Human Tissue Kallikrein Induces Hypotension in Transgenic Mice

Jing Wang, William Xiong, Zhirong Yang, Tia Davis, Michael J. Dewey, Julie Chao, Lee Chao

Abstract We investigated the role of the kallikrein-kinin system in blood pressure control by developing transgenic mice overexpressing human tissue kallikrein. Two lines of transgenic mice carrying the human tissue kallikrein gene under the control of the mouse metallothionein metal-responsive promoter were established. Human tissue kallikrein was identified in pancreas, salivary gland, kidney, liver, and spleen of the transgenic mice by a specific radioimmunoassay for human tissue kallikrein. The immunoreactive human tissue kallikrein reached high levels in the circulation. The linear displacement curves for the transgenic product were parallel with the human tissue kallikrein standard curve, indicating their immunologic identity. The expression of human tissue kallikrein transcript in the transgenic mice was further confirmed by Northern blot analysis and by reverse transcription–polymerase chain reaction followed by Southern blot. Both lines of transgenic mice overexpressing human tissue kallikrein had significantly lowered blood pressure (86.4±13.5 mm Hg [mean±SD], n=8 and 78.9±12.4 mm Hg, n=8) compared with control mice (100.9±5.0 mm Hg, n=8). Induction with zinc did not lower the blood pressure further despite elevated expression of the transgene. Administration of aprotinin, a potent tissue kallikrein inhibitor, restored the blood pressure of the transgenic mice but had no significant effect on control littermates. Our findings raise the possibility of tissue kallikrein being a powerful modulator of blood pressure and provide a new animal model for the study of blood pressure regulation.

Key Words • kallikrein • mice, transgenic • hypotension • gene expression • aprotinin

The kallikrein gene family is a set of tightly clustered, closely related serine proteinases that exhibit limited proteolysis. Members of the family are involved in processing of various polypeptide precursors for production of biologically active forms. The best-known physiological substrate for tissue kallikrein is hepatic-derived kininogen, which exists in both high molecular weight and low molecular weight forms in the circulation and from which the vasodilator peptide kinin is released. The regulation of the kallikrein-kinin system has been reviewed recently.1

Despite the difficulties in ascertaining the physiological functions of tissue kallikrein, extensive evidence has associated kallikrein with blood pressure regulation. The observation that urinary excretion of tissue kallikrein was significantly reduced in hypertensive individuals was recorded as early as 19342 and confirmed more than three decades later.3,5 The results of a recent study involving 57 Utah pedigrees indicate that a dominant allele expressed as high urinary kallikrein excretion may be associated with a decreased risk of essential hypertension.6 In fact, the blood pressure of hypertensive patients can be lowered temporarily by oral administration of pig pancreatic kallikrein.7,8 Tissue kallikrein has been linked with blood pressure regulation by restriction fragment length polymorphisms9 and the cosegregation of high blood pressure with a restriction fragment length polymorphism marking the kallikrein gene family in a hypertensive rat model.10 Reduced urinary kallikrein excretion also has been described in a number of genetic hypertensive rats.11-14 Taken together, these findings suggest that the reduction in renal kallikrein activity may contribute to the pathogenesis of hypertension and that high urinary kallikrein could have a protective effect against hypertension.6 However, molecular evidence documenting the direct link between a kallikrein gene and alteration of blood pressure is still lacking because of the difficulty in eliciting and maintaining high levels of either kinin or kallikrein in experimental animals.

The recent advent of transgenic technology has been instrumental in providing new insights into the mechanisms of development, gene regulation, and the physiological functions of genes. Transgenic mice created by germ line transfer of exogenous genes offer valuable animal models for human diseases. In the present study, we have established two lines of transgenic mice that carry the human tissue kallikrein gene under the control of the mouse metallothionein metal–responsive promoter and showed that the transgenic mice overexpressing human tissue kallikrein have a significant reduction in blood pressure. This transgenic animal model should provide an excellent opportunity for researchers to investigate the role of tissue kallikrein in blood pressure regulation.

