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Detection of immunoreactive napsin A in human urine

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Abstract

Human napsin A is an aspartic proteinase highly expressed in kidney and lung. To elucidate whether napsin A is excreted in the urine we have performed an immunochemical study using anti-napsin A polyclonal antibody. As a result an immunoreactive band at approx. 38 kDa was detected which corresponds to the molecular mass of recombinant active human napsin A. A deglycosylation study showed that excreted napsin A is *N*-glycosylated on apparently all of the three potential glycosylation sites. Immunoreactive napsin A was also observed in urine from patients with a transplanted kidney whose kidney function appeared half to fully normal. On the other hand, no or very low immunostaining was detected in samples from patients with diseased kidneys. The urinary excretion pattern correlates well with the enzymatic activity of napsin A. These data show that human napsin A is excreted as functional proteinase in the urine. Furthermore, immunochemical studies suggest a relation between urinary excretion of napsin A and renal function. More specifically, lack of urinary excretion of napsin A could potentially serve as a tool for the detection of kidney dysfunction.

Keywords: Human napsin A; Urine; Human kidney; Napsin antibody; Aspartic proteinase

1. Introduction

Aspartic proteinases belong to a subclass of endopeptidases with two essential Asp residues in the active site [1,2]. To this group belong enzymes such as cathepsins D and E, pepsin, gastricsin, renin and β-site APP-cleaving enzyme (BACE). Nucleotide sequences of another two human aspartic proteinases, napsins A and B, have been reported by Tatnell et al. [3]. Also, orthologues of human napsin A have been described in mouse [4] and rat [5]. While the napsin A gene encodes a functionally active enzyme, the napsin B gene lacks a stop codon and, thus, it is not clear whether napsin B can be expressed as a functional enzyme [3]. The gene encoding napsin A contains a tentative sequence for a signal peptide and propeptide preceding mature protein sequence [3]. Purification and characterization studies of recombinant human napsin A showed that this enzyme is in many respects similar to the other members of the aspartic proteinase family [6]. Northern blot [3] and immunohistochemical studies using anti-napsin A antibody [7] further demonstrated that napsin A is exclusively expressed in kidney and lung. More specifically, napsin A was detected in proximal and distal convoluted tubules, collecting duct and Henley loops of kidney and in type II pneumocytes in normal lung. Until now only one aspartic proteinase, namely renin, is known to be highly expressed in kidney. Considering the importance of the renal-angiotensin system (RAS) on cardiovascular homeostasis and renal hemodynamics, it is of interest to investigate the properties of this new, kidney-localized, aspartic proteinase. Recently, a role of human napsin A as a marker in the diagnosis of primary adenocarcinoma and a possible candidate proteinase for the proteolytic processing of surfactant proteins has been proposed [8]. However, no indication of its potential role in kidney has been reported until now.

It is known that protein excretion in the urine can serve as a diagnostic tool for the prediction of the progression of renal functional loss [9]. Considering the high expression level of napsin A in renal tubular cells, it was of interest to determine whether napsin A can be detected in the urine by immunochemical methods and if so, if its excretion can be related to kidney injury.

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2. Materials and methods

2.1. Reagents

POROS 20 R1 reverse-phase packing material was from PerSeptive Biosystems (Framingham, MA, USA). 1 ml Mobicols columns and 10 μm lower and upper filters were from Molecular Biologische Technologie (Göttingen, Germany). Acetonitrile was obtained from Merck (Whitehouse Station, NJ, USA) and trifluoroacetic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). SDS-PAGE was performed using 4–20% Tris-glycine gels from Invitrogen (San Diego, CA, USA). Nitrocellulose membrane was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Endoglycosidases F and H were obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). Anti-napsin A antibody was prepared by Genosys Biotechnology (Cambridgeshire, UK) [7]. Recombinant human napsin A was expressed and purified as previously described [6,7]. Synthesis of fluorogenic substrate K(Dabsyl)-TSVLMAAPQ-Lucifer yellow has been previously described [6].

