Tissue-specific expression and promoter analyses of the human tissue kallikrein gene in transgenic mice

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The expression of the tissue kallikrein gene is tissue-specific and exhibits a complex pattern of transcriptional and post-translational regulation. Information concerning the mechanism of its tissue-specific expression has been limited owing to the lack of suitable cell lines for the expression study. We approached this problem by introducing human tissue kallikrein gene constructs into mouse embryos, creating transgenic lines carrying its coding sequence with varying lengths of the promoter region. One construct (PHK) contained 801 bp in the 5'-flanking region and two deletion constructs contained either 302 bp (D300) or 202 bp (D200) of the promoter region. The expression of human tissue kallikrein in these transgenic mice was monitored by Northern

blot, reverse transcriptase-PCR followed by Southern blot, and radioimmunoassay. In all three lines, human tissue kallikrein was expressed predominantly in the pancreas and at lower levels in other tissues, including salivary gland, kidney and spleen. This pattern was similar to that of tissue kallikrein expression in human tissues. The D300 line has higher levels of transgene expression than the D200 and PHK lines. The results indicate that the 202 bp segment immediately upstream of the translation starting site is sufficient to direct a tissue-specific expression pattern of the human tissue kallikrein gene, and that regulatory elements might exist between -801 and -202.

INTRODUCTION

Tissue kallikrein (EC 3.4.21.35) is a serine proteinase that cleaves kininogen substrates to release vasoactive kinin peptides [1]. Kinins are involved in a number of important biological processes such as inflammation, blood pressure regulation, smooth muscle contraction and relaxation [2–4]. The tissue-specific expression of tissue kallikrein has been studied in several species [5–9]. In the rat, tissue kallikrein is detected in the pancreas [10], salivary gland [11,12], kidney [13,14], spleen [15], brain [16,17], skeletal muscle [18], heart [19] and pituitary gland [17,20]. In humans, tissue kallikrein expression has been identified in various tissues including pancreas [21,22], salivary gland [23], kidney [5,24,25] and neutrophils [26], with pancreas possessing the highest levels of tissue kallikrein expression [23]. The complex pattern of kallikrein gene expression suggests an intricate regulatory mechanism that might be linked to its biological function in various tissues. The diverse functions of kinins strongly implicate the kallikrein-kinin system in a plethora of physiological and pathophysiological events [1,3,6,27].

There are few cell lines that are currently available for studying tissue kallikrein gene expression by transfection assay *in vitro*. Despite an extensive search, we have not been able to identify any kidney or salivary gland cell lines that are actively expressing tissue kallikrein. The use of transgenic technology has thus offered a unique way to study regulatory mechanisms of gene expression *in vivo*. We adopted this technology to investigate the transcriptional regulation of human tissue kallikrein gene expression by promoter deletion analysis. Three gene constructs containing various lengths of the 5' promoter region and the complete human tissue kallikrein coding sequence were used. The first construct (PHK) includes the 801 bp of the 5'-flanking region, the D300 construct includes 302 bp, and the D200

construct includes 202 bp of the 5' region (from the translation starting site). The D200 construct contains the minimal promoter elements, a TATA-box and a CAAT-box, whereas D300 includes an upstream glucocorticoid-binding site and cAMP site. The full-length PHK contains two additional putative hormonal responsive elements, oestrogen- and progesterone-binding sites [7]. Transgenic mice were established with the three constructs, and tissue-specific expression of human tissue kallikrein was characterized.