Methods

Transgene Construction and DNA Preparation

We excised the mouse metallothionein metal–responsive element (MRE) in mMT-115 and fused it with a 5.6-kb human kallikrein gene16 containing the entire coding sequence along with 300 bp of the 3' flanking sequence (HK) to generate the tissue kallikrein transgene MRE-PHK. First, a 5'-region frag-
ment including exon 1 and part of intron 1 of the HK gene, with a Bgl II at its 5' end and two BamHI sites at its 3' end, was amplified by polymerase chain reaction (PCR). This fragment (PCR-HK) then was made cohesive at the 5' end with the Bgl II half site. Second, a fragment of the mMT-1 gene promoter region was released with the Kpn I (−650) and Bgl II (+65) sites (the last 262 bp form the metal-responsive element) and was ligated to the PCR-HK fragment through the Bgl II site. The resulting fragment was inserted into pM13 mp18 at Kpn I and HinclI sites for propagation (mp18-MRE), and the fragment from Kpn I to the first BamHI site in the HK gene was released with these two enzymes. The Kpn I--BamHI fragment was then ligated to a plasmid hosting the complete HK gene to generate the final plasmid construct pHK-MRE. All constructs, intermediate or final, were confirmed by either restriction mapping or sequencing.

The transgene fragment MRE-PHK was isolated from the plasmid pHK-MRE by HindIII/EcoRI double digestion and agarose gel electrophoresis. The fragment was purified with the USB Clean kit (USB, Cleveland, Ohio), using the procedures suggested by the manufacturer. DNA concentration was measured by absorbance at 260 nm and confirmed by comparing with a λ HIndIII marker on electrophoresis. The DNA was then digested with exonuclease injection buffer (5 mmol/L Tris-HCl, pH 7.4, 0.1 mmol/L EDTA) to a final concentration of 2 mg/mL and centrifuged at 100 000g for 1 hour to remove detergent particles before injection.

**Generation and Identification of Transgenic Mice**

The purified MRE-PHK DNA was microinjected into C57BL/6 × DBA/2 F1 embryos that were allowed to develop to term in the uterus of pseudopregnant females. Tail DNA from each offspring was screened by Southern blot analysis with a 1.5-kb human tissue kallikrein cDNA. Among 52 mice born from microinjected embryos, two transgenic founders, No. 483 and No. 519, were identified. These were bred with C57BL/6, and each founder transmitted the transgene to its progeny. The results reported were from mice born of the first or second backcross generations to C57BL/6. The control mice were nontransgenic littermates from these matings.

Tails from prepubertal mice were obtained and each cut into approximately 10 to 12 pieces with a razor blade. The fragments from each tail were then suspended in 600 μL of “tail buffer” (50 mmol/L Tris-HCl, pH 8.0, 100 mmol/L EDTA, 100 mmol/L NaCl, 1% sodium dodecyl sulfate), and 37 μL proteinase K (10 mg/mL) was added to the suspension. The suspension was incubated at 55°C overnight with occasional mixing. Additional proteinase K was added if necessary until all the clumps disappeared and only hair was left. The suspension was then extracted two to three times with equal volumes of phenol/chloroform and one milliliter of room-temperature 100% ethanol was added to the final supernatant, and the suspension was allowed to form strings of DNA. The DNA was then spun out with a micropipette yellow tip and rinsed with 80% ethanol. The washed DNA was allowed to air dry for approximately 30 minutes and subsequently was dissolved in 100 μL TE buffer (10 mmol/L Tris-HCl, pH 7.4, 0.1 mmol/L EDTA). Generally, 100 to 300 μg DNA can be obtained from each tail.

For identification of transgenic mice or gene dosage analysis, DNA extracted from tails was digested with either EcoRI or Pst I, followed by agarose gel electrophoresis and Southern blot hybridization to a human tissue kallikrein cDNA probe labeled with [α-32P]dATP. The labeling was performed with a nick translation kit (GIBCO-BRL, Gaithersburg, Md). The human cDNA probe does cross-hybridize with mouse tissue kallikrein genes, but the transgenic animals showed intense unique human transgene banding patterns in a mouse tissue kallikrein gene background.

All experimental protocols were approved by the Institutional Animal Research Committee of the Medical University of South Carolina and were carried out according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

**Expression of the Human Tissue Kallikrein Transgene mRNA**

Total RNA was isolated from mouse tissues by homogenization in guanidine isothiocyanate and centrifugation through CsCl essentially as described. Northern blot was carried out using a human tissue kallikrein cDNA probe labeled with [α-32P]dATP by nick translation. A human tissue kallikrein-specific oligonucleotide (HK-1) with the sequence 5'-GCCCAAGGGACCTAGGC-3' was used to direct reverse transcription (RT) of human tissue kallikrein mRNA using 1 μg total RNA. PCR was carried out with a kit supplied by Perkin-Elmer Cetus (Norwalk, Conn) with the reverse-transcribed product as the template. The HK-1 oligonucleotide was used as the 3' primer. A second human tissue kallikrein-specific oligonucleotide (HK-10) with the sequence 5'-CATTTCAGCACCCTTTCCA-3' was used as the 5' primer. The product of amplification using this pair of primers is a 700-bp DNA fragment. Amplification was performed over 30 cycles with 1 minute of denaturation at 94°C, 2 minutes of annealing at 55°C, and 3 minutes of extension at 72°C. The RT-PCR products were analyzed subsequently by Southern blot using a 32P-labeled oligonucleotide probe (HK-3) with the sequence 5'-AGCACCTTCACAGGCTC-3', which hybridizes to a specific region between HK-1 and HK-10.

