Expression and cellular localization of kininogens in the human kidney

ANDREA HERMANN, ANDREAS BRAUN, CARLOS D. FIGUEROA, WERNER MÜLLER-ESTERL, HANS FRITZ, and JOACHIM REHBOCK

I. Frauenklinik der Universität München, and Abteilung für Klinische Chemie und Biochemie im Dr. von Hauner’schen Kinderspital der Ludwig-Maximilians-Universität München, Munich, Germany; Instituto de Histología & Patología, Universidad Austral de Chile, Valdivia, Chile; and Institut für Physiologische Chemie und Pathobiocemie, Johannes-Gutenberg-Universität Mainz, and Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik, Klinikum Innenstadt der Universität München, Munich, Germany

Expression and cellular localization of kininogens in the human kidney.

Human high (H) and low (L) molecular weight kininogens are encoded by distinct mRNAs derived from a single kininogen gene. Previous studies have demonstrated the presence of L-kininogen but not of H-kininogen in the distal nephron structures of the kidney. Using the highly sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) we have been able to demonstrate the expression of both H-kininogen mRNA and L-kininogen mRNA in kidney and liver. The presence of H- and L-kininogen antigen was shown immunohistochemically by applying specific antibodies that discriminate between the two types of kininogens. Immunoreactive kininogens were localized in the cortical and medullary collecting ducts. Our results indicate that both types of kallikrein-bearing kininogens are expressed in the human kidney where they might contribute to the suggested roles of the kallikrein-kinin system in the regulation of renal blood flow and electrolyte excretion.

Human plasma contains two types of kininogens originating from a single kininogen gene that is alternatively spliced within exon 10 [1]. These two forms, high (H) and low (L) molecular weight kininogens, are single-chain (glyco)proteins comprised of three main portions: the heavy chain, the bradykinin moiety and the light chain. The first two segments and the following twelve amino acids are identical for both kininogens, whereas the remaining carboxy-terminal light chains differ in molecular size, structure and functional properties [1–3]. Thus, while the light chain of L-kininogen has an apparent molecular mass of 5 kDa, the corresponding light chain of H-kininogen is 12-fold larger with an apparent mass of 58 kDa [1–3]. This latter light chain harbors a cell binding site and cofactor binding sites to which prekallikrein and factor XI bind with high affinity. In this way H-kininogen is endowed with unique functional properties not expressed by L-kininogen.

The two kininogens differ in their susceptibility to cleavage by plasma and tissue kallikreins [1–3]. Plasma kallikrein readily releases the nonapeptide bradykinin from H-kininogen whereas L-kininogen is a relatively poor substrate for this enzyme [4]. In contrast, tissue kallikrein, an enzyme occurring in various mammalian organs including the kidney, rapidly generates kallidin (Lys-bradykinin) from both H- and L-kininogens [5].

Early studies of Proud et al [6] suggested that human kidney is an extrahepatic site for kininogen production, and that renal kininogen corresponds to L-kininogen. Specific antibodies directed to the unique light chain of H-kininogen failed to produce significant immunoreactivity in kidney sections. Their immunofluorescence analysis showed that renal L-kininogen was diffusely distributed in distal tubules as well as cortical and medullary collecting ducts [6]. Subsequent immunohistochemical studies revealed that renal kininogen is specifically localized in the collecting ducts, and that the initial portion of this segment contains cells expressing kininogen juxtaposed to cells containing tissue kallikrein [7]. The antibodies used in this study did not discriminate between the two types of kininogens [7]. The notion that the human kidney is an extrahepatic site for L-kininogen synthesis was confirmed by Iwai et al [8] who detected mRNA for L-kininogen but not for H-kininogen in this organ by Northern blotting. Controls indicated that H-kininogen mRNA was present in the liver but not in the kidney [8].

In view of the functional roles conferred by H-kininogen and lacked by L-kininogen, we reinvestigated the presence of kininogens in the human kidney using a sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) assay and antibodies that can discriminate between the H- and L-kininogens. We demonstrate that H-kininogen mRNA and immunoreactive H-kininogen protein are present in the distal portions of the nephron. The finding that both types of kininogens are expressed by the human kidney sheds new light on the possible functional role of the kinin system in the kidney.