2.2. Subjects

A study using 10 ml of urine sample from three healthy volunteers (one man and two women; aged 35 ± 5 years), six patients with renal disease (six men and one woman; aged 63 ± 12 years), one patient with normal renal function (a man; aged 35 years) and 11 patients with a transplanted kidney (seven men and four women; aged 50 ± 10 years), was performed. The diagnosed renal diseases of these patients consisted of polycystic kidney disease, advanced glomerulonephritis (GN), extracapillary glomerulonephritis, lupus nephritis, diabetic nephropathy, and nephrosclerosis. All patients had increased albuminuria consistent with glomerular disease. Patients with a transplanted kidney were under immunosuppressant treatment and their kidney function was diagnosed as half to completely normal.

2.3. Concentrating total urine proteins

In order to concentrate total proteins from urine POROS 20 R1 beads were packed between 10 μm lower and upper filters in 1 ml Mobicols columns. The columns were prewashed sequentially with 5 ml 70% acetonitrile/0.1% trifluoroacetic acid (TFA) and 5 ml 0.1% TFA. A 10 ml urine sample acidified with 1 ml 0.1% TFA was applied to the column using a 5 ml syringe. After washing the column with 4 ml 0.1% TFA, bound proteins were eluted using 2 ml 20% acetonitrile/0.1% TFA followed by elution with 2 ml 70% acetonitrile/0.1% TFA. Lastly, the acetonitrile solution was evaporated by centrifugation in the SpeedVac Concentrator (Savant Instruments, New York, NY, USA). The resulting pellets containing urinary proteins were reconstituted in 50 μl phosphate-buffered saline (PBS) buffer (the final samples were 200-fold concentrated) and immediately used for immunochemical analysis.

2.4. Electrophoresis and Western blot analysis

SDS-PAGE was carried out by following the method of Laemmli using 4–20% Tris-glycine gels [10]. The detailed experimental procedure of SDS-PAGE and Western blot analysis including the generation of anti-napsin A antibody has been described elsewhere [7]. In order to determine whether native napsin A found in urine is *N*-glycosylated, concentrated proteins were incubated for 1 h at pH 5.5 with endoglycosidases F and H as recommended by the manufacturer and subsequently analyzed by Western blot.

2.5. Enzymatic activity measurements

The cleavage of the fluorogenic substrates was monitored by the increase in fluorescence per minute using a Fluostar (BMG LabTechnologies, Offenburg, Germany) and 96-well plates (Dynex Technologies, Chantilly, VA, USA) at room temperature. The reactions were performed in 100 mM sodium acetate reaction buffer, pH 4.3, containing 20 mM EDTA. A volume of 39 μl of reaction buffer was mixed with 1 μl of concentrated urine and the reaction was initiated by the addition of 10 μl of fluorogenic substrate K(Dabsyl)-TSVLMAAPQ-Lucifer yellow (the final substrate concentration was 20 μM) [6]. The increase in fluorescence using excitation at 390 nm and emission at 538 nm was recorded as a function of time. Under these conditions the enzymatic activity of approx. 0.5 nM human napsin A can be determined.

3. Results and discussion

3.1. Immunochemical detection of human napsin A in urine

Human napsin A is highly expressed in kidney with predominant localization in proximal and distal convoluted tubules [7]. Although subcellular localization has not yet been determined, immunohistochemical studies showed disperse staining for napsin A in the renal cortex, which may be indicative of a cytosolic localization. Furthermore, membrane association has been observed in the cells of collecting duct and human embryonic kidney 293 cells (HEK 293) transfected with recombinant human napsin A. Considering the high tubular expression it was of interest to investigate whether napsin A is excreted in the urine and if it can be detected by immunochemical means using previously described anti-napsin A polyclonal antibody [7]. To this end we have concentrated total urine proteins from three healthy volunteers using POROS 20 R1 beads. These beads are designed for general reversed-