**Tissue Preparation and Urine Collection for Direct Radioimmunoassay**

Tissues (salivary gland, liver, pancreas, kidney, spleen, and others) obtained from transgenic and control mice were immersed in phosphate-buffered saline, pH 7.0, and homogenized immediately with a polytron. The homogenate was centrifuged in a table-top centrifuge at 1000 to 1500 rpm for 10 minutes. The supernatant was incubated with 0.5% sodium deoxycholate and then centrifuged at 10 000 rpm for 30 minutes. The content of human tissue kallikrein in each tissue extract was determined by a direct radioimmunoassay specific for human tissue kallikrein as described previously. Total protein was determined by the method of Lowry et al. Urinary excretion of human tissue kallikrein by transgenic and control mice, four transgenic mice and four negative littermates were fed regular food for 3 hours before they were placed in metabolic cages supplied with drinking bottles. Urine was collected 24 hours later and centrifuged at a microfuge at 10 000 rpm to remove particles. The supernatants were analyzed for the levels of human tissue kallikrein. Urine samples were collected after the completion of blood pressure measurements to avoid stress.

**Animal Treatments**

The transgene was constructed such that the expression of the human tissue kallikrein gene is under the control of the mouse metallothionein promoter, which is inducible by zinc. Zinc induction of the transgene expression was accomplished by supplying 76 mmol/L ZnSO4 in the drinking water for 2 weeks before blood pressure measurement.

Aprotinin (Bayer AG, Wuppertal-Elberfeld, Germany) was dissolved in sterilized phosphate-buffered saline, pH 7.2, at 5 mg/mL and was administered subcutaneously at 0.5 mg per mouse per day per injection. Both transgenic and control mice were injected daily with aprotinin for 2 days. The aprotinin regimen was adapted from the procedure devised by Jaffa et al in administering aprotinin in rats. After the aprotinin treatment, the blood pressure of the transgenic and control mice was determined.
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target sites of action, such as the kidney, brain, and the animal body because kallikrein has several potential neutralized by a number of compensatory mechanisms moderately elevated tissue kallikrein level could be

consideration was that the transgene had to be ex-

conscious stages, each blood pressure value was the average of four to five readings with at least 3-minute intervals between two readings.

Statistical Analysis

Group data are expressed as mean \( \pm \) SD. Comparison of parameters between control and transgenic mouse groups was made by the Student's \( t \) test. Differences were considered significant at a value of \( P < .05 \).

Results

Generation of Transgenic Mice

The human tissue kallikrein transgene was constructed based on several considerations. The major consideration was that the transgene had to be expressed at very high levels because the effect of a moderately elevated tissue kallikrein level could be neutralized by a number of compensatory mechanisms or inhibitors. The second consideration was to ensure the wide availability of the transgene product in the animal body because kallikrein has several potential target sites of action, such as the kidney, brain, and the vascular system. In addition, we wished to incorporate the possibility of regulating the transgene expression by dietary manipulations for physiological studies. Accordingly, we chose the mouse metallothionein promoter, which directs high-level expression primarily in the liver with many additional sites of expression. The transgene synthesized in the liver could be secreted into the circulation and exert its effect directly on the vascular system or be transported throughout the body and act at secondary sites. The ability to induce the level of the metallothionein promoter expression with zinc in drinking water also was attractive. Based on these considerations, we constructed a fusion gene in which the human tissue kallikrein gene along with 800 bp of the 5' flanking region and 300 bp of the 3' flanking sequence was linked to the mouse MRE \((\text{Fig 1)}\). Two independent founder mice, No. 519 (male) and No. 483 (female), were identified from the initial progenies by Southern blot analysis as described in "Methods."

