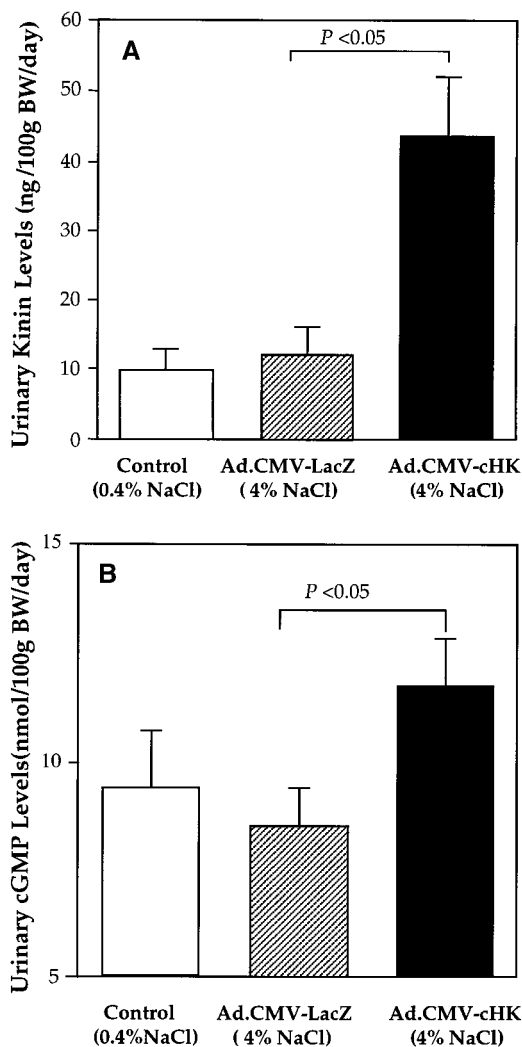


Figure 4. Urinary kinin (A) and cGMP (B) levels in rats after adenovirus-mediated kallikrein gene delivery. Values for each group are reported as mean \pm SEM ($n=6$). $P<0.05$ indicates a significant difference between the groups indicated. See the legend to Figure 1 for explanation of abbreviations.

Effect of Kallikrein Gene Delivery on Urinary Kinin and cGMP Levels

Figure 4A shows that urinary or renal kinin levels increased 3.5-fold after kallikrein gene delivery compared with rats receiving control virus AdCMV-LacZ (43.5 ± 8.4 versus 12.2 ± 3.9 ng \cdot 100 g body weight $^{-1} \cdot$ d $^{-1}$, mean \pm SEM; $n=6$, $P<0.05$). Kinin levels of DS rats on a 0.4% NaCl diet was 9.8 ± 3.0 ng \cdot 100 g body weight $^{-1} \cdot$ d $^{-1}$ ($n=6$). Figure 4B shows that urinary cGMP levels also increased significantly in rats receiving the kallikrein gene compared with rats injected with the AdCMV-LacZ virus (11.7 ± 1.1 versus 8.5 ± 0.8 nmol \cdot 100 g body weight $^{-1} \cdot$ d $^{-1}$, mean \pm SEM; $n=6$, $P<0.05$). cGMP levels of DS rats on a 0.4% NaCl diet were 9.6 ± 1.3 nmol \cdot 100 g body weight $^{-1} \cdot$ d $^{-1}$ ($n=6$).

Mortality Rate of DS Rats With Stroke

DS rats began to show symptoms of stroke, including lethargy, poor grooming, convulsive repetitive forearm movement, and/or semiplegia at 5.5 weeks after high salt

