

Kallikrein gene therapy in hypertension, cardiovascular and renal diseases

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Summary

Somatic gene delivery approaches have received wide attention in recent years as a new technique for studying gene expression and as a potential therapeutic tool in treating both inherited and infectious diseases. Hypertension, which is a polygenic disease influenced by environmental and dietary factors, shows abnormality of the tissue kallikrein-kinin system in its pathogenesis. To demonstrate potential therapeutic effects of gene delivery in treating hypertension, we introduced the human tissue kallikrein gene in the form of naked DNA or an adenoviral vector into hypertensive rats. A single injection of the kallikrein gene caused a sustained blood pressure reduction for several weeks in spontaneously hypertensive rats (SHR), two kidney-one clip (2K1C) Goldblatt hypertensive rats, and Dahl salt-sensitive (Dahl-SS) rats. The expression of human tissue kallikrein in rats receiving gene delivery was identified in tissues relevant to cardiovascular function including the kidney, heart, aorta, lung and liver. Adenovirus-mediated kallikrein gene delivery attenuated cardiac hypertrophy and renal damage in 2K1C and Dahl-SS rats fed on a high salt diet. Kallikrein gene delivery also caused significant increases in renal blood flow, glomerular filtration rate and urine flow as well as in water intake, urine excretion, urinary electrolyte output, kinin, nitrite/nitrate (NO_x) and cGMP levels. These findings are consistent with the mechanisms of blood pressure reduction and enhanced renal function mediated via kinin through a NO-cGMP dependent signal transduction pathway following kallikrein gene delivery. The ability of kallikrein gene delivery to produce a wide spectrum of beneficial effects makes it an excellent candidate in treating hypertensive, cardiovascular and renal diseases.

I. Tissue kallikrein and hypertension

Essential hypertension is a polygenic disease which is governed by the combined action of several genes and results in an increase in blood pressure. Hypertensive subjects are more likely to develop other cardiovascular diseases such as coronary heart disease, congestive heart failure and peripheral vascular and renal diseases. There is ample evidence documenting the role of the tissue kallikrein-kinin system in the pathogenesis of hypertension (Katori and Majima, 1996; Margolius, 1995). Extensive epidemiological studies showed that urinary kallikrein levels are inversely correlated with blood pressure (Zinner et al., 1978; Margolius et al., 1974). Furthermore, a large family pedigree study has shown that

a dominant allele expressed as high urinary kallikrein excretion may be associated with a decreased risk of essential hypertension (Berry et al., 1989). Since renal kallikrein originates from the kidney, these studies suggest that renal kallikrein defects may contribute to the development of human hypertensive diseases. In addition, reduced urinary kallikrein levels have been observed in a number of genetically hypertensive rats (Ader et al., 1985; Margolius et al., 1972). Several restriction fragment length polymorphisms (RFLP)s have been mapped in the tissue kallikrein gene and their regulatory regions in spontaneously hypertensive rats (SHR) (Woodley-Miller et al., 1989). These findings indicate a possible difference in the tissue kallikrein gene locus between SHR and normotensive Wistar-Kyoto (WKY) rats. Furthermore, a

tissue kallikrein RFLP has been shown to cosegregate with high blood pressure in the F2 offspring of SHR and normotensive Brown Norway crosses suggesting a close linkage between the kallikrein gene locus and the hypertensive phenotype of SHR (Pravenec et al., 1991). These findings combine to suggest that low renal kallikrein levels may contribute to hypertension and that high urinary kallikrein may offer a protective effect against the development of high blood pressure and renal diseases.