Methods

Tissues

Human liver and kidney tissue was obtained from the Surgical Hospital, Munich, Germany and the Department of Pathology, Regional Hospital, Valdivia, Chile. After resection due to liver cirrhosis and renal adenocarcinoma, normal segments taken from both organs were immediately put in liquid nitrogen and kept frozen at −80°C until used.
Fig. 1. Restriction maps for the L-kininogen or H-kininogen cDNAs. The restriction sites for HaeIII, DdeI, and StyI are depicted, and the length (bp) of the resulting restriction products are indicated. Portions of the human kininogen gene covering exons 9 to 11 are shown; introns have been omitted from the graph. LHK>, common 3'-primer; <LK-1, <LK-2 and <HK-1, <HK-2, specific 3'-primers for L- and H-kininogen mRNA, respectively.

RNA preparation

Total cellular RNA was extracted by a method already described [9]. Briefly, 500 mg of liver or renal tissue were homogenized with an Ultraturrax (IKA Labortechnik, Germany) in 500 μl nucleic acid extraction buffer (4 mM guanidine isothiocyanate, 5 mM N-lauroylsarcosine sodium salt, 0.1 mM 2-mercapto-ethanol, 25 mM sodium citrate, pH 7) and 50 μl of 2 mM sodium acetate, pH 5.2, were added. The suspension was extracted with 500 μl phenol and 100 μl of a chloroform/isooamyl alcohol mixture (49:1, vol/vol) and centrifuged at 13,000 × g. The RNA dissolved in the aqueous phase was precipitated with ethanol and then resuspended in diethylpyrocarbonate-treated water. The isolated RNA was quantified spectrophotometrically at 260 nm.

PCR primer and RT-PCR

All primers were synthesized by MWG-Biotech (Germany) and designed from the sequence of the human kininogen gene [10]: LHK> 5’-CTGATTTGCAACGCTGAAG-3’ (nt 1045-1063), <HK-1 5’-GTCAGTGCTACGATGC-3’ (nt 1290-1272), <HK-2 5’-CGCTCTTCATCTTTGTGAC-3’ (nt 1250-1232), <LK-1 5’-CCCTCTGATGCTGGCCTC-3’ (nt 1273-1255), <LK-2 5’-TCGACCCGTCTATGCAGCAG-3’ (nt 1236-1218). The localization of the primers in the sequence of the human kininogen gene is depicted in Figure 1. The cDNAs for both H- and L-kininogen were specifically synthesized using the “First Strand cDNA Synthesis Kit” from Pharmacia (Germany). Approximately 1 μg of total RNA was incubated with 1 μl of 200 mm dithiothreitol, 50 ng of the <HK-1 or the <LK-1-primer and RNAase-free water in a final volume of 10 μl. The incubation was performed at 65°C for 10 minutes and then the mixture was directly placed on ice. Five microliters of the “Bulk First Strand Reaction Mix” (Pharmacia), containing murine reverse transcriptase, RNAGuard, bovine serum albumin and deoxynucleotide triphosphates, were added and the mixture incubated for one hour at 37°C.

To exclude the amplification of remaining DNA 1 μg of total RNA was incubated with 1.5 units of RNAase A (Boehringer, Germany) for one hour at 37°C and then used for cDNA-synthesis with either the <HK-1 or the <LK-1-primer as described above.

PCR protocol

The PCR was performed with 1 μl of the cDNA synthesis samples or the RNAase treated control samples using a Perkin Elmer Thermocycler. Five μl of Taq DNA polymerase buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM MgCl2, 1 g/liter gelatin, 1% Triton X-100), 1 μl of 20 mM dNTP solution (Pharmacia LKB, Germany), 50 ng of reverse-primer (<HK-1 or <LK-1) and 50 ng of forward-primer (LHK>, autoclaved water up to 50 μl and 0.5 units of Taq DNA polymerase (Boehringer) were added. Each of the 20 cycles consisted of a denaturation step at 92°C for one minute, an annealing step at 52°C for one minute, and an elongation step at 72°C for one minute. The PCR with the nested reverse-primers <LK-2 or <HK-2 and the forward-primer LHK> (Fig. 1) were performed with 1 μl of the 1 to 10 diluted PCR-reaction. Thirty-five cycles were run with the profile described above.