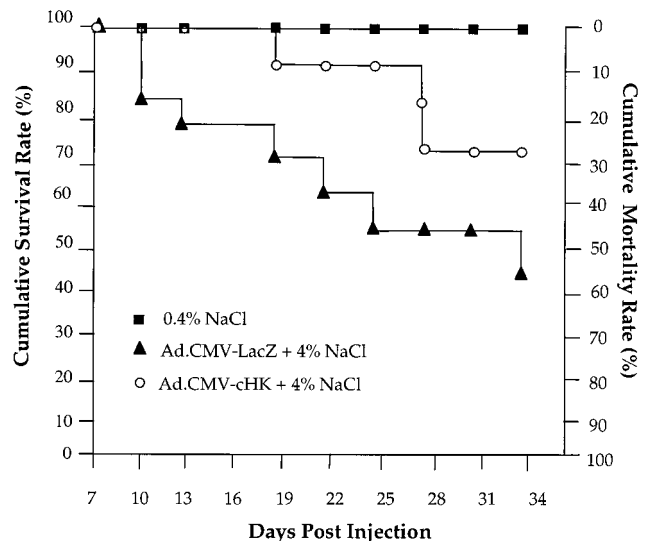


Figure 5. Survival rate of DS rats after adenovirus-mediated kallikrein gene delivery. Survival was monitored in the following group of rats: control ($n=7$), on a normal-salt diet (0.4% NaCl) (■); 4% NaCl group ($n=12$) included DS rats with or without having received the control adenovirus AdCMV-LacZ (▲); 4% NaCl group ($n=13$), having received adenovirus carrying the human kallikrein gene AdCMV-cHK (○). χ^2 analysis of these data generated $P<0.05$ (AdCMV-LacZ group vs AdCMV-cHK group). See the legend to Figure 1 for explanation of abbreviations.

loading. Some rats died rapidly after the appearance of the first behavioral symptoms of stroke. Among 12 rats in the high salt-diet group (with or without control virus injection), 3 rats exhibited hemiplegia and convulsive rhythmic movement of 1 forelimb and shoulder, 1 rat displayed symptoms of repetitive head bobbing, and the other 4 rats exhibited the sign of hemiplegia only. In the group of 12 rats given the high-salt diet and injected with AdCMV-cHK, 1 rat exhibited hemiplegia and repetitive movement of 1 forelimb and shoulder, 3 rats showed repetitive movements of 1 forelimb and shoulder, and 1 rat exhibited the sign of hemiplegia only. Figure 5 shows Kaplan-Meier survival plots for DS rats after kallikrein gene delivery. At 3 weeks after kallikrein gene delivery (7 weeks after high-salt loading), the survival rates were 100% in the control group (0.4% NaCl diet), 91% in the AdCMV-cHK group (4% NaCl), and 62% in the high salt loading group (4% NaCl diet with or without AdCMV-LacZ) (Figure 5). The salt-induced mortality rate in DS rats at 5 weeks after kallikrein gene delivery was 27%, whereas 54% of control DS rats that were fed with the high-salt diet had died of stroke. Figure 6 shows the effects of AdCMV-cHK delivery on cerebral infarction in DS rats after high salt loading. The brain sections of DS rats on a normal-salt diet appeared reddish, as stained by TTC, whereas focal infarction regions in the brain sections from rats in the high-salt plus AdCMV-LacZ group appeared whitish. Similar results were also seen in rats on a high-salt diet without injection of control Ad (data not shown). After kallikrein gene delivery, brain sections of DS rats appeared reddish and relatively normal. The results showed that Ad-mediated kallikrein gene delivery significantly reduced the infarct areas compared with those in control rats fed with a high-salt diet alone ($2.1 \pm 1.0\%$ versus $10.3 \pm 0.6\%$; $n=3$, $P<0.01$).