II. Vasodilating kallikrein-kinin system counter-balances vasoconstricting renin-angiotensin system

Tissue kallikrein (E.C. 3.4.21.35) belongs to a subgroup of serine proteinases which process kininogen substrates and release vasoactive kinin peptides (**Figure 1**). The well recognized function of tissue kallikrein is mediated by kinins. Kinins are cleaved by kinin degrading enzymes to produce a number of kinin metabolites or inactive fragments. Intact kinins bind to B₂ receptors while kinin metabolites, such as Des-Arg⁹-bradykinin or Des-Arg¹⁰-Lys-bradykinin, bind to B₁ receptors. The binding of kinins to the B₂ receptor activates second messengers which trigger a broad spectrum of biological effects such as vasodilation, smooth muscle contraction

and relaxation, inflammation, pain and cell proliferation (Bhoola et al., 1992). Activation of the B₁ receptor may induce biological effects such as inflammation, cell proliferation and vasoconstriction or vasodilation (Marceau, 1995). The vasodilating kallikrein-kinin system is linked to the vasoconstricting renin-angiotensin system by angiotensin converting enzyme (ACE), also known as kininase II, a kinin degrading enzyme. Renin converts angiotensinogen to angiotensin I which is then cleaved by ACE to produce the potent vasoconstrictor, angiotensin II. Administration of ACE inhibitors causes inhibition of angiotensin II production as well as accumulation of kinin. Therefore, the anti-hypertensive effect of ACE inhibition could be attributed, in part, to increased kinin levels (**Figure 1**). The renin-angiotensin system is well known for its important role in the development and maintenance of hypertension in both essential hypertensive patients and in animal models of hypertension (Rosenthal, 1993; Fyhrquist et al., 1995). Interruption of the renin-angiotensin system by pharmacological manipulations can control high blood pressure and other cardiovascular complications (Nicholls et al., 1994; Linz et al., 1995). Hypertension could result from either an excess of vasoconstrictive substances or a deficiency of vasodilating substances. Therefore, pharmacological and/or genetic manipulation of the vasodilating kallikrein-kinin system could potentially counter-balance the vasopressor renin-angiotensin system in blood pressure regulation.

Figure 1. Mechanisms of tissue kallikrein in blood pressure regulation.

III. Kallikrein protein therapy in hypertension

Intravenous infusion of tissue kallikrein or kinin results in a transient reduction of blood pressure which lasts only 1-2 min (Chao and Chao, 1997; Schachter, 1969). The short duration of tissue kallikrein/kinin in the circulation is due to the presence of tissue kallikrein inhibitors in the circulation as well as kinin degrading enzymes in the vasculature. Oral administration of purified pig pancreatic kallikrein has been used to temporarily lower both the supine and upright blood pressures of hypertensive patients (Overlack et al., 1981; Ogawa et al., 1985). However, continuous oral kallikrein intake three times daily was required to maintain the blood pressure-lowering effect. The benefit of kallikrein-induced blood pressure reduction disappeared quickly upon the termination of oral kallikrein intake. Therefore, protein therapy is not considered a practical approach for antihypertensive therapy. Gene delivery would be the only alternative designed to circumvent these difficulties. To evaluate the role of the tissue kallikrein-kinin system and potential therapeutic effects in hypertensive and cardiovascular diseases, we employed molecular genetic approaches by manipulating the expression of the genes encoding tissue kallikrein-kinin system components in intact animals.

IV. Transgenic mice expressing human tissue kallikrein or bradykinin B₂ receptor are hypotensive

Transgenic technologies were employed for the development of transgenic mouse lines expressing the human tissue kallikrein or the human bradykinin B₂ receptor gene under the control of various promoters (Wang et al., 1994; Song et al., 1996; Wang et al., 1997a). The human tissue kallikrein gene under the control of the mouse metallothionein promoter, a metal-responsive element (MRE), was first introduced into mouse embryos via microinjection, and transgenic mouse lines expressing human tissue kallikrein were established (Wang et al., 1994). The transgenic mice overexpressing human tissue kallikrein were permanently hypotensive throughout their lifetime, compared to their control littermates (Chao and Chao, 1996). In order to determine the role of circulating tissue kallikrein in blood pressure regulation, transgenic mice with liver-targeted expression of human tissue kallikrein under the control of a mouse albumin enhancer and promoter were developed (Song et al., 1996). Three lines of independently established transgenic mice were