MATERIALS AND METHODS

Transgene construction and DNA preparation

The PHK construct was generated by cloning the complete human tissue kallikrein gene including 801 bp in the 5'-flanking region [28] into the vector pUC 18. For the two deletion constructs, D200 and D300, a DNA fragment containing the appropriate promoter length was created by PCR with 5' primers located at -213 to -193 (5'-ACAGGTGCGAATTCTCCCTCC-3') and -315 to -295 (5'-GCCTCACTGAATTCTCCCCAG-3') respectively, and 3' primer located at an internal *Bam*HI site (5'-AGGAGAAAGTACTGAGGCTTCC-3'). The fragment was excised with *Eco*RI and *Bam*HI, and subsequently cloned into mp18 for sequence verification. The fragment replaced the original full-length *Eco*RI-*Bam*HI fragment of the PHK construct by ligating with PHK, which was digested with *Eco*RI and *Bam*HI. The final constructs were confirmed by DNA sequencing to rule out PCR artifacts.

All three DNA constructs were released from plasmids by *Hind*III/*Eco*RI double digestion and separated by agarose gel electrophoresis. The fragment was purified with the USBiClean kit (USB, Cleveland, OH, U.S.A.) with the procedures suggested by the manufacturer. The concentration of DNA was measured

Abbreviation used: RT-PCR, reverse transcriptase PCR.

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by absorbance at 260 nm and confirmed by comparison with lambda/*Hind*III marker on electrophoresis. The DNA was then diluted with injection buffer [5 mM Tris/HCl (pH 7.4)/0.1 mM EDTA] to a final concentration of 4 µg/ml and centrifuged at 100 000 *g* for 1 h to remove dust particles before injection.

Generation and identification of transgenic mice

All experimental protocols were approved by the Institutional Animal Research Committee of the Medical University of South Carolina and were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

The detailed procedures for generation and identification of transgenic mice have been described previously [29]. Briefly, the DNA was microinjected into (C57BL/6 × DBA/2) F₂ embryos that were allowed to develop to term *in utero* in pseudopregnant females. Tail DNA from each offspring was screened by genomic Southern blot with a human tissue kallikrein cDNA probe. Founder mice were identified in each line and were bred with C57BL/6 mice. Each founder transmitted the transgene to its progeny for lines PHK, D300 and D200.

For identification of the transgenic mice expressing human tissue kallikrein, DNA extracted from tails was digested with *Pst*I, and then subjected to agarose gel electrophoresis and Southern blot hybridization with a human tissue kallikrein cDNA probe labelled with [α -³²P]dATP as previously described [29]. The labelling was performed with a nick translation kit (Gibco-BRL, Gaithersburg, MD, U.S.A.). Hybridization was performed at 65 °C overnight. The membrane was then washed starting at 2 × SSPE [1 × SSPE is 0.15 M NaCl/0.01 M NaH₂PO₄/1 mM EDTA (pH 7.4)] containing 0.1 % SDS at room temperature. The final wash condition was 0.5 × SSPE containing 0.1 % SDS at 65 °C.

Northern blot analysis of human tissue kallikrein mRNA in transgenic mice

Total RNA was isolated from mouse tissues by homogenization in guanidine isothiocyanate and centrifugation through CsCl as previously described [30]. Northern blot analysis was performed as described [31] with a human tissue kallikrein cDNA probe labelled with [α -³²P]dATP by nick translation. Each lane contained 40 µg of total pancreatic RNA. The hybridization buffer consisted of 6 × SSC [1 × SSC is 0.15 M NaCl/0.015 M sodium citrate (pH 7.0)], 5 × Denhardt's solution, 0.5 % SDS and 100 µg/ml of herring sperm DNA. The hybridization was performed at 60 °C overnight with the labelled probe at 3 × 10⁵ c.p.m./ml. After hybridization the membrane was washed in 6 × SSC with 0.1 % SDS at 50 °C and exposed to Kodak X-Omat film at -80 °C.