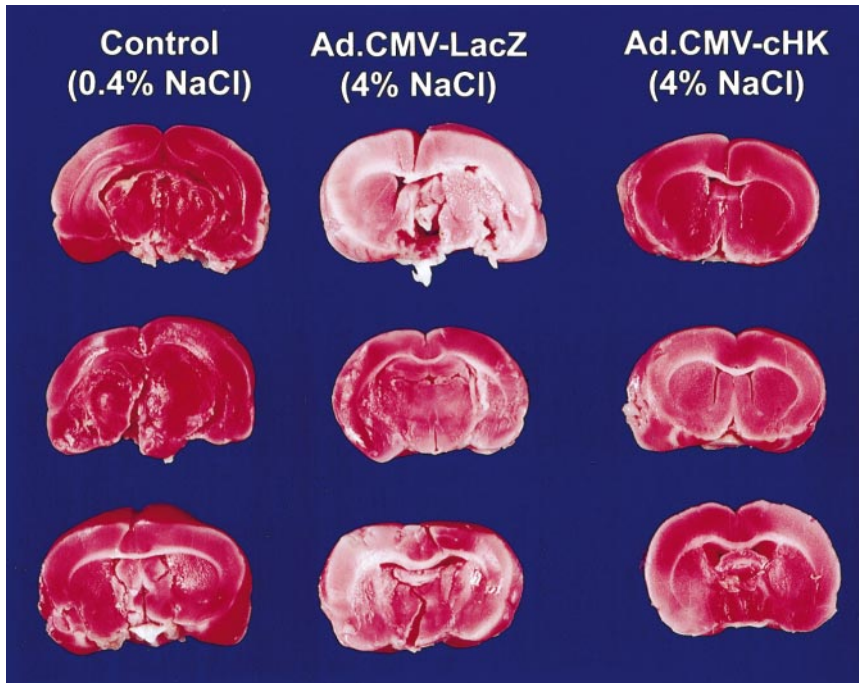


Figure 6. Effects of human tissue kallikrein gene delivery on cerebral infarction of DS rats after high salt loading. Rats were killed 35 days after gene delivery, and brain sections were stained with 2,3,5-triphenyltetrazolium chloride (TTC). Control indicates rats that were fed with the normal-salt diet (0.4% NaCl); AdCMV-LacZ (4% NaCl), received control adenovirus carrying the LacZ gene; and AdCMV-cHK (4% NaCl), received adenovirus carrying the human tissue kallikrein gene. Five weeks after gene delivery, rat brain sections were prepared and stained with 2% TTC solution at 37°C for 30 minutes. Infarcted regions in brain sections appear white.

Effect of Kallikrein Gene Delivery on Salt-Induced Aortic Thickening in DS Rats

Figure 7 shows the effect of kallikrein gene delivery on salt-induced aortic thickening in DS rats. The thickness of the aortic wall was significantly reduced in the AdCMV-cHK group at 5 weeks after gene delivery compared with that of the AdCMV-LacZ group (134.9 ± 1.7 versus 161.3 ± 1.3 μm , mean \pm SEM; $n=5$, $P<0.01$), whereas aortic wall thickness for DS rats on a normal-salt diet (0.4% NaCl) was 114.8 ± 1.5 μm ($n=5$, $P<0.01$). These results indicate that Ad-mediated kallikrein gene delivery can attenuate, at least in

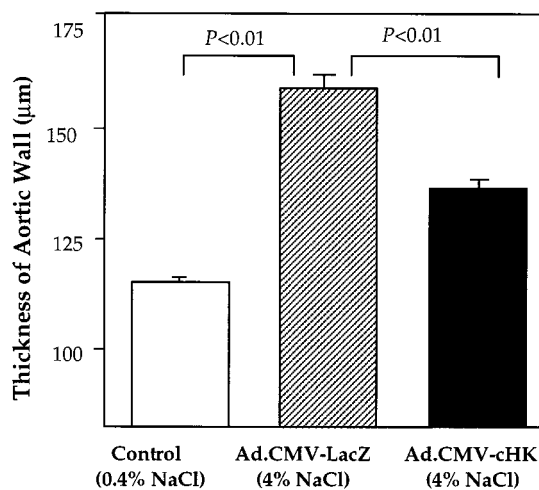


Figure 7. Effects of human kallikrein gene delivery on aortic wall thickness in DS rats after high salt loading. Control indicates rats that were fed with the normal-salt diet (0.4% NaCl); AdCMV-LacZ (4% NaCl), received control adenovirus carrying the LacZ gene; and AdCMV-cHK (4% NaCl), received adenovirus carrying the human kallikrein gene. The thickness of aortic wall is expressed as mean \pm SEM ($n=5$). $P<0.01$ indicates a significant difference between the groups indicated.