hypotensive. The blood pressure of these transgenic mice expressing human tissue kallikrein can be restored by aprotinin, a tissue kallikrein inhibitor, or by incatibant (Hoe 140), a specific bradykinin B₂ receptor antagonist (Song et al., 1996). Since human tissue kallikrein is capable of processing mouse kininogen to produce kinins (Wang et al., 1994), these results suggest that hypotension in kallikrein transgenic mice is mediated by binding of kinin to bradykinin B₂ receptors. This notion is further supported by the finding that heterozygous transgenic mouse lines expressing human bradykinin B₂ receptor under the control of Rous sarcoma 3'-LTR are hypotensive (Wang et al., 1997a). Together, these results provide direct molecular evidence linking the physiological function of the tissue kallikrein-kinin system in blood pressure regulation. Since it is not possible to introduce the human tissue kallikrein gene into hypertensive patients by the transgenic approach, we explored the potential of gene therapy in treating hypertension by introducing the human tissue kallikrein gene into hypertensive animal models by somatic gene delivery.

V. Systemic delivery of the naked human tissue kallikrein gene reduces blood pressure in spontaneously hypertensive rats

To evaluate potential therapeutic effects of tissue kallikrein in hypertension, SHR were subjected to somatic gene therapy. The human tissue kallikrein gene or cDNA constructs were created under the promoter control of MRE, albumin, cytomegalovirus or Rous sarcoma virus 3'-LTR (Wang et al., 1994; Xiong et al., 1995; Chao et al., 1996). The human tissue kallikrein gene in the form of naked plasmid DNA was introduced into SHR via intravenous, intraportal vein or intraperitoneal injections. A single injection of the naked human kallikrein plasmid DNA caused a significant delay in blood pressure increase in SHR for more than 6 weeks, as compared to control SHR injected with the vector DNA (Xiong et al., 1995; Chao et al., 1996; Wang et al., 1995). The extent of blood pressure reduction was dependent on the dose of DNA injected, time post injection, gender of the animals, the promoter directing kallikrein expression and the route of injection (Chao et al., 1996; Chao et al., 1997a). Although intravenous delivery of the kallikrein gene into young adult or adult male SHR consistently produced a delay in blood pressure increase in SHR, kallikrein gene delivery did not have significant effects on the blood pressure reduction of adult female SHR (Chao et al., 1997a).

The gender difference in response to kallikrein gene therapy in SHR was not expected and it may be attributed to a higher basal expression level of tissue kallikrein in female rats than in male rats. This notion is supported by the observation that tissue kallikrein mRNA levels are significantly higher in the kidney of adult female rats than in male rats (Gerald et al., 1986). Ovariectomized rats showed a significant reduction in tissue kallikrein mRNA levels and in immunoreactive tissue kallikrein content in the kidney which can be corrected by estrogen and progesterone replacement (Gerald et al., 1986). Furthermore, tissue kallikrein levels in humans are apparently regulated by sex hormones, as urinary kallikrein levels in women are higher than in men, decrease with age, and peak at the progestinic phase of the menstrual cycle (Albano et al., 1994). The regulation of human tissue kallikrein by sex hormones is consistent with the identification of potential estrogen response elements in the promoter region of the human tissue kallikrein gene (Murray et al., 1990; Madeddu et al., 1991). Therefore, a lack of response to kallikrein gene therapy in female SHR may be attributed to high expression of tissue kallikrein at the transcriptional level in females. Moreover, sex dimorphism of bradykinin B₂ receptor mRNA and differential cardiovascular responses to early blockade of bradykinin receptors in male vs. female rats indicates a gender difference in the regulation of cardiovascular function (Madeddu et al., 1996). Collectively, these results suggest that higher expression of tissue kallikrein-kinin system components in females than in males may be a contributing factor to vascular function and responsiveness in hypertensive animal models.