Reverse transcriptase PCR (RT-PCR) followed by Southern blot analysis

The reverse transcription reaction mixture (20 µl) contained 1 µg of total RNA, 10 pmol of the 3' primer (5'-CTTCACATAAG-ACAGCAC-3'), 2 µl of 2.5 mM dNTP, 4 µl of 5 × reverse transcription buffer [250 mM Tris/HCl (pH 8.3)/375 mM KCl/15 mM MgCl₂] and 10 units of avian myeloblastosis virus reverse transcriptase. The reaction mixture was incubated at 37 °C for 1 h to allow the synthesis of the first strand of cDNA. The products were then amplified in accordance with the Perkin Elmer AmpliWax protocol (Roche Molecular Systems, Branchburg, NJ, U.S.A.). The 5' primer (5'-AACACAGCCCA-GTTTGT-3', 50 pmol) and 3' primer (as mentioned above,

50 pmol), 10 µl of 10 × PCR buffer, 5 µl of 2.5 mM dNTP and 2.5 units of *Taq* DNA polymerase were added to the reverse transcription mixture which was then subjected to 30 cycles (94 °C, 1 min; 55 °C, 2 min; 72 °C, 3 min) in a thermal cycler. The 5' and 3' primers were designed in such a way that they corresponded to a portion of the sequence at exons 3 and 5 of the human tissue kallikrein gene respectively. The product of amplification with this pair of primers is a 503 bp DNA fragment. Samples were subjected to electrophoresis in a 0.8 % agarose gel and analysed by Southern blot as previously described [29]. The blot was hybridized with the end-labelled oligonucleotide (5'-GACCTCAAATCCTGCC-3'), of which the sequence was derived from the fourth exon of the human tissue kallikrein gene. The filter was washed in 2 × SSC at 45 °C and exposed to Kodak X-Omat film at -80 °C.

Genomic Southern blot for gene dosage analysis

Genomic DNAs from PHK, D300 and D200 (0.2 µg of each) were loaded directly on to the 0.8 % agarose gel. Samples were subjected to electrophoresis for 30 min at a constant 30 V to achieve a relatively narrow band of genomic DNA. Southern blot analysis was performed as previously described [29]. The blot was hybridized with the ³²P-labelled human tissue kallikrein cDNA. The membrane was washed in 0.5 × SSPE containing 0.1 % SDS at 65 °C before exposure to Kodak X-Omat film at -80 °C. Densitometric scannings of the band intensity in agarose gel and autoradiogram were performed with a Hewlett Packard ScanJet II CX/T with Macintosh application NIH Image version 1.60.

Tissue preparation and urine collection

Tissues collected from transgenic and control mice were homogenized immediately in PBS, pH 7.0. The homogenate was centrifuged at 800 *g* for 10 min. The supernatant was incubated with 0.5 % sodium deoxycholate and then centrifuged at 20 000 *g* for 30 min at 4 °C. The level of human tissue kallikrein in tissue extracts was determined by a direct radioimmunoassay of human tissue kallikrein [32]. Total protein was determined by Lowry's method [33]. For urinary excretion of human tissue kallikrein by transgenic and control mice, transgenic mice and negative littermates were fed with their usual food for 3 h before they were placed in metabolic cages supplied with drinking bottles. Urine was collected 24 h later and centrifuged in a microcentrifuge at 10 000 *g* to remove particles. The supernatants were analysed for the amount of human tissue kallikrein by a radioimmunoassay [32].

Statistical analysis

Group data are expressed as mean ± S.E.M. Comparison of parameters between groups was made by Student's *t* test. Differences were considered significant at a value of *P* < 0.05.

RESULTS

Identification of transgenic mouse lines

Three human tissue kallikrein gene constructs, PHK, D300 and D200, were generated and the sequences confirmed (Figure 1). The PHK construct contains the complete human tissue kallikrein gene with 801 bp of its own 5'-flanking region, whereas the 5' promoter region in D300 is decreased to 302 bp, and that in D200 to 202 bp. The DNA constructs were used to generate transgenic mouse lines PHK, D300 and D200 and the transgenes were identified by the extraction of tail DNA and digestion by

Figure 1 Human tissue kallikrein transgene constructs

The black regions represent five exons of the human tissue kallikrein gene. The white regions indicate the introns or flanking sequence. The locations of CAAT, TATA box and other hormonal responsive elements are illustrated. The translation starting site is indicated by +1. Abbreviations: GRE, glucocorticoid response element; CRE, cAMP response element; PRE, progesterone response element; ERE, oestrogen response element; hKLK1, human tissue kallikrein gene.

