Mannan-binding lectin (MBL)-associated plasma protein present in human urine inhibits calcium oxalate crystal growth

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Received 17 September 1999; received in revised form 1 November 1999

Abstract Mannan-binding lectin (MBL)-associated plasma protein (MAp19) is an alternatively spliced form of MBL-associated serine protease-2, a component of a complement activation cascade. We observed that MAp19 is excreted in human urine. Interestingly, the amount of MAp19 was higher in urine of renal cell carcinoma patients than healthy people. Pretreatment of urine dialysate with 50 mM EDTA increased the recovery of MAp19, suggesting that MAp19 is a calcium-binding protein. The recombinant MAp19 showed a strong inhibition of calcium oxalate crystal growth in vitro in a concentration-dependent manner. Thus, we conclude that MAp19 plays a role in the inhibition of calcium oxalate renal stone formation.

Key words: Mannan-binding lectin-associated plasma protein; Human urine; Renal cell carcinoma; Calcium oxalate crystal; Renal stone

1. Introduction

Nephrolithiasis is a common disorder, and calcium oxalate is the most common crystalline component of human renal stones [1]. The nucleation and growth of calcium oxalate or phosphate occur because urine in normal people is supersaturated with these salts. However, the fraction of people who form severe renal stones is relatively small. One reason why urine supersaturation does not lead to stone formation and nephrolithiasis is that urine contains inhibitors of crystallization of calcium oxalate. Inhibitory proteins found in urine include nephrocalcin [2,3], Tamm-Horsfall protein [4], uropontin/osteopontin [5,6], crystal matrix protein (F prothrombin fragment) [7], and uronic rich-acid protein [8]. Most of these molecules are anionic with many acidic amino acid residues, and appear to exert their effects by binding to the surface of calcium oxalate [9]. Although a Ca^{2+}-binding property is important for their inhibitory function, the specific structural motifs that favor crystal binding and inhibition are not yet known. Some of the urinary inhibitor proteins are made by renal epithelial cells, whereas others gain access to the urine by glomerular filtration.

Mannan-binding lectin (MBL) binds to many pathogens via surface carbohydrates and induces cell lysis via complement activation [10,11]. This MBL-induced activation of the complement cascade was shown to occur via MBL-associated serine protease-1 (MASP-1), which consumes C2 and C3, and has been proposed as the third pathway of complement activation [12–15]. MBL is related to the complement C1 subcomponent, C1q, and MASP-1 is similar to C1r and C1s [11]. A second form of MBL-associated serine protease-2 (MASP-2) was also identified, which shows sequence homology with previously reported MASP (MASP-1) and two C1q-associated serine proteases, C1r and C1s [16]. Similarly to MASP-1, MASP-2 is composed of an N-terminal C1r/C1s-like domain, followed by an epidermal growth factor (EGF)-like domain, a second C1r/C1s-like domain, two complement control protein (CCP) domains, and a serine protease domain [16]. Upon activation, it is cleaved into two disulfide-linked chains, an N-terminal 52 kDa polypeptide chain composed of the first five domains and a C-terminal catalytically active 31 kDa polypeptide chain [16,17]. Recently, the alternatively spliced form of MASP-2, MBL-associated plasma protein (MAp19), was found to be associated with MASP-1 in plasma. It consists of the first two domains of MASP-2 with four different C-terminal amino acids [18]. MAp19 is also shown to be the major translational product of the MASP-2 gene in plasma although it is enzymatically inactive as it lacks the serine protease domain. This result implies that there may be some unknown roles of this protein in addition to as a component of the MBL-MASP complex [17].

Although several urinary inhibitors were identified, there is a report suggesting the existence of other unknown proteins in urine with strong inhibitory action of stone formation [19]. Therefore, we attempted to find a novel inhibitor of renal stone formation in urine. Since nephrocalcin is excreted more in urine of renal cell carcinoma patients [20,21], we screened urine proteins from both patients with renal cell carcinoma and normal adults.