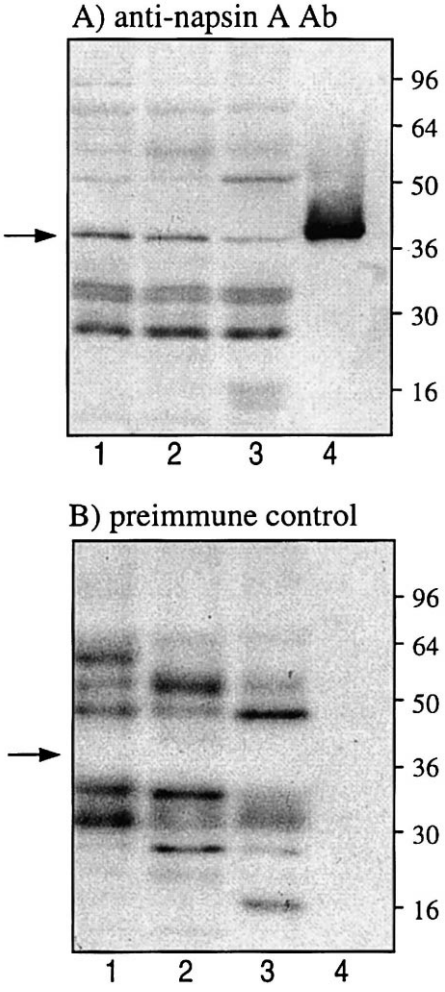

Fig. 1. Immunochemical detection of human napsin A in urine of healthy volunteers. Samples containing concentrated urine proteins (see Section 2 for details) were subjected to SDS-PAGE and Western blotting. (A) Blots were probed with an anti-napsin A polyclonal antibody raised in rabbits and detected by the chemiluminescence detection method. (B) Blots were probed with a preimmune serum from rabbits. Arrows indicate the position of napsin A (in A) and the expected position of napsin A (in B). Lanes 1–3 contain concentrated urine samples from three healthy volunteers. Lane 4 contains recombinant human napsin A expressed in HEK293 cells [6,7] as a positive control. Positions of molecular mass standards are indicated.

phase chromatography of peptides, proteins, polynucleotides and other biomolecules. Elution of bound proteins can be achieved using organic solvent such as acetonitrile. More specifically, 20% and 70% solutions of acetonitrile/0.1% TFA were employed, respectively. Consequently, eluted proteins were further concentrated by solvent evaporation. Finally, the remaining protein pellet was dissolved in a minimal volume of the PBS buffer and these solutions were used for immunochemical studies using polyclonal anti-napsin A antibody. As seen from Fig. 1A, the immunoreactive serum did not appear to be very specific since several bands with different molecular masses were observed in all the test samples. We should point out that this antibody has been raised against napsin A-specific peptidic epitope, which should provide antibody specificity, which has been already successfully show in immunohistochemical detection of native napsin A in human tissues and for the detection of recombinant human napsin A stably expressed in human embryonic kidney (HEK293) cells [6,7]. Considering that human urine contains a variety of proteins whose concentration increases 200-fold following the above described concentrating procedure, it is likely that non-specific interactions are responsible for the detection of the additional bands. This assumption was confirmed by Western blot analysis using preimmune serum. As shown in Fig. 1B, the positive control (purified recombinant napsin A, lane 4) as well as bands at 38 kDa in the test samples could not be observed while the other bands were still visible. These results suggest that the 38 kDa immunoreactive band, found in all three urine samples, represents human napsin A. Therefore, native human napsin A was detected in urine. The native enzyme has a molecular mass identical to the recombinant mature napsin A and, thus, it is likely that napsin A excreted in urine represents the same form of the enzyme. This was further confirmed by an activity assay for napsin A using a previously described fluorogenic substrate K(Dabsyl)-TSVLMAAPQ-Lucifer yellow [6]. The observed cleavage