part, salt-induced aortic hypertrophy in DS rats. To evaluate whether the greater wall thickness in the DS rats was related to a structural or functional change, we also measured the number of elastic fiber and smooth muscle layers in the media of aortas. Our results showed that kallikrein gene delivery significantly reduced salt-induced aortic thickening by reducing the number of elastic fiber layers (8 ± 0.2 versus 10 ± 0.2 ; $n=4$, $P<0.01$) and smooth muscle cell layers (10 ± 0.2 versus 12 ± 0.4 ; $n=4$, $P<0.01$).

Discussion

This is the first study to show that a continuous supply of tissue kallikrein by Ad-mediated gene delivery reduces stroke mortality rate and partially attenuates aortic hypertrophy in DS rats fed a high-salt diet. A single injection into the tail vein of the cHK gene caused a significant reduction in blood pressure for >3 weeks. Expression of human tissue kallikrein mRNA was detected in the kidney, aorta, heart, and liver after systemic delivery of Ad carrying human tissue kallikrein.^{24,33,34} The ability of kallikrein gene transfer to improve the survival rate of and protect against structural changes in the vasculature in hypertensive DS rats may provide a new approach for studying the function of tissue kallikrein in vascular tissues. These results also suggest the feasibility of kallikrein gene therapy in salt-induced stroke or vascular disorders.

Previous study showed that hypertensive DS rats on a high-salt diet were affected by severe renal damage, exhibited cerebral infarction and hemorrhage, and eventually died spontaneously.^{23,35} These findings suggest that stroke development in DS rats is due to fulminating hypertension.^{36,37} Stroke development in DS rats could also be associated with renal failure, because arteriolar changes in the kidney may be related to the pathogenesis of cerebrovascular lesions.³⁸ Morphological analysis of brain sections showed hemorrhagic sites in the cerebrum of 4 of 8 control rats fed with a

high-salt diet (n=8) but not in the group receiving kallikrein gene (n=8). Moreover, in the control rats on a high-salt diet, loosened tissue was identified around the hemorrhagic areas, suggesting cerebral infarction. By using TTC staining, we found that AdCMV-cHK gene delivery significantly reduced the infarct areas compared with those of control rats fed with a high-salt diet (Figure 6). The hemorrhage and infarction may be attributed to rupture or closure of microcerebral vessels, as well as vasospasm associated with the accumulation of blood in the cerebrum.

Recombinant human tissue kallikrein was detected in rat serum and urine after kallikrein gene delivery. Expression of human tissue kallikrein results in increased kinin-releasing activities in the brain and elevated urinary kinin levels in rats. Binding of kinin to its receptors activates the signal transduction pathway and triggers a broad spectrum of biological effects, such as vasodilation and increased microvessel permeability. In this study, we observed increased kinin-releasing activities in the brain after kallikrein gene delivery. Kinin has additionally been shown to stimulate the release of tissue plasminogen activator, which converts plasminogen to plasmin, and which may contribute to the inhibition of thrombotic cerebral ischemia.³⁹ The possibility that kallikrein attenuation of the ischemic stroke-induced mortality rate in hypertensive DS rats is mediated by stimulation of plasminogen activator release awaits further studies. Another possibility for attenuation of salt-induced mortality in DS rats after kallikrein gene delivery is through enhanced sodium excretion. Increased kinin levels in the kidney or urine were accompanied by significant increases in urinary sodium excretion in salt-loaded DS rats.²³ Increased sodium excretion into urine may indirectly reduce sodium concentration in the circulation as well as sodium influx from the blood to brain via the blood-brain barrier. Because kallikrein gene delivery may prevent salt-induced cell swelling in the brain through regulation of sodium transport, this protective effect of kallikrein on cerebrovascular dysfunction could account for the lower incidence of stroke. However, it is not clear whether the protection is primarily due to protection of the brain or of the kidney. A previous study by Nagaoka et al³⁸ suggested that the pathogenesis of cerebrovascular lesions in SPSHR is related to renal dysfunction. In our recent studies, we reported that kallikrein gene delivery significantly enhanced renal function as evidenced by increases in glomerular filtration rate and renal blood flow in DS rats fed with a high-salt diet.²⁴ These results supported the notion that the kallikrein-mediated protective effect on stroke-induced mortality rate may be attributed, in part, to blood pressure reduction and renal protection.