VI. Adenovirus-mediated kallikrein gene delivery reduces blood pressure in genetically and experimentally-induced hypertensive rats

Although somatic delivery of the human tissue kallikrein gene in the form of plasmid DNA produces a prolonged delay in blood pressure increase, the efficiency of cellular uptake of the naked DNA and the expression of the gene product are limited. To improve the efficiency of foreign gene expression in animal models following somatic gene delivery, we constructed an adenovirus vector carrying the human tissue kallikrein gene under the control of cytomegalovirus or Rous sarcoma virus 3'-LTR. Adenovirus-mediated gene delivery results in high efficiency expression of human tissue kallikrein. A profound and rapid blood pressure reduction was observed

1 to 2 days post gene delivery. The delay in blood pressure increase lasted for 4-6 weeks in spontaneously hypertensive rats, (SHR), two kidney-one clip (2K1C) Goldblatt hypertensive rats and Dahl-SS rats fed on a high salt diet (Yayama et al., 1997; Chao et al., 1997b; Jin et al., 1997). When the same amounts of the adenovirus carrying the human tissue kallikrein gene or the control adenovirus carrying the LacZ gene under the cytomegalovirus (CMV) promoter control were injected into normotensive WKY rats, the blood pressure remained normotensive during 7 weeks post gene delivery in both the experimental and control groups (Jin et al., 1997). Human tissue kallikrein levels in sera and urine of WKY rats were similar to those of SHR following kallikrein gene delivery. Therefore, the differential effects of gene delivery on blood pressures between hypertensive and normotensive rats may be attributed to their different sensitivities to the exogenous kallikrein in the vasculature. **Table 1** summarizes the comparison of kallikrein gene delivery based on naked DNA or adenovirus vector in blood pressure reduction.

VII. Local delivery of the tissue kallikrein gene reduces high blood pressure in hypertensive rats

Similar to systemic delivery, local delivery of the human tissue kallikrein gene causes a sustained blood pressure reduction in SHR (Xiong et al., 1995). For example, intramuscular delivery of the naked DNA into SHR produced a prolonged reduction of blood pressure which lasted for more than 8 weeks (Xiong et al., 1995). Central administration of the human tissue kallikrein gene via intracerebroventricular (ICV) injection caused a delay in blood pressure increase in SHR, as compared to control rats receiving the vector DNA or injected with adenovirus containing the LacZ gene (Wang et al., 1997b). Adenovirus-mediated delivery of the human tissue kallikrein or kallistatin gene into rat salivary gland via direct intracapsular injection results in expression of human kallikrein or kallistatin in the salivary gland (Wang et al., 1997c; Xiong et al., 1997). Human tissue kallikrein can also be detected in sera and saliva after direct gene delivery into salivary glands, demonstrating that locally synthesized kallikrein in the salivary gland can be secreted into both the vascular compartment and saliva. Therefore, local delivery of the kallikrein gene into salivary glands may provide a unique opportunity for studying the role of the kallikrein-kinin system in the salivary gland.

Table 1. Kallikrein gene delivery based on naked DNA or adenovirus vector in blood pressure reduction

Vector	Naked DNA	Adenovirus
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	CMV-cHK	Ad.CMV-cHK
Onset	1-2 weeks	1-2 days
Duration	6-8 weeks	4-6 weeks
Repeated Administration	yes	no
Expression Efficiency	low	high
Serum Kallikrein Levels	n.d.	up to 500 ng/ml
Site of Expression	liver, kidney, heart, lung	liver>>kidney>aorta>heart
Immune Response	n.d.	yes

n.d.: not detectable.

Table 2. Kallikrein gene delivery attenuates hypertension, cardiac hypertrophy, renal injury and stenosis

Rat Models	Reference	Blood Pressure	Cardiac Hypertrophy	Renal Injury	Stenosis
SHR	Xiong et al, 1995	↓	-	-	-
Dahl-SS	Chao et al, 1997b	↓	↓	↓	-
2K1C Goldblatt	Yayama et al, 1997	↓	↓	-	-
Nephrotoxicity	Murakami et al, 1997	-	↓	↓	-
Angioplasty		-	-	-	↓

SHR: spontaneously hypertensive rats; Dahl-SS: Dahl salt sensitive rats; 2K1C Goldblatt: two kidney, one clip Goldblatt hypertensive rats. "-" not observed.