2. Materials and methods

2.1. Materials

Diethylaminoethyl-cellulose (DEAE-Sephacell) was purchased from Pharmacia. [^{14}C]Oxalic acid was obtained from New England Nuclear (NEN) Du Pont Co. Urine specimens were collected from patients with renal cell carcinoma and normal adults. pET-21a plasmid was from Novagen and Ni-NTA affinity resin was from Qiagen. Hybond membrane and ECL kit containing anti-rabbit IgG conjugated with horseradish peroxidase (HRP) were purchased from Amersham. All other chemicals were the highest grade available.

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Abbreviations: MBL, mannan-binding lectin; MAp19, MBL-associated plasma protein; MASP, MBL-associated serine protease; DEAE, diethylaminoethyl; IPTG, isopropyl-β-thiogalactoside; HPLC, high performance liquid chromatography; EGF, epidermal growth factor

PII: S0014-5793(99)01509-4

2.2. Partial purification of MAp19

One liter of the collected urine was dialyzed against 12 l of deionized water for 24 h at 4°C with two changes. The dialysate was adjusted to 50 mM NaCl, Tris-HCl, pH 7.3, and then mixed with 1/10 volume of DEAE-cellulose preequilibrated with 50 mM Tris-HCl, pH 7.3, 50 mM NaCl. The suspension was stirred for 30 min at room temperature. After the resin had settled down, it was filtered through a glass wool fiber in the column. The DEAE-cellulose resin was washed with a 50 mM Tris-HCl buffer, pH 7.3, containing 50 mM NaCl to remove non-specifically bound proteins. The proteins were eluted using 180 mM NaCl, 50 mM Tris-HCl, a buffer, pH 7.3. For EDTA treatment of urine samples, the dialysate was first adjusted to 50 mM EDTA, pH 8.0, and incubated for 24 h at 4°C. After this EDTA treatment, these dialysates were subjected to DEAE-cellulose chromatography as above. The eluates were pooled and dialyzed against 4 l of deionized water and lyophilized. Protein concentration was determined by the Lowry method (Bio-Rad) using bovine serum albumin as a standard.

2.3. SDS-polyacrylamide gel electrophoresis and peptide sequencing

The partially purified MAp19 was separated on 15% SDS-polyacrylamide gel and stained with Coomassie blue. After destaining, the MAp19 band was excised. The peptides were eluted from the gel after in situ tryptic digestion, and separated by HPLC. The amino acid sequencing was performed using Applied Biosystems Division Precise Protein/Peptide Sequencer. The resulting sequences were searched via the BLAST Network Server.

2.4. Preparation of polyclonal antibody against MAp19

Peptide (LASPGFPGEYAND QER) was synthesized and coupled to keyhole limpet hemocyanin using a bifunctional coupling reagent, glutaraldehyde. New Zealand White rabbits were initially immunized with 1 mg of peptide conjugate in 50% (v/v) Freund's complete adjuvant (FCA). Five weeks after immunization, the rabbits were boosted with 1 mg of peptide conjugate. The animals were bled 7 weeks after the initial immunization. Serum was collected by centrifugation after clotting, and MAp19 antibody was purified from this serum by ammonium sulfate precipitation and an antigen-bound affinity column.

2.5. Immunoblotting

Samples were separated by 15% SDS-PAGE and transferred electrophoretically to Hybond nitrocellulose membrane (Amersham Co.) at 400 mA for 3 h. The membrane was blocked with 5% skim milk, and incubated with anti-MAp19 antibody. After washing for 1 h, it was incubated with anti-rabbit IgG conjugated with HRP. The immunoadsorbed proteins were finally detected with a chemiluminescence kit.

