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Localization and inhibitory activity of α_2HS-glycoprotein in the kidney

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Abstract α_2HS-Glycoprotein (HS), a crystal surface binding substance extracted from human urine, is considered to be one of the urinary macromolecular inhibitors in urolithiasis. In the present study, reverse transcription–polymerase chain reaction was used to examine HS mRNA expression, and immunohistochemical staining was used to reveal its localization in the human kidney. The inhibitory effects of recombinant human HS and native human HS on calcium oxalate crystal growth were examined in a seed crystal system. HS mRNA was found to be expressed in the human kidney, and it was located in the epithelial cells of distal and proximal renal tubular cells. However, neither recombinant HS nor native HS had an inhibitory effect on crystals in the protein concentration of urine of healthy humans. HS in the urine, therefore, does not seem to be a potent inhibitor in stone formation.

Key words Calcium oxalate · Crystallization · α_2HS-Glycoprotein · Protein inhibitor · Crystal surface binding substance

Introduction

Urinary stones contain an organic matrix that accounts for 2–3% of their weight [10]. Many studies have investigated the relationship between urinary stone formation and urinary proteins. Urinary proteins are currently thought to play a more important role in stone formation than small molecular substances, because of their strong inhibitory effect on calcium oxalate (CaOx) crystal growth and aggregation. The urinary proteins, nephrocalcin [13], calprotectin [19], osteopontin [10, 15, 20], crystal matrix extract [3, 17], and uronic-acid-rich protein [1] have been identified and described as important proteins affecting urinary stone formation. α_2HS-Glycoprotein (HS), extracted from human urine, has recently been shown to be a crystal surface binding substance (CSBS) [8] and is thought to be one of the urinary macromolecular inhibitors of urolithiasis. The aims of the present study were to characterize the effect of HS on CaOx crystallization and to examine the existence and localization of HS in the human kidney, by means of reverse transcription–polymerase chain reaction (RT-PCR) [9] and immunohistochemical staining.

Materials and methods

First-strand cDNA synthesis

Total RNA was extracted from human kidney tissue using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. We developed primers specific to human HS mRNA as follows: outer upstream primer, 5′-CTTTGTCTTGCTCAGC-TC-3′ corresponding to nucleotides 70–87; outer downstream primer, 5'-GGCCCCCCACCCATGCCCA-3′ corresponding to nucleotides 1216–1234; inner upstream primer, 5′-GAGAAT-TCGCCCCACATGGCCCACA-3′ corresponding to nucleotides 103–117; inner downstream primer, 5′-GGAAGCTTCTAG-ACCTTGAAGTG-3′ corresponding to nucleotides 1137–1151. The inner upstream primer has an *Eco*RI site (underlined above) immediately followed by the mature HS sequence, while the inner downstream primer spans the 3′-noncoding region flanked by a *Hind*III site (underlined above).

The RT reaction of HS was performed in an RT reaction mixture (total 20 µl) as follows (RNA LA PCR Kit, TaKaRa, Tokyo, Japan): 1 µg human kidney RNA, 5 mM $MgCl_2$, 10 × RNA PCR buffer, 8.5 µl RNase free dH_2O, 1 mM dNTP mixture, 1 U/µl RNase inhibitor, 0.25 U/µl reverse transcriptase, 1.0 µM outer downstream primer specific to human HS or β-actin. The RT reaction mixture was incubated at 25°C for 10 min, at 42°C for 50 min, and at 90°C for 5 min, then placed on ice for 10 min.

Nested-PCR analysis

Of this complementary DNA, 1 µl served as the starting template for the outer upstream PCR. The 100 µl PCR reaction mixture

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(total 100 μl) included 20 μl of the cDNA products, 2.5 U/100 μl Taq polymerase, 1 × PCR reaction buffer, 2.5 mM $MgCl_2$, 63.5 μl H_2O and 0.2 μM outer upstream primer for HS. This mix was then transferred to a thermal cycler (9600 DNA, Perkin Elmer, Norwalk, Conn.) and incubated for 25 cycles. The PCR profile was 94°C for 15 s, 60°C for 15 s, and 72°C for 45 s. After 25 cycles the samples were placed on ice, and 2 μl of this reaction mixture served as the template for another round of PCR using inner primer with the same profile for 25 cycles using the Program Temp Control System PC-700 (Astec, Tokyo, Japan).