In this study, we showed that urinary kinin and cGMP levels increased significantly after Ad-mediated kallikrein gene delivery. Binding of kinin to the B₂ receptor triggers the release of nitric oxide, which subsequently activates guanylyl cyclase to produce cGMP. This notion is supported by our previous report that systemic delivery of the human tissue kallikrein gene increased urinary nitrite/nitrate levels in addition to kinin and cGMP levels in salt-induced hypertensive DS rats.²⁴ We observed that high salt loading caused severe aortic thickening in DS rats, as evidenced by marked increases in thickness of the

aortic wall, whereas kallikrein gene delivery significantly attenuated salt-induced aortic thickening by reducing the number of elastic fiber and smooth muscle cell layers, indicating a structural change in the vascular wall. A possible mechanism for these findings is that expression of recombinant human kallikrein resulted in increased kinin levels. Binding of kinins to bradykinin receptors may thus activate a cGMP-dependent signal transduction pathway, and elevated cGMP levels may result in inhibition of smooth muscle cell growth and proliferation in the aorta, due to its antimitogenic and antihyperplastic properties.^{40,41}

In the present study, we showed that at 5 weeks after gene delivery, survival rate in the kallikrein group was 2-fold higher than in the high-salt groups, with or without injection of the control Ad. Ad-mediated gene transfer can achieve a high efficiency of expression, but this technique is also associated with the problem of inflammation, which will limit the persistence of transgene expression. However, the observations that kallikrein gene delivery attenuated aortic hypertrophy and ischemic infarction in the brain sections cannot be attributed to inflammatory responses, because control rats were injected with the same amounts of the AdCMV-LacZ gene. First-generation adenoviral vectors with E1 and E3 deletions produce cytolytic effects and immunological responses and are cleared by the host within 3 to 4 weeks after gene delivery.⁴² Recently, second-generation adenoviral vectors were shown to produce prolonged transgene expression and markedly reduce inflammation.⁴³ Thus, the development of improved adenoviral vectors with reduced cytolytic effects and minimal immunological responses would be useful tools for further investigations of the role of kallikrein in stroke and cerebrovascular diseases by gene transfer approaches. On the other hand, systemic gene delivery via intravenous injection undergoes relatively limited efficiency to target end-organs, like the brain, due to the obstacle of the blood-brain barrier. Intracisternal or intracerebroventricular injection of adenoviral vectors carrying desirable genes may locally improve expression efficiency in the brain.⁴⁴ However, safety is an important consideration in injection of adenovirus into the brain. Another safe and effective gene delivery protocol is to open the vascular endothelium by osmotic disruption of the blood-brain barrier and to inject vectors into the carotid artery.⁴⁵

In conclusion, this report shows that Ad-mediated gene delivery of cHK not only attenuates salt-induced aortic hypertrophy but also significantly lowers stroke incidence and death rate in hypertensive DS rats. These results suggest that systemic delivery of the kallikrein gene may have beneficial effects in protecting salt-induced cerebrovascular damage, such as hemorrhagic or ischemic infarction and brain edema. Moreover, kallikrein gene therapy may have applications in treating individuals at high risk for stroke and cerebrovascular diseases.