2.6. Expression and purification of MAp19 protein in Escherichia coli

cDNA encoding 170 amino acids of the N-terminal region (MAp19) of MASP-2 except the signal peptide was amplified by polymerization chain reaction (PCR) using the liver cDNA library as a template. The PCR primers were designed to include *Bam*HI and *Hin*dIII sites at the 5′- and 3′-end of the PCR product, respectively. The sequences of the primers were as follows: 5′-CGGGATCCACCCCCTTGGGCCCGAAG-3′, 5′-GGAAGCTTGAGGCTCTGCTCTGAGCA-3′. The PCR reactions were performed at the standard condition. The PCR product was digested with restriction enzymes (*Bam*HI and *Hin*dIII) and subcloned into pET-21a expression vector. BL21 competent bacteria were transformed with this vector and cultured until the optical density (OD) reached 1 at 600 nm. For induction of MAp19 in transformed *E. coli*, IPTG was added to a final concentration of 0.5 mM to this bacterial culture and bacterial cells were further incubated for 4 h at 37°C. After this induction of recombinant MAp19, bacterial cells were centrifuged and the pellet was resuspended with a column buffer containing 50 mM sodium phosphate, pH 7.5, 100 mM KCl, 0.1% Tween 20, 10 μM phenylmethylsulfonyl fluoride (PMSF) and 20 mM imidazole. After sonication, only soluble extracts were subjected to the Ni-NTA column that was preequilibrated with this buffer. The column was washed, and MAp19 was finally eluted by adding 400 mM imidazole.

2.7. Assay of calcium oxalate monohydrate (COM) crystal growth inhibition

Calcium oxalate crystal growth inhibition was measured as previously described with some modifications [3]. This assay was performed in a 1.5 ml microcentrifuge tube containing 0.67 ml of reaction buffer (2 mM $CaCl_2$ in barbituric acid, 133 mM NaCl, 0.03% acetic acid, pH 5.7), and 0.134 ml of calcium oxalate crystal slurry (1.5 mg/ml). 25 μl of protein samples was added to the tubes. Elution buffer containing 400 mM imidazole was used as a control. The reaction was started by adding 0.67 ml of 0.4 mM sodium oxalate containing 40 nM [^{14}C]oxalic acid as a tracer in a 50 mM barbiturate-acetate buffer. The assay solutions were incubated at 37°C with shaking. After the indicated period of incubation, the crystals were pelleted by centrifugation for 30 s, and 100 μl aliquots of supernatant were withdrawn. These aliquots were counted in a liquid scintillation counter after dissolving in 4 ml of scintillation cocktail (Wallac-Pharmacia).

3. Results

3.1. 19 kDa MAp19 was detected in human urine

To find an inhibitor of calcium oxalate crystallization from human urine, we screened urine protein samples from patients with renal cell carcinoma and normal adults. After partial purification of urine by DEAE-cellulose ion exchange chromatography, the eluates were separated on 15% SDS-PAGE and stained with Coomassie blue. The results showed that two out of five urine protein samples obtained from patients with renal cell carcinoma show protein bands in the region of 19 kDa, which was not detectable in the urine of normal subjects (Fig. 1). In order to reveal the identity of this protein, we attempted to determine the amino acid sequence of this protein. To do this, we cut out a 19 kDa protein band from the 15% SDS-PAGE gel after Coomassie blue staining, eluted from the gel and sequenced using the Applied Biosystems sequencer after in situ trypsin digestion. The results showed that amino acid sequences of three peptides were identical to the N-terminal region of the recently identified MAp19 [16,17]. The amino acid sequences of the three peptides, with the positions of the three peptides in MAp19 in parentheses, are as follows: peptide 1, LASPGFPGEYANDQER

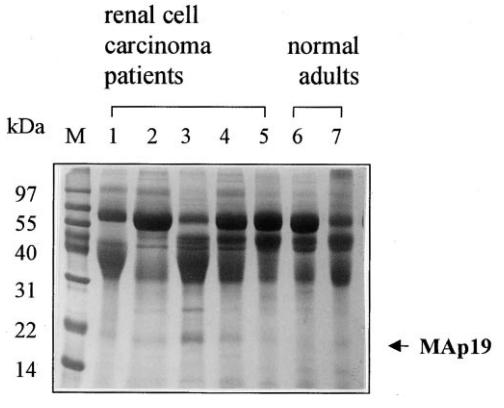

Fig. 1. SDS-PAGE analysis of partially purified MAp19 from urine. The MAp19 protein mixtures were partially purified from urine samples of five renal cell carcinoma patients (lanes 1–5) and two normal adults (lanes 6,7) by DEAE-cellulose anion exchange chromatography. 100 μg of these mixtures were subjected to 15% SDS-PAGE and stained with Coomassie blue. The arrow indicates a protein of molecular mass approximately 19 kDa. M: protein molecular weight marker.