VIII. Adenovirus-mediated kallikrein gene delivery protects cardiovascular and renal function

Long-term infusion of purified rat tissue kallikrein via a minipump has been shown to attenuate glomerular sclerosis without affecting the blood pressure of Dahl-SS rats fed on a high salt diet (Uehara et al., 1997). This finding indicates that a continuous supply of tissue kallikrein might provide protective effects on salt-induced renal injury. Our recent studies showed that adenovirus-mediated kallikrein gene delivery not only caused a prolonged blood pressure reduction but also reduced left ventricular weight and cardiomyocyte size as well as attenuated glomerular and tubular damage in Dahl-SS rats fed on a high salt diet (Chao et al., 1997b). Moreover, kallikrein gene delivery into 2K1C Goldblatt hypertensive rats significantly attenuated cardiac hypertrophy and improved renal function by increasing glomerular filtration rate, renal blood flow and urine flow (Yayama et al., 1997). The protective effects of kallikrein gene delivery in renal tubular injury was also observed morphologically in rats with gentamycin-induced nephrotoxicity (Murakami et al., 1997). Moreover, kallikrein gene delivery inhibited neointimal thickening in balloon-injured rat artery. Collectively, these results lend strong support to an important role of tissue kallikrein in cardiovascular and renal function. **Table 2** summarizes

the beneficial effects of kallikrein gene delivery on hypertension, cardiac hypertrophy, renal injury and stenosis.

IX. Expression and localization of human tissue kallikrein in rats post gene delivery

Expression of human tissue kallikrein mRNA in rats following injection of naked plasmid DNA was identified by reverse transcription-polymerase chain reaction (RT-PCR) followed by Southern blot analysis using specific oligonucleotide probes for human tissue kallikrein. Human tissue kallikrein mRNA can be identified in heart, aorta, kidney, adrenal gland, lung and liver of rats injected with the kallikrein gene but not in the corresponding tissues of rats injected with the control DNA. Low levels of immunoreactive human tissue kallikrein can be detected in rat tissues following kallikrein gene delivery by a specific enzyme-linked immunosorbent assay (ELISA) (Chao et al., 1996; Wang et al., 1995). Expression of adenovirus-mediated gene delivery via intravenous injection was readily detectable in the hepatocytes 3 days post delivery of the LacZ gene by blue staining with demonstrated β -galactosidase activity. The expression of recombinant human tissue kallikrein in rat liver or kidney was rapidly secreted into the circulation and urine. Although an accurate evaluation of the level of

adenovirus-mediated transduction can not be determined, following the time-course of human tissue kallikrein levels in the serum could well serve as an indicator of efficiency in the expression of the foreign gene. We observed that human tissue kallikrein levels in the serum reached a peak at days 3-5 and declined gradually following adenovirus-mediated kallikrein gene delivery into SHR. Adenovirus-mediated gene delivery also produced similar serum profiles of human tissue kallikrein levels in hypertensive Dahl-SS rats and Goldblatt hypertensive rats. The reason for the decline of human tissue kallikrein levels 1 week after kallikrein gene delivery is not clear at this time, but two likely causes are inactivation or clearance of the adenovirus by the immune system. Immunoreactive human tissue kallikrein was not detected in control rats injected with the control adenovirus containing the lacZ gene. These results show that the human tissue kallikrein gene is expressed in tissues relevant to cardiovascular and renal function post systemic gene delivery.