Figure 2 Identification of PHK, D300 and D200 transgenic lines by Southern blot analysis

Genomic DNA (5 µg) was used for Southern blot analysis. Lane 1, PHK DNA; lane 2, D300 DNA; lane 3, D200 DNA; lane 4, negative non-transgenic mouse DNA control.

restriction enzymes followed by genomic Southern blot analysis. Figure 2 demonstrates the results of a genomic Southern blot for identification of transgenic lines. The animals harbouring human transgenes PHK, D300 and D200 showed intense and unique individual banding patterns of the transgenes in the genomic Southern blot with a human tissue kallikrein cDNA probe. There was no cross-reactivity between the mouse tissue kallikrein gene and the human tissue kallikrein cDNA probe under these conditions, as indicated by the negative result of non-transgenic mouse DNA in Figure 2.

Expression of immunoreactive human tissue kallikrein in transgenic mice

The expression of human tissue kallikrein in transgenic mice was analysed by a direct radioimmunoassay specific for human

Table 1 Tissue distribution of human tissue kallikrein in transgenic and control mice

Values from tissues and sera are means ± S.E.M., *n* = 4; urine values are from single-mouse 24 h urine collections. Abbreviation: n.d., not detected.

(Units) and source	Human tissue kallikrein			
	PHK	D300	D200	Control
(ng/mg of protein)				
Pancreas	53.5 ± 7.04	353.5 ± 16.32	167.3 ± 28.15	n.d.
Spleen	4.35 ± 1.21	27.75 ± 19.83	24.24 ± 14.62	n.d.
Salivary gland	0.68 ± 0.49	0.97 ± 0.77	1.55 ± 0.67	n.d.
Kidney	2.49 ± 0.33	5.72 ± 0.91	4.70 ± 0.77	n.d.
(ng/ml)				
Serum	n.d.	1.93 ± 2.14	n.d.	n.d.
(ng/24 h)				
Urine	19.7	1635.4	247.9	n.d.

Figure 3 log–logit transformation of a typical radioimmunoassay standard curve of human tissue kallikrein and serial dilutions of pancreatic extracts from a PHK transgenic mouse

B/B₀ is the ratio (expressed as a percentage) of bound radioactivities in the presence and in the absence of unlabelled kallikrein, with rabbit anti-(human tissue kallikrein) antiserum.

tissue kallikrein. The results in Table 1 show that human tissue kallikrein was expressed predominantly in the pancreas, with lower levels in other tissues such as spleen, salivary gland and kidney. Human tissue kallikrein was also detected in serum and urine of the transgenic mice. The distribution patterns in various tissues were similar in the three transgenic mouse lines. Note that the expression levels of human tissue kallikrein in the PHK line were considerably lower than those in the D300 or D200 lines.

Figure 3 shows that a serial dilution of the pancreatic tissue extract from transgenic mice parallels the human tissue kallikrein standard curve, indicating their immunological identity. Immunoreactive human tissue kallikrein was not detected in the pancreas or other tissues of control mice, indicating the specificity of the human tissue kallikrein radioimmunoassay.

Human tissue kallikrein mRNA levels in transgenic mouse lines

Northern blot analysis showed the expression of human tissue kallikrein mRNA in the pancreas of PHK, D300 and D200 mice (Figure 4). D300 had the highest level of expression, as judged by

Figure 4 Northern blot analysis of human tissue kallikrein

Total pancreatic RNA (40 μ g) from transgenic mice was added in each lane. 32 P-labelled human tissue kallikrein cDNA was used as the hybridization probe.