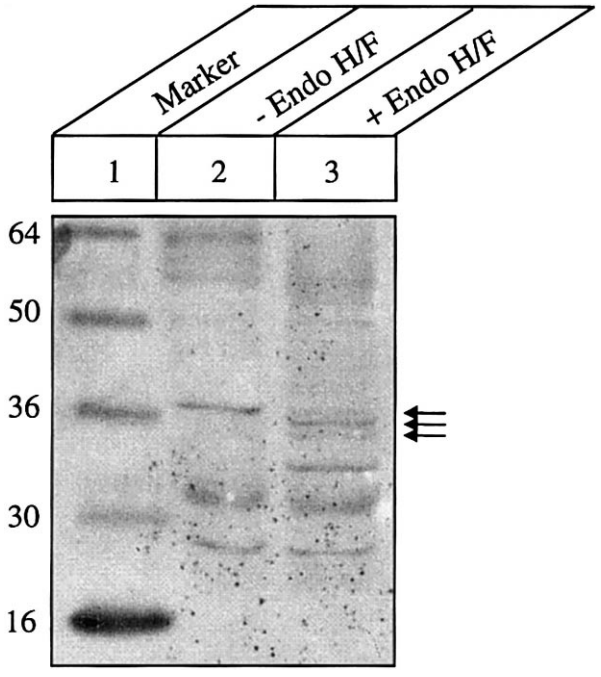

Fig. 2. Deglycosylation of human napsin A excreted in the urine. Lanes 2 and 3 contain concentrated urine proteins from a healthy volunteer before and after treatment with a mixture of endoglycosidases H and F, respectively. Western blot using anti-napsin A antibody revealed three additional bands in an endoglycosidase-treated sample (lane 3) corresponding to di-, mono-, and non-glycosylated forms of napsin A (arrows indicate the position of deglycosylated forms of napsin A). The additional band with a molecular mass of approx. 34 kDa is the result of a non-specific interaction between antibody and endoglycosidase H [6]. The molecular mass standards are shown in lane 1.

of the substrate was inhibited by pepstatin, a general aspartic proteinase inhibitor (data not shown).

3.2. Glycosylation of napsin A

During our studies with recombinant human napsin A it was observed that the enzyme is *N*-glycosylated with a sugar moiety contributing approx. 2 kDa to the apparent molecular mass [7]. It was of interest to determine whether the native enzyme from the urine is also glycosylated and how it compares to recombinant napsin A. For this reason, concentrated urine proteins were treated with endoglycosidases H and F and analyzed by Western blot analysis. Treatment with endoglycosidases resulted in the appearance of three additional bands, with lower molecular masses, and disappearance of the 38 kDa band (Fig. 2). Human napsin A has three putative N-linked oligosaccharide attachment sites as deduced from the amino acid sequence [3]. The presence of the three bands in endoglycosidase-treated napsin A strongly indicates that in the native form all three sites are glycosylated, the upper two bands representing partially glycosylated and the lower band complete deglycosylated forms of napsin A. The band at approx. 34 kDa results from non-specific interactions between the immunoreactive serum and endoglycosidase H (see [6]). This indicates that napsin A is *N*-glycosylated in vivo. Considering the fact that the enzyme contains a signal peptide, which could be involved in translocation to the endoplasmic reticulum via the signal recognition particle (SRP) route, it is likely that this route is responsible for the processing of the *N*-glycosylation sites. However, further studies would be needed to confirm these assumptions.

3.3. Human napsin A in urine of kidney patients

Analysis of proteins in urine is a standard method of addressing issues of renal (dys)function. Namely, in healthy individuals, the glomerular filtration barrier provides an effective mechanism for preventing the passage of excess protein from the plasma to the urine. Impairment in renal function associated with loss of functioning nephron units leads to structural changes in the glomerulus. Consequently plasma proteins can freely pass across the glomerular membrane resulting in proteinuria. Since proteinuria correlates well with the tubulointerstitial damage