Complementary DNA quality was verified by performing control reactions derived from the β-actin gene sequence, yielding a 1058 base pair RT-PCR product. The sequence for the outer upstream primer was 5′-CATGGATGATGATATCGC-3′ (41–58), and that for the outer downstream primer was 5′-CGCAACTAAGTCATAGTC-3′ (1172–1189). The sequence for the inner upstream primer selected was 5′-AGGCCAACCGCGAGAAGATGA-3′ (73–93), and that for the inner downstream primer sequence was 5′-ATGTCACACTGGGGAAGC-3′ (1148–1175).

The entire HS mix and β-actin mix were run on 1.5% agarose gels, stained with ethidium bromide and photographed. DNA standards (100 Base-Pair Ladder, Pharmacia Biotech, Uppsala, Sweden) were also electrophoresed as controls. We also sequenced the PCR products and compared them with those of cDNA clones.

Preparation of recombinant HS

After DNA sequencing, the PCR product of HS was cloned as an *Eco*RI to *Hind*III fragment with a pMOSBlue T-vector kit (Amersham, Buckingham, UK). The expression vector pMAL-c was ligated with HS DNA, and used to transform the competent cells, *E. coli* JMI109. The cells were grown, harvested by centrifugation, and lysed by sonication [7]. The supernatant was subjected to Amirose resin affinity chromatography, and the fusion protein, maltose binding protein, was digested with clotting factor Xa [20], and purified by chromatography on a Q Sepharose column [7]. Recombinant HS (rHS) was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a discontinuous Tris/glycine buffer system with 10% cross-linked linear gel [19]. NH_2-terminal analysis of 20 amino acids using 400–600 pmol samples of rHS was carried out using a gas phase protein sequencer 477A (Applied Biosystems, Foster, Calif.).

Immunohistochemical staining

Antibodies were generated against purified HS [22, 23] in rabbits using Freund's adjuvants (Difco Laboratories, Detroit, Mich.). Rabbits were injected intramuscularly with 50 μg protein, followed by repeated injections 30 and 60 days later, and immune serum was collected 10 days after the last boost. Immunohistochemical staining of the human kidney was performed by Vectastain ABC Kit (Vector Laboratories, Burlingame, Calif.) according to the manufacturer's instructions.

Assay of crystal growth inhibitory activity of HS by the seed crystal method

The inhibitory effects of HS (human native α_2HS-glycoprotein, Chemosorb, Calif.), rHS and pyrophosphoric acid (for positive control) on CaOX crystal growth were determined with a seed crystal system according to a modified version of the method employed by Robertson et al. [14]. Metastable synthetic urine was composed of 1 mM $CaCl_2$, 0.2 mM sodium oxalate, 150 mM NaCl and 10 mM sodium cacodylate (pH 6), with a tracer dose of [^{14}C]oxalate. Seed crystals were prepared by adding 100 mg of commercially available calcium oxalate monohydrate crystals to 100 ml of distilled water and stirring with a magnetic stirrer for 2 h. Then 250 μl of seed crystals was added to 10 ml of synthetic urine containing the test sample, or distilled water as a control, and incubated for 3 h at 37°C with shaking in a water bath. The mixture was filtered through a 0.22 μm Millipore filter to remove crystals. The radioactivity in the filtrate was measured with a scintillation counter, and the amount of oxalate consumed for crystal growth was calculated ($n = 4$). Inhibitory activity was determined by the formula $(1 - C_s/C_c) \times 100\%$, where C_s is the amount of oxalate consumed with the use of the test sample and C_c is the amount of oxalate consumed under control conditions.

Results

RT-PCR

β-Actin and HS mRNA were expressed in normal human kidney as single bands of 1058 bp and 1020 bp respectively (Fig. 1). The PCR product of HS was sequenced and showed complete homology with HS [18, 22, 23].

Purification of recombinant HS

The results of the SDS-PAGE analysis of rHS eluted from the Q Sepharose column are shown in Fig. 2. The protein in lane 2 was purified rHS (53 kDa). It was