X. Mechanisms of kallikrein gene therapy on blood pressure reduction

Tissue kallikrein cleaves LMW kininogen substrate to release the kinin product by limited proteolysis. Kinins are capable of binding to B₂ receptors, activating second messengers in target tissues via a G-protein coupled signal transduction pathway and triggering biological effects such as vasodilation or vasoconstriction (Bhoola et al., 1992; Linz et al., 1995). There are multiple steps to inhibit or block the tissue kallikrein-kinin system, such as aprotinin, a potent tissue kallikrein inhibitor or inactibant (Hoe 140), a bradykinin B₂ receptor antagonist. Kallikrein gene delivery caused a prolonged delay in the blood pressure increase in SHR and the hypotensive effect of kallikrein gene delivery was abolished by aprotinin, a potent tissue kallikrein inhibitor. This suggests that the hypotensive effect of kallikrein gene delivery is due to the expression of functional kallikrein (Wang et al., 1995). The antihypertensive effect in SHR post kallikrein gene delivery was reversed by inactibant (Hoe 140), a specific bradykinin B₂ receptor antagonist, suggesting that the blood pressure-lowering effect following somatic gene delivery of human tissue kallikrein is mediated by a bradykinin B₂ receptor-mediated signal transduction pathway (Xiong et al., 1995). Adenovirus-mediated kallikrein gene delivery into various hypertensive rat models led to significant increases of urinary kinin, nitrite/nitrate (NO_x) and cGMP levels, suggesting that blood pressure reduction was mediated via kinin through a NO-cGMP dependent signal transduction pathway (Figure 1).

XI. Antisense inhibition of the tissue kallikrein-kinin system

An antisense inhibition strategy, based on interference of information flow from genes to proteins, was used to determine the role of the tissue kallikrein-kinin system in blood pressure regulation. Acute intracerebroventricular (ICV) injection of antisense oligonucleotides which block rat kininogen mRNA or bradykinin B₂-receptor mRNA translation caused a significant blood pressure increase in SHR which returned to basal levels within 24 hours (Madeddu et al., 1996). Prolonged vasopressor effects were observed after repeated injections of antisense oligonucleotides. Mean arterial blood pressure was not altered by intravenous injection of antisense oligonucleotides or by central injection of sense or scrambled oligonucleotides. Uptake of the antisense oligonucleotides of rat kininogen mRNA or bradykinin B₂-receptor mRNA was detected in the hippocampus, thalamus and hypothalamus periventricularis one hour after the central injection of fluorescein isothiocyanate-conjugated antisense oligonucleotides. Kininogen levels were significantly lower in the brain of SHR injected with antisense kininogen oligonucleotides via the ICV delivery compared with controls injected with the sense oligonucleotides. These results indicate that the brain kallikrein-kinin system plays a role in the central regulation of blood pressure and suggest that this system may exert a protective action against further elevation of blood pressure in SHR. In contrast, ICV injection of the antisense oligonucleotides targeted to rat B₁ receptor mRNA induced a profound blood pressure reduction in SHR while similar administration of sense or scrambled oligonucleotides had no effect on their blood pressure (Emanueli et al., 1997). The fact that B₁ receptor blockade can decrease blood pressure in SHR suggests that activation of B₁ receptors by brain kinin metabolites exerts a vasoconstrictor activity. These findings suggest that bradykinin B₁ and B₂ receptors play different roles in the central regulation of blood pressure.

XII. Concluding remarks

We showed that kallikrein gene delivery into various rat models exhibits protection such as reduction of high blood pressure, attenuation of cardiac hypertrophy, inhibition of renal damage and restenosis (Table 1). The ability of kallikrein gene transfer to produce such a wide spectrum of beneficial effects makes it an excellent candidate for treating salt-related hypertension as well as cardiovascular and renal diseases. Somatic gene therapy has several unique advantages over traditional pharmaceuticals. For example, gene therapy produces long-lasting effects. It is inexpensive and simple to administer. Gene therapy has the potential to offer

permanent gene replacement or to treat diseases not treatable by drugs. Several important factors should be taken into consideration in gene delivery using animal models. These factors include animal age, gender, species, strains, and the choice of local or systemic delivery. Under certain conditions, it might be necessary to deliver genes into specific organs or tissues. Catalytic vs. stoichiometric actions of the therapeutic proteins and availability of the substrate in vivo for an enzyme should be considered. Attention should also be directed to potential immune responses to DNA, gene products, and vectors. Also, in order for the therapeutic proteins or peptides to exhibit their function in vivo, the half-life of these gene products should be sufficiently long and they should not have any significant side-effect. Information obtained from kallikrein gene delivery studies in genetically and experimentally hypertensive animal models is crucial for future clinical applications in treating hypertensive, cardiovascular and renal diseases by gene therapy.

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