Restriction analysis

The PCR products were treated with RNAase A (Boehringer) and digested with restriction enzymes. Three aliquots of the PCR products were incubated with five units of three different restriction enzymes [HaeIII, DdeI and StyI (Boehringer)], cleavage buffer and autoclaved water. A fourth aliquot remained undigested and was diluted to the same volume. The PCR products, restriction analysis fragments and a standard length DNA (123 bp
DNA Ladder, Gibco BRL, USA) were run on a 3% Metaphor\textsuperscript{TM}. Agarose gel (Biozym, Germany) in TBE (100 mM Tris, 100 mM boronic acid, 2.5 mM EDTA, pH 8). The DNA was analyzed by direct visualization after ethidium bromide staining.

**Immunohistochemistry**

Normal segments of human liver and kidney tissue samples were fixed by immersion in 3.7% formaldehyde, diluted in 0.9% NaCl, for 24 hours at room temperature. After fixation the tissue was dehydrated through a graded series of ethanol and embedded in Histosec (Merek, Germany). Immunoreactivity for kininogens was demonstrated using the peroxidase/antiperoxidase (PAP) technique of Sternberger et al [11] with modifications already described [12–14]. Tissue sections, 5 to 7 μm thick, were dewaxed, rehydrated and treated with absolute methanol and 5% hydrogen peroxide to block endogenous pseudoperoxidase activity. Successful peroxidase inhibition was confirmed in control sections.

The two types of kininogens were visualized using antibodies that discriminate between them by recognizing epitopes of their distinct light chain portions [15, 16]. L-kininogen was detected using a polyclonal antibody, raised in rabbits, against an epitope at the unique carboxyterminal segment (residues 389-409 of the light chain portion of human L-kininogen), NH\textsubscript{2}-Cys-Glu-Tyr-Lys-Gly-Arg-Pro-Pro-Lys-Ala-Gly-Ala-Glu-Pro-Ala-Ser-Glu-Arg-Glu-Val-Ser-COOH [15, 16]. H-kininogen was visualized using an affinity-purified polyclonal antibody produced against human H-kininogen in sheep, and preabsorbed with human L-kininogen. The antiserum had less than 5% cross reactivity with L-kininogen [16]. Prior to immunohistochemistry the sections were treated for 10 to 15 minutes at 37°C with 0.5 g/liter porcine trypsin (Type II, Sigma Chemicals, St. Louis, MO, USA) dissolved in 50 mM Tris-HCl, pH 7.8, supplemented with 0.1% CaCl\textsubscript{2}, or were immersed in 0.01 M citrate buffer, pH 6.0, and microwaved for 10 minutes at 85°C [17].

Following unmasking of the antigens the sections were incubated with sheep anti-H-kininogen (1:500 to 1:1000) or rabbit anti-L-kininogen (1:300) antibodies overnight at 22°C in a thermostated water bath that was used as a moisture chamber. When sheep primary antiserum was used, a second step with rabbit anti-sheep IgG (Sigma), 1:500, for 30 minutes was included. Next, the sections were sequentially incubated with swine anti-rabbit IgG (Dako), 1:80, and PAP complex (Dako), 1:100, for 30 minutes each. Peroxidase activity was demonstrated with 0.1% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) in the presence of 0.03% hydrogen peroxide for 15 minutes at room temperature [12–14]. The sections were counterstained with Harris’ hematoxilin (Merek), washed with tap water, dehydrated and coverslipped. None of the secondary antibodies used cross reacted with human immunoglobulins.

The specificity of the immunostaining was monitored: (i) by prior absorption of diluted L-kininogen and H-kininogen antisera with their respective antigens (20 to 50 μg/ml); (ii) by omission of the anti-kininogen antibody; and (iii) by its replacement with non-immune serum of the same species [12–14].