Expression of the Human Tissue Kallikrein Transgene

The expression of the human tissue kallikrein mRNA was analyzed by Northern blot and RT-PCR. \( \text{Fig 2) shows that both male and female transgenic mice gave rise to a specific product of 700 bp in the liver and kidney that was not detected in any of the negative control mice by RT-PCR (Fig 2A, indicating that the human tissue kallikrein gene was successfully transcribed in the transgenic mice. Northern blot analysis showed that the liver of both male and female transgenic mice gave rise to a single hybridization band that corresponded in size to that of the predicted mRNA initiated from the MRE promoter (Fig 2B). Administration of \( \text{ZnSO}_4 \) for 2 weeks significantly increased the transcriptional level of the transgene in the liver (Fig 2B).

Tissue distribution of human tissue kallikrein mRNA in the transgenic mice was analyzed by RT-PCR followed by Southern hybridization using a human kallikrein–specific oligonucleotide. The results indicated that the transgene mRNA was expressed in a large number of tissues as expected (Fig 3). A direct radioimmunoassay using specific anti-human tissue kallikrein antibodies was used to detect the transgene product in various tissues. The results in Table 1 show that human tissue kallikrein was predominantly found in the pancreas, salivary gland, kidney, liver, and spleen. Low levels of the transgene product also were detected in the lung, heart, brain, and skeletal muscle. Human tissue kallikrein was not detected in tissues of the control mice. The linear displacement curves for the transgene products were parallel with the human tissue kallikrein standard curve, indicating their immunologic identity (Fig 4). The tissue distribution pattern was in accordance with the report of the expression pattern of the
metallothionein gene, indicating that the human kallikrein transgene was controlled by the metal-responsive promoter. Furthermore, transgenic mice treated with ZnSO₄ have elevated levels of human tissue kallikrein in the pancreas, kidney, and liver compared with transgenic mice without ZnSO₄ induction (Table 1). However, the response to ZnSO₄ treatment was not as significant in the salivary gland and spleen. The overall transgene expression level was twofold to threefold higher in mice of line 519 than those of line 483.

All of the transgenic mice analyzed showed high levels of circulating human tissue kallikrein, 11-fold to 115-fold higher than those in normal human serum. Transgenic mice treated with ZnSO₄ expressed 1.6-fold to 3-fold higher levels of circulating human tissue kallikrein over untreated transgenic controls (Table 1).

Urinary kallikrein level has been considered a valid marker for evaluating diseases associated with hypertension. Analysis of 24-hour urine samples showed considerable urinary excretion of human tissue kallikrein in both lines of transgenic mice (Table 2). Human tissue kallikrein was not detected in the urine of the control mice.

**Blood Pressure Analysis of Transgenic Mice**

Measurement of systolic blood pressure of the transgenic lines by the tail-cuff method revealed that the progeny inheriting the human tissue kallikrein transgene had significantly lowered blood pressure (Table 3). Both lines of transgenic mice developed hypotensive phenotypes. Transgenic mice carrying the human tissue kallikrein transgene exhibited systolic blood pressures of 86.4±13.5 mm Hg (line 483) and 78.9±12.4 mm Hg (line 519) (mean±SD, n=8), an average of 14.5 and 22 mm Hg lower than their negative siblings (100.9±5.0 mm Hg, mean±SD, n=8). Blood pressure of the trans...
TABLE 1. Tissue Distribution of Human Tissue Kallikrein

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mouse, ng Human Tissue Kallikrein/mg Total Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls, Zn (-) ng/mg</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>Serum, ng/mL</td>
<td>n.d.</td>
</tr>
<tr>
<td>Pancreas</td>
<td>n.d.</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>n.d.</td>
</tr>
<tr>
<td>Kidney</td>
<td>n.d.</td>
</tr>
<tr>
<td>Liver</td>
<td>n.d.</td>
</tr>
<tr>
<td>Spleen</td>
<td>n.d.</td>
</tr>
<tr>
<td>Lung</td>
<td>n.d.</td>
</tr>
<tr>
<td>Heart</td>
<td>n.d.</td>
</tr>
<tr>
<td>Brain</td>
<td>n.d.</td>
</tr>
<tr>
<td>Muscle</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d. indicates not detected; Zn (-) or Zn (+), with or without ZnSO₄ induction.

genic and control mice did not change significantly after ZnSO₄ induction, despite elevated levels of human tissue kallikrein in various tissues and the circulation (Table 1). The difference in blood pressure between transgenic and control mice was unperturbed by ZnSO₄ induction. There was no significant difference between blood pressure values of these two transgenic lines, either before or after ZnSO₄ induction.