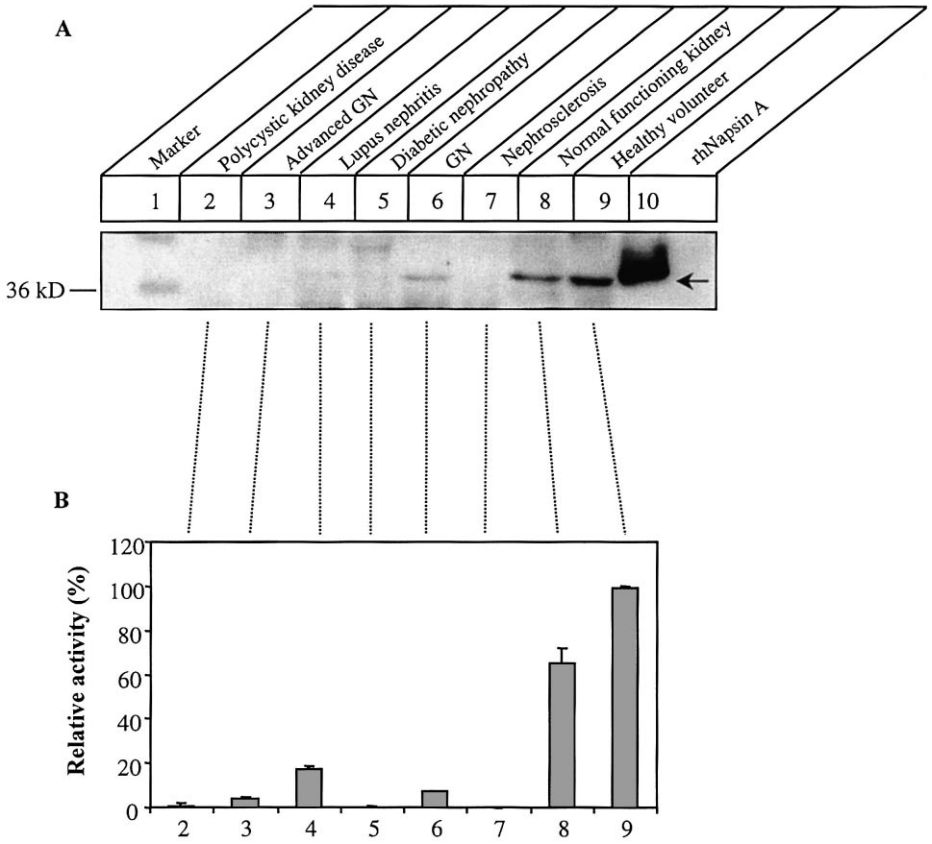

Fig. 3. Detection of napsin A in urine of kidney patients. (A) Western blot analysis. Lane 1 contains molecular mass markers. Lanes 2–7 were loaded with 10 μl of concentrated urine solution from patients diagnosed with different kidney diseases as indicated. Lanes 8–10 contain concentrated urine sample from a patient not diagnosed with kidney disease, a healthy volunteer and recombinant human napsin A, respectively, as positive controls. An arrow indicates the position of an immunoreactive band of napsin A. GN, glomerulonephritis. (B) Measurement of enzymatic activity. The reaction was performed in assay buffer containing 100 mM sodium acetate pH 4.3 and 20 mM EDTA (see text for details). The velocity corresponding to the maximum activity of the sample from a healthy volunteer was set as 100%.

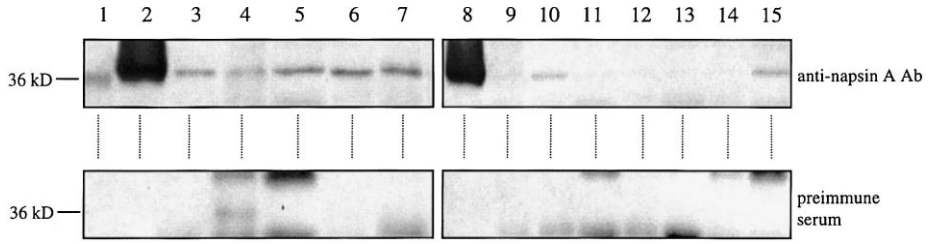

Fig. 4. Immunochemical detection of napsin A in patients with transplanted kidneys. Urine samples from patients with a transplanted kidney (lanes 3–7 and 9–14) and one healthy volunteer (lane 15) were concentrated and subjected to SDS-PAGE and Western blotting as described in the text. Blots were probed with anti-napsin A antibody and preimmune serum as indicated. Lane 1 contains molecular mass marker and lanes 2 and 8 contain recombinant human napsin A.