**Results**

**RT-PCR analysis**

The PCR products with their restriction sites for DdeI, HaeIII and StyI are schematically shown in Figure 1. Results of the PCR and restriction enzyme analyses are shown in Figure 2. As expected, in human liver the PCR products (upper portion of Fig. 2) appeared at a length of 192 bp and 206 bp for L-kininogen and H-kininogen, respectively (Fig. 2, lanes 2 and 6). Both products were cleaved with the restriction enzyme HaeIII, resulting in one broad band of about 95 bp for L-kininogen (theoretically 98 and 94 bp) (Fig. 2, lane 3) and two scarcely distinguishable bands of about 100 bp for H-kininogen (theoretically 98 and 108 bp) (Fig. 2, lane 7). This cleavage pattern is in accordance with the known sequence because HaeIII cleaves within exon 10a, which is identical for L- and H-kininogen mRNAs (Fig. 1). DdeI cleaved the L-kininogen cDNA to two fragments of about 170 bp and 20 bp (very faint), but not the H-kininogen cDNA (Fig. 2, lanes 4 and 8). The cleavage site is located within exon 11 of L-kininogen, which is not present in H-kininogen (Fig. 1). According to this
scheme, two fragments of 166 and 26 bp should be expected for L-kininogen cDNA. The enzyme SspI that recognizes exon 10b present in the H-kininogen but not in the L-kininogen mRNA (Fig. 1) did not cleave the the L-kininogen PCR product (Fig. 2, lane 5), but cleaved the H-kininogen PCR product (Fig. 2, lane 9). The predicted fragments of approximately 180 bp and 30 bp (very faint) were found (Fig. 2, lane 9).

A similar pattern of restriction fragments was obtained in the experiments performed with human renal tissue (Fig. 2, lower portion).

After RNAsase treatment of liver or kidney mRNA the samples did not show any PCR products, neither in the L-kininogen (Fig. 2 lane 1) nor in the H-kininogen (Fig. 2 lane 10) assay. Thus, contamination of the samples with the expected PCR products and with DNA can be ruled out. As shown in Figure 1, the PCR primers flank one or two exon-intron boundaries (H-kininogen or L-kininogen). Therefore the lengths of the PCR products should grossly diverge in the presence of contaminating DNA. As all products had the expected length, a contamination of the isolated RNA with genomic DNA can be excluded.

**Immunohistochemistry**

To prove that the renal H-kininogen mRNA is indeed translated into the corresponding protein we performed an immunohistochemical analysis using antibodies that specifically differentiate between H- and L-kininogen. This approach revealed the presence of both kinin-releasing substrates in the human kidney. Immunostaining localized the kininogens in cortical collecting ducts of the medullary rays and in outer and inner medullary collecting ducts (Figs. 3 and 4). In cortical collecting ducts, the cells containing immunoreactive kininogens were intermingled with cells that showed no staining (Fig. 4A). The kininogen-containing cells displayed the morphological features of collecting duct cells (formerly called principal cells) known to have a convex and smooth luminal surface with the nucleus situated in the midportion of the cell (Fig. 4B). By comparison, the morphological appearance of the unstained cells, that is, a smaller size and a nucleus located in the basal portion of the cell, corresponded to those characteristics described for intercalated cells. As predicted, these cells (intercalated cells) diminished in number from cortex to medulla where collecting ducts are known to be formed only by collecting duct cells [18]. At the medulla, all collecting duct cells contained immunoreactive kininogens (Figs. 3B and 4). Immunoreactivity for H- and L-kininogen proteins excluded the nucleus and was concentrated in the upper one-third of collecting duct cells though some immunoreactivity was also observed in the basal portion of the cells (Fig. 4). No substantial differences in the staining pattern of the two kininogens (intensity of the staining and localization) were observed.

In addition to the immunoreactivity present in cortical and medullary collecting ducts, a focal immunostaining was also observed in the lumen of some blood vessels (Fig. 3) that included glomerular capillaries, arteries and veins, and in proximal tubule cells where it concentrated in the microvilli and reabsorption droplets (not shown).