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References

- Byrom FB. The pathogenesis of hypertensive encephalopathy and its relation to the malignant phase of hypertension. *Lancet*. 1954;2:201–211.
- Robertson DM, Dinsdale HB, Ayashi T, Tu J. Cerebral lesions in adrenal regeneration hypertension. *Am J Pathol*. 1970;59:115–131.
- Skelton FR. Development of hypertension and cardiovascular renal lesions during adrenal regeneration in the rat. *Proc Soc Exp Biol Med*. 1955;90:342–346.
- Okamoto K, Yamori Y, Nagaoka A. Establishment of the stroke-prone spontaneously hypertensive rat. *Circ Res*. 1974;34(suppl 1):I-143–I-153.
- Nagaoka A, Iwatsuka H, Suzuoki Z, Okamoto K. Genetic predisposition to stroke in spontaneously hypertensive rats. *Am J Physiol*. 1976;230:1354–1359.
- Volpe M, Camargo MJ, Mueller FB, Campbell WG Jr, Sealey JE, Pecker MS, Sosa RE, Laragh JH. Relation of plasma renin to end-organ damage and to protection of K⁺ feeding in stroke-prone hypertensive rats. *Hypertension*. 1990;15:318–326.
- Tagami M, Nara Y, Kubota A, Sunaga T, Maezawa H, Fujino H, Yamori Y. Ultrastructural characteristic of occluded perforating arteries in stroke-prone spontaneously hypertensive rats. *Stroke*. 1987;18:733–740.
- Knudsen KD, Dahl LK, Thompson K, Iwai J, Heine M, Leitl G. Effects of chronic excess salt ingestion: inheritance of hypertension in the rat. *J Exp Med*. 1970;132:976–1000.
- Scicli AG, Carretero OA. Renal kallikrein-kinin system. *Kidney Int*. 1986;29:120–130.
- Margolius HS. Tissue kallikrein and kinins: regulation and roles in hypertensive and diabetic diseases. *Annu Rev Pharmacol Toxicol*. 1989;29:343–364.
- Clements JA. The glandular kallikrein family of enzymes: tissue-specific expression and hormonal regulation. *Endocr Rev*. 1989;10:393–419.
- Bhoola KD, Figueroa CD, Worthy K. Bioregulation of kinins: kallikrein, kininogens, and kininases. *Pharmacol Rev*. 1992;44:1–8.
- Carretero OA, Scicli AG. Local hormonal factors (intracrine, paracrine, autocrine) in hypertension. *Hypertension*. 1991;18(suppl 1):I-158–I-169.
- Ahmad S. Angiotensin receptor antagonists delay nitric oxide-deficient stroke in stroke-prone rats. *Eur J Pharmacol*. 1997;333:39–45.
- Toda N. Actions of bradykinin on isolated cerebral and peripheral arteries. *Am J Physiol*. 1977;232:H267–H274.
- Whalley ET, Fritz H, Geiger R. Kinin receptors and angiotensin converting enzyme in rabbit basilar arteries. *Naunyn Schmiedebergs Arch Pharmacol*. 1983;324:296–301.
- Whalley ET, Whal M. The effects of kininase II inhibition on the response of feline cerebral arteries to bradykinin and angiotensin. *Pflugers Arch*. 1983;398:175–177.
- Hori S. The presence of bradykinin polypeptides, kinin-releasing and destroying activity in brain. *Jpn J Biol Chem*. 1968;18:772–787.
- Shikimi T, Kema R, Matsumoto M, Yamahata Y, Miyata S. Studies on kinin-like substances in brain. *Biochem Pharmacol*. 1973;22:556–573.
- Wang C, Chao C, Madeddu P, Chao L, Chao J. Central delivery of human tissue kallikrein gene reduces blood pressure in hypertensive rats. *Biochem Biophys Res Commun*. 1998;244:449–454.
- Madeddu P, Parpaglia PP, Glorioso N, Chao J, Chao L. Antisense inhibition of brain kallikrein-kinin system in spontaneously hypertensive rats. *Hypertension*. 1996;28:980–989.
- Emanueli C, Chao J, Rogoli D, Chao L, Geppetti P, Madeddu P. Role of brain bradykinin B1 receptor in the central regulation of blood pressure in rats. *Br J Pharmacol*. In press.
- Chao J, Zhang JJ, Lin KF, Chao L. Human kallikrein gene delivery attenuates hypertension, cardiac hypertrophy, and renal injury in Dahl salt-sensitive rats. *Hum Gene Ther*. 1998;9:21–31.