Figure 5 Gene dosage comparison of PHK, D300 and D200 by Southern blot analysis

The same quantity of genomic DNA species (0.2 μ g) from PHK, D300, D200 lines and a control mouse was used. Upper panel, 0.8% agarose-gel electrophoresis; lower panel, autoradiogram of the corresponding lanes after Southern blot analysis. Lane 1, D200 DNA; lane 2, D300 DNA; lane 3, PHK DNA; lane 4, negative control.

the intensity of the hybridization band. The expression of human tissue kallikrein was lowest in the PHK mouse. These results are consistent with the expression levels of immunoreactive human tissue kallikrein shown in Table 1.

Transgenic dosage in PHK, D300 and D200 mouse lines

To observe the gene dosages of these three transgenes, a modified genomic Southern blot was performed. Figure 5 illustrates the results of this genomic Southern blot with DNA species extracted from D200, D300 and PHK transgenic mouse lines. For the purpose of quantification, the genomic DNA was not enzyme-digested and was subjected to a short duration of electrophoresis. The end result was one relatively densely packed genomic DNA

Figure 6 RT-PCR followed by Southern blot analysis

RT-PCR was performed with 1 μ g of total RNA from tissues of a D200 transgenic mouse. Oligonucleotides specific to human tissue kallikrein were used as primers. The RT-PCR products were then hybridized with another specific oligonucleotide probe. In the pancreas, only 5 μ l of the PCR product was used in the subsequent Southern blot, compared with 20 μ l in other tissues.

band on the agarose gel, as seen in the upper panel of Figure 5. The subsequent Southern blot is shown in the lower panel. Densitometer scanning was employed to compare the transgene dosages between the PHK, D300 and D200 lines. The gene dosage analysis in these three lines showed a PHK/D300/D200 ratio of approx. 1:3.0:3.2 after normalization by the total amount of genomic DNA from the agarose gel.

Tissue distribution of human tissue kallikrein mRNA in transgenic lines

The tissue distribution of human tissue kallikrein mRNA in the transgenic mice was analysed by RT-PCR followed by Southern hybridization with human kallikrein-specific oligonucleotides. Figure 6 shows that human tissue kallikrein is expressed in the D200 transgenic mouse line. Owing to the high level of human tissue kallikrein expression in the pancreas, only 5 μ l of pancreatic PCR product was used in the subsequent Southern blot analysis compared with 20 μ l of PCR products from all other tissues. All tissues gave rise to a specific band of approx. 500 bp. The results show that the human kallikrein transgene mRNA is expressed at the highest level in the pancreas and in much smaller amounts in spleen, salivary glands, heart, lung, brain, kidney, muscle and prostate. Signals could not be detected in the liver of the transgenic mouse. The results indicate that the tissue-specific expression pattern of human tissue kallikrein directed by its own promoter in transgenic mice is similar to that in human tissues [23].

Electrolyte analysis of PHK, D300 and D200 transgenic mice

The results of electrolyte analysis (Na^+ , K^+ and Cl^-) from urine samples of PHK, D300 and D200 transgenic mice showed no significant difference from those of normal control mice (results not shown). There was also no apparent abnormality observed in these animals in their body weight, growth pattern or gross morphology.

DISCUSSION

Regulation of human tissue kallikrein gene expression is governed by a complex array of transcriptional control elements. Although

the 5'-flanking region of tissue kallikrein genes proved to be important in regulating its expression, the precise mechanisms of its regulation are not well understood [6,7,34]. Evidence indicates that kallikrein gene expression in different species might have its own unique regulatory mechanisms [8,9,22,35]. As regards the rat, a previous report analysed the tissue distribution pattern of rat tissue kallikrein genes by linking various promoters of rat tissue kallikrein to an SV40 T-antigen as the reporter and introducing these constructs into mouse and rat [8]. Their results suggested that the expression of the rat kallikrein gene family members is controlled by the proximal 5'-flanking region of each gene as well as by possible dominant control regions distal to the rat kallikrein family gene locus. Transgenic mice carrying prostate-specific antigen promoter with rasT24 developed unexpected salivary gland neoplasm instead of prostate tumour [36].