and progression of renal failure, a measurement of protein (mostly albumin) filtered in urine is used as a marker for prognosis of progression of renal functional loss [11,12]. Given that napsin A can be detected in urine of healthy individuals we investigated whether napsin A is also excreted in urine of patients diagnosed with renal diseases. For this reason urine samples from six patients diagnosed with renal diseases and urine from one patient with normal renal function were analyzed as described above. As a positive control a recombinant human napsin A and a urine sample from a healthy volunteer were employed. As shown in Fig. 3A, an immunoreactive band corresponding to human napsin A was observed in positive control samples (lanes 8–10). Samples from patients with impaired renal function contained no or very weak immunoreactive napsin A. The exception was a sample in lane 6, which showed an immunoreactive band of napsin A (although less pronounced than in control samples). Measurement of napsin A activity in these samples showed excellent correlation with the detection of the immunoreactive napsin A band (Fig. 3B). Therefore, it appears that napsin A cannot be detected (or at very low levels) in the urine of patients diagnosed with different kidney diseases. It is tempting to speculate that lack or reduction in excretion of napsin A in urine can be indicative of renal injury.

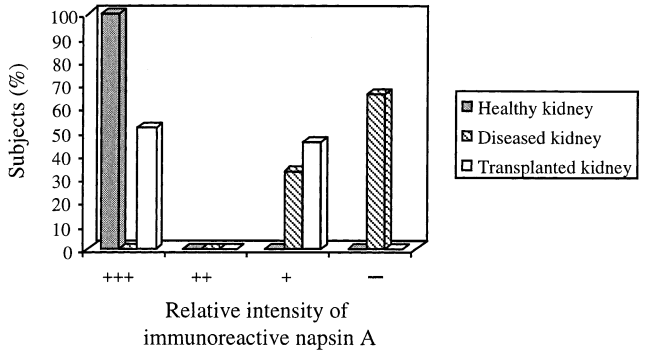

Fig. 5. Correlation between kidney function and relative intensity of immunoreactive bands of napsin A in urine samples. The intensity of the napsin A band was arbitrarily assigned: +++, as in healthy volunteers and six patients with transplanted kidney; ++, as in no subjects; +, as in two patients with renal disease and five patients with transplanted kidney; −, as in four patients with renal disease.

3.4. Human napsin A in urine of patients with a transplanted kidney

In order to further test this hypothesis, we have performed immunochemical analysis of urine samples from patients with transplanted kidneys. All the patients were diagnosed after transplantation with half to fully normal kidney function and, therefore, the expectation was that these samples would contain napsin A. Indeed, an immunochemical study (Fig. 4) showed that most of the samples contained a strong, immunoreactive band corresponding to napsin A. However, in five out of 11 samples the immunoreactive signal was weaker than in the rest of the samples (lanes 9 and 11–14). Even so, all presented data support a correlation between renal function and the level of urinary excretion of napsin A as shown in Fig. 5.

The renal proximal tubular epithelial cells are highly metabolic cells which support extremely rapid, active transport processes. These cells also have an important role in the development of renal diseases [13]. The amount of protein found in the urine, taken as an indication of the primary abnormality in the glomerular permeability, is considered by most nephrologists as a marker of the severity of renal lesions. Based on the results of our study it would appear that the opposite effect, namely absence of a protein (napsin A) in urine, may lead to the conclusion of the presence of renal injury. Prescott has shown that, under physiological conditions, almost 2×10^6 tubular epithelial cells slough into the urine each day [14]. Renal injury increases the rate of cell loss beyond the capacity of basic physiological proliferative mechanisms to preserve the structural integrity of the kidney. Since napsin A is predominantly expressed in these cells, it is plausible that the lack of its excretion in urine is indicative of tubular injury. More specifically, all kidney patients who lacked napsin A in their urine were diagnosed with tubular interstitial diseases. It should be added that no immunoreactive band for napsin A was detected in human plasma (data not shown). Therefore, it would appear that napsin A in the urine is of renal origin, which is further supported by high mRNA and protein levels in the kidney [3,7]. The results of many studies [15–17] indicate that proteins filtered through the glomerular capillary may induce intrin-

sic renal toxicity, which together with other independent risk factors such as hypertension may contribute to the progression of renal damage. Obviously, further studies aimed to investigate the physiological significance and the regulation of napsin A gene expression will be necessary to clarify the present pattern of napsin A excretion in the urine. In particular it would be of interest whether lack of napsin A in urine precedes or follows the progression of renal functional loss and increase in proteinuria, namely, could it be an early indicator or rather a marker of these processes.

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