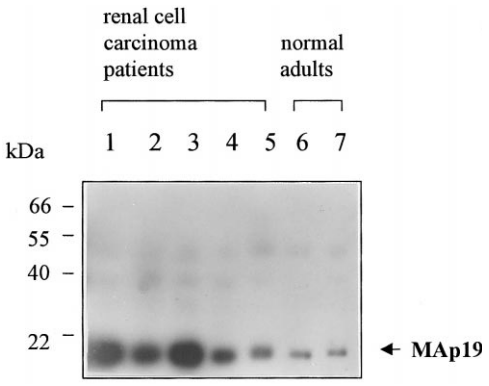

Fig. 2. Immunoblot analysis of MAp19 in urine after partial purification. MAp19 partially purified from urine samples of five renal cell carcinoma patients (lanes 1–5) and two normal adults (lanes 6,7) was semiquantified after separation on 15% SDS-PAGE by immunoblot using polyclonal anti-MAp19 antibody. In each lane, 10 μg of protein was loaded.

(29–45); peptide 2, WTLTAPPGYR (47–56); peptide 3, SDYSNEKPFTGFEAF (119–133).

In order to determine the amount of MAp19 in urine, we next raised antibody against MAp19 by immunizing a rabbit with peptide synthesized based on the peptide 1 sequence, and then purified by an antigen-conjugated affinity column following ammonium sulfate precipitation. Using this antibody, we semiquantified the MAp19 amount in patients with renal cell carcinoma and normal subjects. The results showed that it is present in urine of both patients with renal cell carcinoma and normal adults, although the amount of MAp19 was higher in urine samples from patients with renal cell carcinoma than normal adults (Fig. 2).

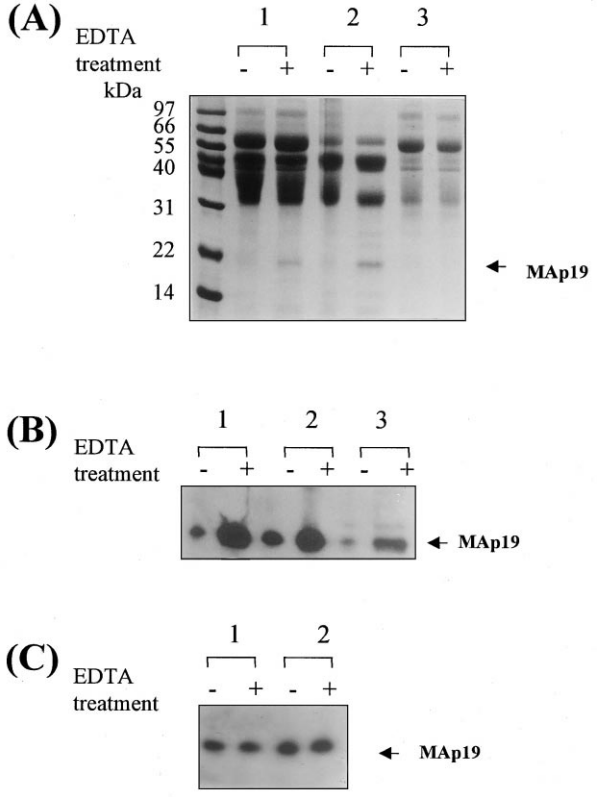

Fig. 3. Increase in the amount of MAp19 with EDTA pretreatment. The effect of EDTA treatment of urine dialysate prior to DEAE-cellulose chromatography on the recovery of MAp19 was examined by SDS-PAGE (A) and immunoblot (B). The partially purified urine proteins were also treated with EDTA and analyzed by immunoblot (C). The urine samples without a MAp19 band on SDS-PAGE were treated with (+) or without (−) 50 mM EDTA for 24 h at 4°C. Each two lanes are a pair of urine samples from the same person.