To examine whether the blood pressure reduction in the transgenic mice was due to tissue kallikrein, we administered aprotinin subcutaneously into male transgenic and control mice. After aprotinin treatment, the blood pressure of the transgenic mice increased by an average of 18.6 mmHg (P<.05), and the control mice exhibited no significant change in blood pressure (Table 4). Administration of aprotinin was able to restore the blood pressure of transgenic mice close to control levels.

To verify the validity of the tail-cuff method for blood pressure measurement, we compared the values of blood pressure obtained from the indirect tail-cuff method with those from direct intra-arterial cannulation (Table 5). The differences in blood pressure between transgenic and control groups were in close agreement when measurements from the tail-cuff method were compared with those of anesthetized and conscious mice after arterial cannulation. The results indicated that the blood pressure values obtained by the tail-cuff method were consistent with direct readings by intra-arterial cannulation.

Discussion

The present study demonstrates that a sustained high level of tissue kallikrein in the circulation induces chronic hypotension. Transgenic mice from both lines
TABLE 2. Urinary Excretion of Human Tissue Kallikrein by Transgenic Mice

<table>
<thead>
<tr>
<th>Urine Collection</th>
<th>Control Mice</th>
<th>Transgenic Mice</th>
<th>Transgenic Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2609</td>
<td>2610</td>
<td>2611</td>
</tr>
<tr>
<td>Concentration, ng/mL</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Total amount, ng/24 h</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

n.d. indicates not detected. Values are mean±SD of 3-day urine collections.

(No. 483 and No. 519) expressing high levels of human tissue kallikrein displayed reduced blood pressure. The observation that both independent lineages exhibited the hypotensive effect of the transgene supports the notion that the phenotype is a direct consequence of elevated tissue kallikrein expression rather than spurious insertional inactivation of an endogenous gene. However, there was no apparent blood pressure difference between the two lines despite the fact that the overall transgene expression levels differed between them by a factor of two to three. This result taken together with the failure of a ZnSO4 induction to further reduce blood pressure might suggest that there is a limit to the effects of kallikrein overexpression on blood pressure reduction. Altering kallikrein levels within a certain range affects blood pressure, whereas increasing the levels over a certain threshold may be without effect. The concentration of human kallikrein in both transgenic lines would then be over that threshold. The saturation effect could be explained by cardiovascular homeostasis, which involves many humoral and neural regulatory systems. These systems have their own individual pathways, targets, and regulating mechanisms. However, they interact by various feedback mechanisms to activate or inhibit each other so that it is almost impossible to identify one component that is solely responsible for a specific consequence. Changing one component within this complex system could trigger a series of agonistic or antagonistic reactions for maintaining the homeostasis of a particular living organism. Therefore, it is conceivable that severe hypotension...
Human tissue kallikrein is both responsible and necessary for the hypotensive phenotype of transgenic mice. The mechanisms involved in blood pressure regulation are complex. The use of genetic approaches has become increasingly popular in studying hypertension and blood pressure control in recent years. One outstanding example was reported by Mullins et al.\(^2\) that the mouse Ren-2 transgene in rats caused fulminating hypertension. In another study Steinhelper et al.\(^26\) reported that hepatic expression of the atrial natriuretic factor fusion transgene in mice caused hypertension. Although we have demonstrated tissue kallikrein-induced hypotension in this transgenic mouse model, the mechanisms responsible for lowering blood pressure still remain to be determined. To determine whether human tissue kallikrein cleaves mouse kininogen and releases kinin, we have incubated human kallikrein with mouse low molecular weight kininogen purified by affinity and Mono Q column chromatography.\(^27\) Kinin released after the incubation was determined by a kinin radioimmunoassay.\(^28\) We found that human tissue kallikrein cleaves mouse kininogen substrate with a specific activity of 32.6±10.8 µg kinin released per milligram kallikrein per 30 minutes (mean±SD, n=3), a value comparable to that of rat tissue kallikrein.\(^27\) The ability of aprotinin to reverse the hypotensive effect of the transgene supports the kinin pathway. On the other hand, the transgene product could regulate blood pressure through its natriuretic and diuretic effects in the kidney or through the central regulation of cardiovascular function in the brain, as human tissue kallikrein was detected at both sites. The hypotensive phenotype might be the result of a combination of the above mechanisms. An extensive examination of the physiology of transgenic mice is warranted to determine the etiology of the hypotensive phenotype. Although the precise mechanism is still to be elucidated, our findings raise a highly promising potential for treating hypertension through gene therapy.

**Acknowledgments**

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