Staining was not observed when each of the antibodies was preabsorbed with its authentic antigens (exemplified for H-kininogen, Fig. 4D) or when the specific antibody was omitted or replaced by nonimmune serum from the homologue species (not shown).

![Fig. 3. Immunohistochemical visualization of human kininogens in renal cortex (A) and medulla (B). A. Low power view of renal cortex showing immunostaining for H-kininogen in collecting ducts (CD) of the medullary ray (MR). The arrow points immunoreactive H-kininogen in the lumen of a blood vessel (95 ×). B. Immunostaining for L-kininogen in medullary collecting ducts (CD; ×95).](image-url)
**Discussion**

Our results demonstrate for the first time that the mRNA of both L- and H-kininogen is expressed in the human kidney.

In a previous report Iwai et al. [8] failed to identify H-kininogen mRNA in the human kidney by applying conventional Northern blot analysis. This discrepancy of findings by Iwai et al. [8] and our group can be explained by the fact that we have used a highly sensitive method, the RT-PCR assay, for mRNA detection which is more likely to pick up even minute amounts of H-kininogen mRNA.

As the two kininogen mRNAs derive from a single gene and have a large common sequence portion, the discrimination of the corresponding RT-PCR products merely by their variant length could be misleading. Amplification of two distinct products for the L- and H-kininogen cDNAs which differ only by 14 bp (192 vs. 206 bp) was further proven by their susceptibility to various restriction enzymes and the use of nested primers. The cleavage pattern observed with the enzymes applied in this study clearly showed that there are two distinct PCR products, and that both H- and L-kininogen mRNAs are expressed in the human kidney.

To identify the specific cell types expressing the kininogens in the kidney we applied immunohistochemistry using antibodies that discriminate between the two types of kininogens [15, 16]. This method revealed the presence of both H- and L-kininogen in cortical and medullary collecting duct cells. This observation
confirms the results reported in a previous study in which a polyclonal antibody cross-reacting with both H- and L-kininogen proteins was used [7].

Remarkably, the enzyme tissue kallikrein has been shown to occur in neighboring cells of the connecting tubule [12–14]. Our present study suggests that kallidin (Lys-bradykinin), and possibly bradykinin, may be locally generated at the site where connecting tubules merge into collecting ducts. Previous studies using electron microscopy and immunocytochemistry have shown that in this portion of human nephron [7, 19], and of the rat kidney [12–14, 19], the characteristic cell types of connecting and collecting tubules are intermingled over varying distances [19]. High concentrations of free kinin(s) have been found in the terminal portions of the distal nephron [20] thus supporting our notion that production and release of kininogens and kallikrein by neighboring cells of the connecting tubules might locally generate high concentrations of kinins.

Immune-reactivity for both kininogens was also detected in the lumen of some blood vessels and in the reabsorption droplets of proximal tubules. The presence of kininogens in renal blood vessels may be explained by the fact that these proteins are abundant in plasma and are crosslinked by conditions of fixatives during sample fixation. Furthermore, some intact L-kininogen and degradation products of plasma H-kininogen could be filtered through the glomeruli and finally trapped by proximal tubules, the main site for protein reabsorption and degradation. Consequently, the kininogen-containing cells of collecting ducts may represent the primary source of renal kininogen from which endogenous kinins will be formed.

Recently the kallikrein-containing cells and the kininogen-immune-reactive cells have been found to display bradykinin B2 receptors [21]. Immunoreactive bradykinin B2 receptors are present in the basolateral infoldings and the luminal portions of connecting tubules and connecting ducts [21]. It is tempting to speculate that locally released kinins might act in an auto-/paracrine manner to exert their well established diuretic and natriuretic effects in the distal portions of the kidney.

Acknowledgments

Part of this work was supported by a grant S/94/45 from Dirección de Investigacion, Universidad Austral de Chile. We would like to thank Drs. Günther Kindermann (I. Frauenklinik der Universität, München) and Edwin Fink (Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik, Klinikum Ienstadt der Universität, München) for their continued support.

Reprint request to Dr. Joachim Rehbock, I. Frauenklinik der Universität München, Meistraße 11, D-80337 München, Germany.

References