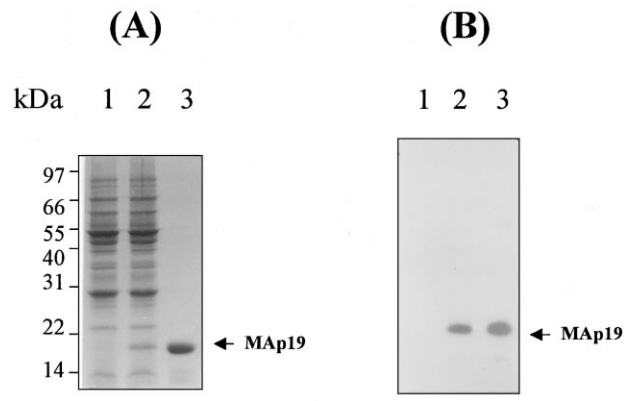

Fig. 4. Expression and purification of MAp19 in *E. coli*. MAp19 was overexpressed after 0.5 mM IPTG induction for 4 h, and it was purified from transformed *E. coli* lysates using Ni-NTA affinity chromatography. Protein was analyzed by 15% SDS-PAGE (A), and immunoblotted with anti-MAp19 antibody (B). Lane 1, soluble fraction of cell lysate; lane 2, soluble fraction of cell lysate after IPTG induction for 4 h; lane 3, MAp19 purified by Ni-NTA affinity chromatography.

3.2. *MAp19 is present in urine as a calcium-binding protein*

MAp19 has common domain structures such as a C1r/C1s-like domain and an EGF-like domain [16,17]. This EGF-like domain in the C-terminal region is predicted to have a calcium-binding helix-loop-helix motif termed an EF-hand [22,23], and it also has the key residues involved in the calcium-binding sites. To test whether MAp19 is a calcium-binding protein, we treated urine dialysates with 50 mM EDTA for 24 h, partially purified by DEAE-cellulose ion chromatography, and analyzed by SDS-PAGE and by immunoblot. When they were treated with EDTA before purification, two urine samples showed increases in band intensity around the 19 kDa region on SDS-PAGE analysis (Fig. 3A). However, all of the urine samples pretreated with EDTA showed a significant increase in MAp19 band intensity on immunoblot analysis (Fig. 3B). Next, we examined whether it is present as an aggregated oligomer like nephrocalcin. When we treated urine samples with 50 mM EDTA for 24 h after partial purification, there was no increase in MAp19 band intensity on immunoblot analysis (Fig. 3C). These results suggest that MAp19 is a calcium-binding protein that exists as a monomeric form in urine.

3.3. *Recombinant MAp19 inhibits calcium oxalate crystal growth in vitro*

Although MAp19 was detected as part of the MBL-MASP complex that activates the complement system, we speculated that MAp19 in urine might have another function related to its calcium-binding property. Therefore, we next investigated whether this protein can inhibit calcium oxalate crystal growth like other urinary proteins such as urinary bikunin/urinary acidic protein/nephrocalcin [24–26], Tamm-Horsfall

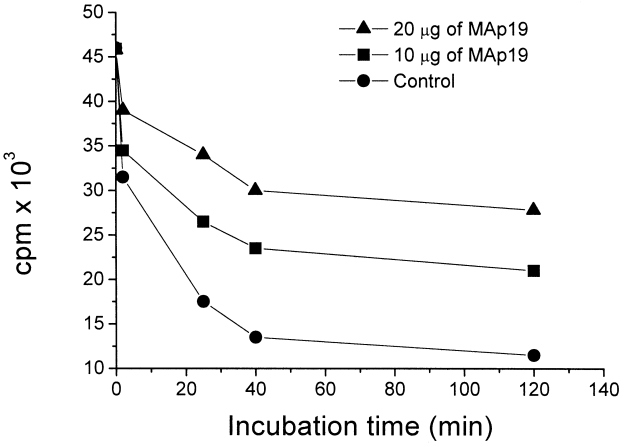

Fig. 5. Time course of calcium oxalate monohydrate crystal growth inhibition by recombinant MAp19. Purified MAp19 protein from *E. coli* was assayed for the inhibitory activity of calcium oxalate monohydrate crystal growth as described in Section 2. Imidazole buffer only without MAp19 (●), 10 μg (■) and 20 μg (▲) of recombinant MAp19.