- Chao J, Zhang JJ, Lin KF, Chao L. Adenovirus-mediated kallikrein gene delivery reverses salt-induced renal injury in Dahl salt-sensitive rats. *Kidney Int*. 1998;54:1250–1260.
- Wang C, Chao L, Chao J. Direct gene delivery of human tissue kallikrein reduces blood pressure in spontaneously hypertensive rats. *J Clin Invest*. 1995;95:1710–1716.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193:265–275.
- Shimamoto K, Ando T, Nakao T, Tanaka S, Sakuma MA, Miyahara M. A sensitive radioimmunoassay method for urinary kinins in man. *J Lab Clin Med*. 1987;91:721–728.
- Lin KF, Chao L, Chao J. Prolonged reduction of high blood pressure with human nitric oxide synthase gene delivery. *Hypertension*. 1997;30:307–313.
- Johnson DA, Salvesen G, Brown MA, Barrett AJ. Rapid isolation of human kininogens. *Thromb Res*. 1987;48:187–193.
- Smeda JS. Hemorrhagic stroke development in spontaneously hypertensive rats fed a North American, Japanese-style diet. *Stroke*. 1989;20:1212–1218.
- Smeda JS. Cerebral vascular changes associated with hemorrhagic stroke in hypertension. *Can J Physiol*. 1992;70:552–564.
- Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL, Bartkowski HM. Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. *Stroke*. 1986;17:1304–1308.
- Yayama K, Wang C, Chao L, Chao J. Kallikrein gene delivery attenuates hypertension and cardiac hypertrophy and enhances renal function in Goldblatt hypertensive rats. *Hypertension*. 1998;31:1104–1110.
- Murakami H, Yayama K, Chao L, Chao J. Human kallikrein gene delivery protects against gentamycin-induced nephrotoxicity in rats. *Kidney Int*. 1998;53:1305–1313.
- Hirawa N, Uehara Y, Kawabata Y. Subpressor dose of angiotensin II increases susceptibility to the haemodynamic injury of blood pressure in Dahl salt-sensitive rats. *J Hypertens*. 1995;13:81–90.
- Werber AH, Baumbach GL, Wagner DV, Mark AL, Heistad DD. Factors that influence stroke in Dahl salt-sensitive rats. *Hypertension*. 1985;7:59–64.
- Von Lutterotti N, Camargo MJF, Campbell WG Jr, Mueller FB, Timmermans PB, Sealey JE, Laragh JH. Angiotensin II receptor antagonist delays renal damage and stroke in salt-loaded Dahl salt-sensitive rats. *J Hypertens*. 1992;10:949–957.
- Nagaoka A, Shino A, Shibota M. Implication of renal perfusion pressure in stroke of spontaneously hypertensive rats. *Am J Physiol*. 1980;238:H317–H324.
- Smith D, Gilbert M, Owen WG. Tissue plasminogen activator release in vivo in response to vasoactive agents. *Blood*. 1983;66:835–839.
- Dixon BS, Dennis MJ. Regulation of mitogenesis by kinins in arterial smooth muscle cells. *Am J Physiol*. 1997;273:C7–C20.
- Wirth KJ, Scholkens BA, Wiemer G. The bradykinin B2 receptor antagonist WIN 64338 inhibits the effect of des-Arg9-bradykinin in endothelial cells. *Eur J Pharmacol*. 1994;288:R1–R2.
- Christ M, Lusky M, Stoeckel F, Dreyer D, Dieterle A, Michou AI, Pavirani A, Mehtali M. Gene therapy with recombinant adenovirus vectors: evaluation of the host immune response. *Immunol Lett*. 1997;57:19–25.
- Wang Q, Greenburg G, Bunch D, Farson D, Finer MH. Persistent transgene expression in mouse liver following in vivo gene transfer with ΔE1/ΔE4 adenovirus vector. *Gene Ther*. 1997;4:393–400.
- Ooboshi H, Welsh MJ, Rios CD, Davidson BL, Heistad DD. Adenovirus-mediated gene transfer in vivo to cerebral blood vessels and perivascular tissue. *Circ Res*. 1995;77:7–13.
- Doran SE, Ren XD, Betz AL, Pagel MA, Neuwelt EA, Roessler BJ, Davidson BL. Gene expression from recombinant viral vectors in the central nervous system after blood-brain barrier disruption. *Neurosurgery*. 1995;36:965–970.