The use of transgenic models to study the tissue-specific expression of human tissue kallikrein has its own advantages. Although many cell lines are now available for the analysis of gene expression *in vitro*, the study of tissue-specific expression of the kallikrein gene requires many cell lines of specific cell types from various sources such as pancreas, spleen, salivary gland and kidney. Currently available cell lines are not suitable for the analysis of tissue kallikrein expression because none of the cell lines from spleen, salivary gland and kidney has been shown to actively express tissue kallikrein. It is also difficult to maintain uniform and optimal transfection conditions for all these cell lines; such conditions are crucial for any meaningful comparisons. In this aspect, studies with transgenic animals are likely to yield more reliable results when studying the tissue-specific expression of genes.

The current study was designed to analyse the functional domain of the human tissue kallikrein gene promoter *in vivo*. Three transgenic mouse lines, which express the human tissue kallikrein gene directed by three constructs containing various lengths of the human kallikrein gene promoter region, were established. On the basis of the protein data obtained from radioimmunoassay, we have demonstrated that all three constructs exhibit the tissue-specific expression of human tissue kallikrein in transgenic mice. The expression pattern reflects the tissue distribution of tissue kallikrein in humans, with the highest level in the pancreas and lower levels in the submandibular gland, pituitary gland, kidney and muscle [5,22–26,37]. This is in contrast with the distribution pattern of the endogenous mouse tissue kallikrein, which has the highest levels in the salivary glands, and lower levels in pancreas, kidney, spleen and other tissues [9]. This discrepancy is most probably caused by the presence of a salivary-specific enhancer element in the mouse tissue kallikrein gene locus.

From the results of human tissue kallikrein protein measurements it is clear that the D200 transgene has the same transgenic expression pattern as the two other transgenic lines with longer promoter regions. Human tissue kallikrein mRNA expression in D200 transgenic mice also follows a similar pattern when analysed by RT-PCR followed by Southern blot analysis. Previous studies suggest that in humans the tissue kallikrein mRNA level is the highest in the pancreas, compared with several other tissues by using Northern blot analysis [22,23]. The results from the current study are in agreement with those observations obtained from human studies at the protein and mRNA levels. In addition we are unable to detect human tissue kallikrein mRNA in the liver of the transgenic mice, which is consistent with the current knowledge that there is no apparent expression of tissue kallikrein in human liver. The results support the notion that the 202 bp promoter is sufficient to mediate the

tissue-specific expression of the human tissue kallikrein transgene. It should be noted that in certain tissues of the transgenic mice, human tissue kallikrein mRNA but not its protein was detected. This was most probably due to the high degree of sensitivity of the RT-PCR Southern blot used in this study. However, the detection by RT-PCR Southern blot analysis is not considered to be a quantitative assay. This might explain the lack of total agreement between the relative levels of RT-PCR products shown in Figure 6 and the protein levels of kallikrein shown in Table 1 among several tissues analysed. Alternatively, the difference might also be caused by differential translational efficiency of the kallikrein mRNA in different tissues.

Although the expression of human tissue kallikrein in these three transgenic lines is within the same magnitude, the results of protein measurements indicate that transgene expression is the highest in D300, lower in D200 and the lowest in PHK. After normalization of the effect of the transgene copy number, the level of expression in D300 is approximately twice the levels in PHK and D200. The results suggest that there might be a negative regulatory element between –801 and –302 and/or an enhancer-like element between –302 and –202. However, caution should be exercised in explaining these results: additional studies such as cell culture experiments need to be performed before any definite conclusion can be reached. In addition to the copy number (gene dosage) effect, there is the possibility of positional effects that might influence the results of this comparison.

This study provides the basis for identifying the precise region responsible for regulating tissue kallikrein promoter activity. We are currently designing gel retardation and footprint assays to identify the regulatory sequences within this region. The information obtained from these studies will be useful to understand further the regulatory mechanisms of human tissue kallikrein expression.

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