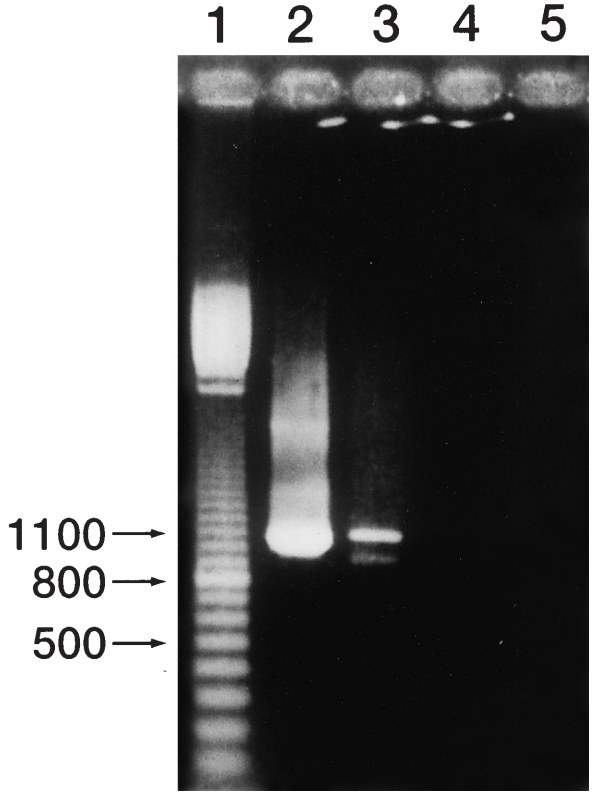

Fig. 1 α_2HS-Glycoprotein (HS) analysis by means of reverse transcription–polymerase chain reaction (RT-PCR). These PCR products were run individually on 1.5% agarose gels. *Lane 2* is amplified HS and *lane 3* β-actin from the human kidney. *Lane 4* (for HS) and *lane 5* (for β-actin) are blank controls without cDNA, and *lane 1* is a molecular weight marker (100 Base-Pair Ladder, Pharmacia Biotech, Buckingham, UK; 1100, 800 and 500 base pairs are indicated by *arrows*)

sequenced and showed complete homology with HS [22]. The second weaker band at around 47 kDa in lane 2 was not determined by NH_2-terminal sequence as its NH_2-terminal may have been blocked. However, the amount of the second protein at 47 kDa was small, so this protein was used as rHS. Western blotting analysis (data not shown), using anti-human HS antibody, showed reactivity in this second protein, as well as in rHS in the main band. Thus, it is plausible the second band is a derivative of rHS.

Immunohistochemical staining

Figure 3 shows light photomicrographs of the immunohistochemical staining of HS. HS was located in the distal and proximal renal tubules in the human kidney.

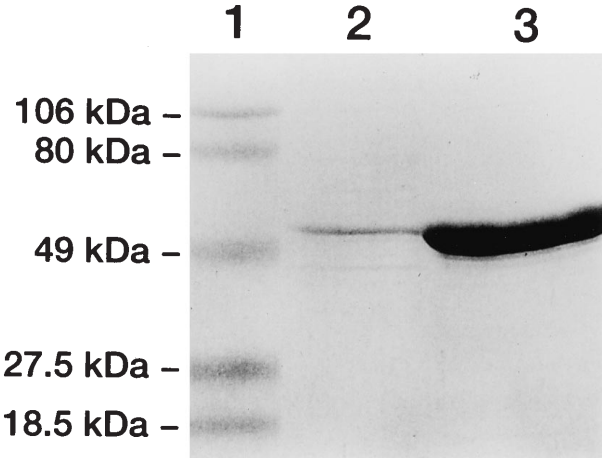

Fig. 2 SDS-PAGE analysis (8 mm thickness, Coomassie Brilliant Blue stain). *Lane 1* molecular weight markers (low range, Bio-Rad, Calif.). *lane 2* recombinant α_2HS-glycoprotein (53 kDa), *lane 3* maltose binding protein (50 kDa)

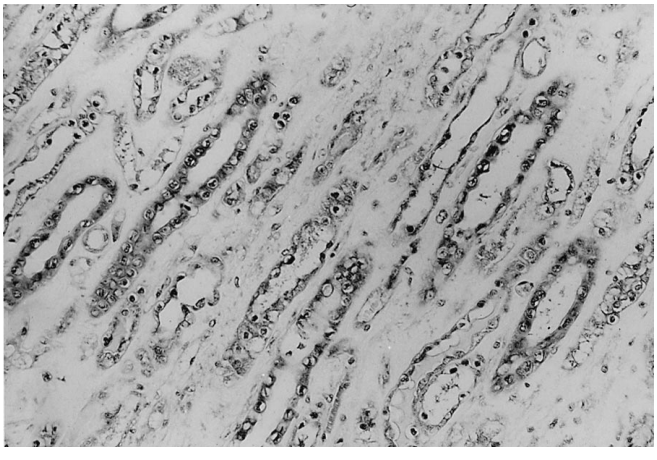

Fig. 3 Immunohistochemical staining for α_2HS-glycoprotein (HS) in a human kidney (original magnification ×80). HS was located in the distal and proximal renal tubular cells in the human kidney