Editorial Comment

The basic concept of this study is simple and attractive. An adenoviral vector can be injected intravenously and, because there is minimal binding of the virus as blood flows rapidly past the endothelium of blood vessels, probably >95% of the virus is trapped in the liver. The recombinant virus then expresses a product

that is released into the blood to reduce arterial pressure. The authors have demonstrated some gene transfer to other tissues after intravenous injection of recombinant adenovirus, but it is likely that the amount of local expression outside the liver is small and plays only a minor role in the effects that are observed.

Many previous studies with intravenous injection of viruses for gene therapy have focused on gene transfer of the LDL receptor to the liver to treat hypercholesterolemia. The authors of this study are pioneers in the use of intravenous injection of adenovirus to treat hypertension. The novel finding in this study is that the reduction of arterial pressure by a single injection of recombinant virus is of sufficient magnitude and duration to reduce cerebrovascular consequences of salt-induced hypertension. Protection against stroke is probably due, in large part, to a reduction in arterial pressure but may also be due in part to other effects of the kallikrein-kinin system.

Gene transfer approaches are currently useful for mechanistic studies of vascular biology. We believe that gene transfer approaches also have the potential for laying the foundation for eventual gene therapy.¹ Because gene therapy is in its early stages of development, with substantial potential risks as well as benefits, gene therapy initially will be attempted for diseases and clinical problems for which there currently are no good therapeutic options. But will gene therapy be useful for common clinical problems, such as hypertension? Certainly, hypertension will not be a prime application for gene therapy initially, but because poor compliance with antihypertensive medications is an enormous problem, the possibility of one or occasional injections of virus to treat hypertension is attractive.

Which vector should be used for gene transfer? "First-generation" adenoviral vectors, such as those used in this study, give rather brief duration of expression, which is sufficient for some applications but detracts from the potential therapeutic value for hypertension. After a flurry of excitement, "second-generation" adenoviruses appear to suffer from similar limita-

tions and, in our opinion, are unlikely to be useful for long-term therapy. But "gutted" adenoviruses,² lentiviruses, and other vectors that are being developed give the promise of providing sufficient duration of expression to be useful for treatment of hypertension. Thus, in the long term, it may be both feasible and attractive to treat common diseases such as hypertension with gene therapy, to minimize the problem of noncompliance with medications.

Where to now? We assume that the authors will continue to use similar approaches to examine mechanisms by which the kallikrein-kinin system protects against hypertension and stroke. In addition, it will be attractive to determine whether the protective effect of gene transfer of kallikrein is due entirely to gene transfer to the liver or whether local gene transfer to the kidney or brain is sufficient to contribute to the protective effect. It will be exciting to follow this line of research to determine whether gene therapy eventually proves to be useful for treatment of hypertension and its complications in patients who are noncompliant with medications.

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1. Heistad DD, Faraci FM. Gene therapy for cerebral vascular disease. *Stroke*. 1996;27:1688-1693.
2. Schneider G, Morral N, Parks RJ, Wu Y, Koopmans SC, Langston C, Graham FL, Beaudet AL, Kochanek S. Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. *Nat Genet*. 1998;18:180-183.