protein [27], and uropontin [28]. To do this work, we first obtained cDNA of MAp19 by polymerase chain reaction (PCR) using the liver cDNA library as a template, and subcloned this PCR product into a prokaryotic expression vector, pET-21a. After subcloning, we induced MAp19 in *E. coli* using 0.5 mM IPTG. Since MAp19 was fused with a 6×His tag, it was purified using Ni-NTA affinity chromatography. This purification method produces a single protein on SDS-PAGE with a molecular mass of approximately 19 kDa as we expected (Fig. 4A). Immunoblot analysis confirmed that the overexpressed protein is indeed MAp19 (Fig. 4B). Next, we tested whether recombinant MAp19 has an inhibitory activity on calcium oxalate crystal growth in vitro. It was not exposed to EDTA for this assay. The result showed that MAp19 inhibits calcium oxalate crystal growth in a concentration-dependent manner in vitro (Fig. 5).

4. Discussion

In this study, we first showed that MAp19 is excreted in urine of patients with renal cell carcinoma and normal adults although its amount is higher in urine of renal cell carcinoma patients. We also showed for the first time that recombinant MAp19 has an inhibitory activity on calcium oxalate crystal growth in vitro. Since MAp19 is an alternatively spliced form of MASP-2 which is a component of a complement system, our finding that MAp19 is excreted more in urine of renal cell carcinoma patients than normal adults suggests complement activation by the MBL-MASP-2/MAp19 complex in renal cell carcinoma. MBL can not only kill exogenous microorganisms, but also destroy mammalian cells with mannose-type oligosaccharides on their surfaces [29]. Consistent with this report, Magyarlaki et al. detected tissue deposition of C1q, IgG and C3 in renal cell carcinoma biopsies indicating that a complement system is activated in renal cell carcinoma. However, they also showed that these renal cell carcinomas with complement activation also express the membrane-bound complement regulatory factors that may inhibit complement-mediated tumor cell lysis [30]. Thus, it is to be determined whether this complement activation by the MBL-MASP-2/MAp19 complex causes any significant damage to renal cell carcinoma.

Pretreatment of urine dialysate with 50 mM EDTA increased the recovery of MAp19, whereas the purified samples did not give larger amounts of MAp19 even after treatment with the same concentration of EDTA. These results suggest that MAp19, unlike nephrocalcin, is present as a monomeric form in the presence of calcium ion. Also, the reason why we recovered more MAp19 with EDTA pretreatment seems to be that it is bound more to DEAE-cellulose anion resin upon removal of Ca^{2+} ion from protein. In fact, MAp19 has a domain rich in acidic amino acids (Asp and Glu) in its C-terminal region, and these residues may provide an ideal electrostatic environment for Ca^{2+} binding. These properties of urinary proteins seem to be responsible for the inhibitory activity of calcium oxalate monohydrate growth [31].

It remains to be determined if MAp19 can inhibit other stages of crystallization, since the formation of renal stones requires nucleation, growth, aggregation of crystals, and crystal attachment to renal epithelial cells. Actually, urinary proteins inhibit more than one stage of this crystallization [24]. For example, Tamm-Horsfall protein inhibits growth and aggregation without inhibition of nucleation, whereas nephrocalcin has been found to inhibit all three stages of crystallization [24]. Thus, we are currently investigating whether it can inhibit other stages of crystallization.

In summary, we showed that MAp19 inhibits calcium oxalate crystal growth in vitro, suggesting that it plays a role as an inhibitor of renal stone formation in urine.

Acknowledgements: We thank Dr. Kenneth Williams, Department of Molecular Biophysics and Biochemistry, Yale University, USA, for protein sequencing of MAp19. We also thank Dr. Chan Y. Jung, Department of Biophysics, State University of New York at Buffalo, USA, for helpful discussion and financial support. This work was partially supported by Grant HMP-97-B-2-0017 and Korean National Cancer Control Program 97-5-11 from the Ministry of Health and Welfare, Korea.

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