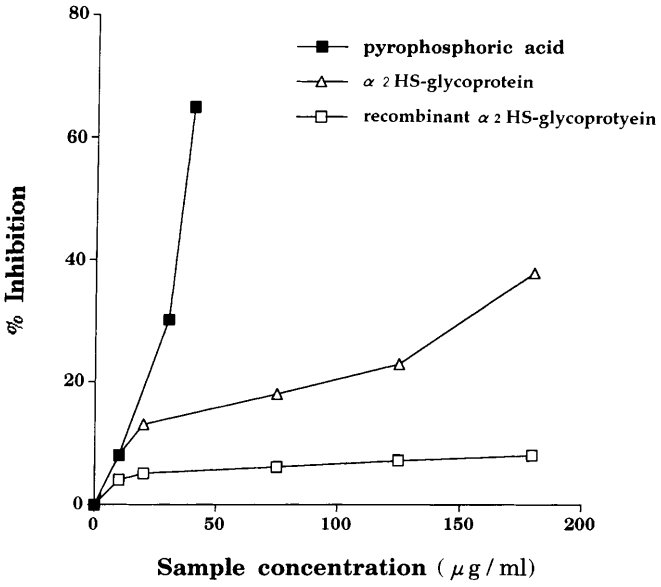

Fig. 4 The inhibitory activity of α_2HS-glycoprotein (HS) and recombinant HS as assessed by the seed crystal method (n = 4). HS activity was significantly higher than that of recombinant HS at all the protein concentrations tested

Inhibitory activity of HS as assessed by the seed crystal method

As shown in Fig. 4, at a protein concentration of 50 μg/ml, the inhibitory activity of pyrophosphoric acid (about 70%) was markedly greater than those of HS (about 15%) and rHS (about 5%). The inhibitory activity of rHS remained low at a protein concentration of 180 μg/ml, but HS showed inhibitory activity of about 40% at the same protein concentration.

Discussion

Immunohistochemical staining for HS was previously found to be particularly strong in the earliest cortical plate cells of the human fetal brain, suggesting an important, but as yet undetermined, function of HS in the development of the brain [5]. It is also known that human HS has a high affinity for calcium ions, and is one of the major noncollagenous proteins in bone matrix, suggesting a potential role in bone formation and resorption [2, 18]. Other activities of HS, such as the stimulation of lipid uptake and adipogenesis [22], phagocytosis [23], and adherence to the substratum [22], as well as the inhibition of phytohemagglutinin-induced lymphocyte activation [22] and insulin receptor tyrosine kinase activity [18], have been reported in a number of papers from various fields. None of these biological functions, however, has yet been sufficiently substantiated, and the molecular basis of the functional diversity of HS remains obscure.

Urinary macromolecules play an important inhibitory role in urinary stone formation [3], and proteins

appear to be responsible for most of this inhibitory activity [11, 12]. Honda et al. [8] showed that CSBS markedly inhibited CaOx crystal growth by the seed crystal method and contained both proteins and glycosaminoglycans [8, 16]. They identified the two most important protein components, prothrombin activation peptide F_1 and osteopontin, which have inhibitory effects on CaOx crystal growth, and they also identified other proteins including HS from CSBS by amino acid sequencing.

The aims of the present study were (1) to characterize and measure the inhibitory effect of HS, which is one of the components of CSBS from human urine, on CaOx crystal growth, by employing the seed crystal method and (2) to identify the localization of HS in the human kidney. Our study, using RT-PCR, demonstrated that human kidney tissues contain HS mRNA. The HS in the kidney was localized in the distal and proximal renal tubular cells as shown by immunohistochemical staining. These results raised the possibility that HS is produced in the renal tubular cells as well as in the liver [21, 23], and is excreted into the urine. However, the inhibitory activity of HS was weak (about 8% at 10 μg/ml) and rHS showed no inhibitory activity by the seed crystal method. On the basis of our results we concluded that HS, which demonstrated little inhibitory activity, does not have a major role as an inhibitor of urinary stone growth in urine.

There are some concerns regarding the validity of experiments conducted using artificial urine (seed crystal method) as compared with undiluted urine. In the undiluted urine method, the conditions are closer to those in the living body than in the seed crystal method, and therefore experiments using the former seem essential for reliable and accurate results. However, according to Doyle et al. [4], crystal matrix extract, for example, inhibits CaOx crystal growth in both aqueous inorganic solution and undiluted urine. On the other hand, there are reports [8] whose conclusions are based solely on results derived from using the seed crystal method. Therefore our results, at the very least, indicate in vitro events and further studies are necessary to validate our findings.

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