Ribonucleases 6 and 7 have antimicrobial function in the human and murine urinary tract

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Recent evidence suggests antimicrobial peptides protect the urinary tract from infection. Ribonuclease 7 (RNase 7), a member of the RNase A superfamily, is a potent epithelial-derived protein that maintains human urinary tract sterility. RNase 7 expression is restricted to primates, limiting evaluation of its antimicrobial activity *in vivo*. Here we identified ribonuclease 6 (RNase 6) as the RNase A superfamily member present in humans and mice that is most conserved at the amino acid level relative to RNase 7. Like RNase 7, recombinant human and murine RNase 6 has potent antimicrobial activity against uropathogens. Quantitative real-time PCR and immunoblot analysis indicate that RNase 6 mRNA and protein are upregulated in the human and murine urinary tract during infection. Immunostaining located RNase 6 to resident and infiltrating monocytes, macrophages, and neutrophils. Uropathogenic *E. coli* induces RNase 6 peptide expression in human CD14$^+$ monocytes and murine bone marrow–derived macrophages. Thus, RNase 6 is an inducible, myeloid-derived protein with markedly different expression from the epithelial-derived RNase 7 but with equally potent antimicrobial activity. Our studies suggest RNase 6 serves as an evolutionarily conserved antimicrobial peptide that participates in the maintenance of urinary tract sterility.

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The urinary tract is one of the most common targets of bacterial infection afflicting humans. An estimated 11% of women in the United States report at least one physician-diagnosed urinary tract infection (UTI) per year, and the lifetime probability that a woman will develop a UTI is 60%.[1] The clinical management of UTI is complicated by the increasing incidence of infections caused by strains of *Escherichia coli* that are resistant to commonly used antimicrobial agents. Recently, antimicrobial peptides (AMPs), a fundamental component of the innate immune response, have been shown to possess antimicrobial activity against uropathogenic bacteria.[2,3] Thus, AMPs may represent new classes of antibiotics against drug-resistant uropathogens. AMPs have many desirable features of a novel antibiotic class: (1) AMPs display antimicrobial activity at low micromolar concentrations; (2) AMPs overcome the shortfalls of conventional antibiotics given their ability to permeabilize microbial membranes; (3) because AMPs target cell wall components and microbes cannot readily alter their cell wall composition, development of resistance to AMPs is limited; and (4) AMPs show synergy with conventional antibiotics.[4] Thus, the study of AMPs as therapeutic targets is indicated.

To date, very few AMPs have been described in the human kidney and urinary tract. Recently, our research group has shown that ribonuclease 7 (RNase 7) is an epithelial-derived AMP that helps maintain human urinary tract sterility.[5–7] RNase 7 belongs to the RNase A superfamily, a vertebrate-specific group of genes encoding proteins with a characteristic signal peptide, ribonuclease catalytic motif (CKXXNTF), and six to eight conserved cysteine residues for disulfide bridging.[8,9] RNase 7 exhibits broad-spectrum antimicrobial activity against Gram-positive and Gram-negative uropathogenic bacteria at micromolar concentrations.[6,10] The uroepithelium of the lower urinary tract and the intercalated cells of the renal collecting tubules constitutively express RNase 7 and secrete it into the urinary stream.[5,6] When urinary RNase 7 is neutralized in human urine specimens *in vitro*, urinary bacterial growth increases.[5]

Three lineages of the RNase A superfamily encode proteins associated with host defense: (1) angiogenins, (2) eosinophil RNases, and (3) RNase 7 and RNase 8; they exist uniquely in primates.[9,11,12] The eosinophil RNases exhibit rapid rates of non-silent amino acid substitutions, with a high degree of interspecies divergence.[13–15] This rapid molecular evolution has occurred through gene duplication and deactivation in a pattern similar to the human major histocompatibility complex and T-cell–receptor loci. These observations attest to a potential central role for rapid evolutionary tailoring of RNase A superfamily members toward host-specific microbes.

Although RNase 7 is most closely related in amino acid sequence to RNase 8, the mouse genome does not encode an ortholog of either protein, thereby limiting our understanding of their roles in urinary tract host defense *in vivo*. Certain members of the Ribonuclease A superfamily, with potential antimicrobial function, are conserved between humans and lower order vertebrates. Rosenberg and Dyer[16] identified orthologous sequences of RNase 6 in both primate and non-primate mammalian species. Human RNase 6 displays 56% amino acid identity to RNase 7 and its mRNA expression has been identified in the human kidney by northern blot.[17] At the cellular level, transcripts encoding *RNASE6* have been detected in human peripheral blood monocytes and neutrophils, suggesting a role for this ribonuclease in host defense.[16] In the mouse, *Rnase6* transcripts have been detected by real-time PCR (RT-PCR) in kidney, bone marrow, and spleen.[18] Murine *Rnase6* is syntenic with human *RNASE6* and their encoded proteins display 85% amino acid identity.[16,18] Thus, this study was designed with three objectives: (1) to determine whether mouse and human RNase 6 exhibits antimicrobial activity against uropathogenic bacteria; (2) to compare the urinary tract expression and antimicrobial activity of human RNase 6 and RNase 7; and (3) to evaluate urinary tract expression of murine RNase 6 at baseline and following experimental UTI. The results from this study develop a foundation to evaluate the role of RNase 6 in maintaining urinary tract sterility *in vivo*.

RESULTS

Primary and predicted tertiary structures of RNase 6 and RNase 7

To identify murine proteins with the greatest identity to human RNase 7, we queried the non-redundant *Mus musculus* protein database with the mature human RNase 7 peptide sequence (amino acids 29–156 of NP_115961 encoding the C terminal 128 amino acids of RNase 7).[17] This identified RNase 6 as the most similar mouse protein (52% identity and 71% similarity). Alignment of the predicted human and mouse RNase 6 mature peptides demonstrated 85% amino acid identity (Figure 1a). A protein-modeling algorithm identified similarities between the predicted tertiary structure of human RNase 6 and the NMR solution structure of RNase 7 (Figure 1b).[19]

RNase 6 exhibits broad-spectrum antimicrobial activity against uropathogenic bacteria

We conducted Live/Dead bacterial viability assays to define the antimicrobial activity of recombinant human and mouse RNase 6 against Gram-positive and Gram-negative uropathogenic bacteria. Our results demonstrate that both human and mouse RNase 6 exhibits rapid, dose-dependent bactericidal activity toward uropathogenic *E. coli* (UPEC), *Enterococcus faecalis*, and *Staphylococcus saprophyticus* at micromolar concentrations, comparable to RNase 7 (Figure 2).[6] To evaluate whether recombinant human RNase 6 and RNase 7 have synergistic antimicrobial activity against UPEC, we used the broth microdilution checkerboard antibacterial assay.[20] Synergistic antimicrobial activity was not detected between these two peptides (data not shown). Recombinant human and mouse RNase 6 (0.1–10 μmol/l) did not demonstrate cytotoxic activity toward immortalized human uroepithelial cells (UROtsa cells) or cause hemolysis of human red blood cells (data not shown).

RNase 6 expression in the human urinary tract differs from RNase 7

To investigate the tissue distribution of *RNASE6* and *RNASE7* mRNA expression, we analyzed complementary DNA (cDNA) obtained from various human body tissues by quantitative RT-PCR (qRT-PCR; Figure 3; Supplementary Figure S1 online). *RNASE7* mRNA expression is significantly greater compared with *RNASE6* mRNA expression in the bladder ($n = 4$, $P = 0.0389$, unpaired Student's *t*-test) and kidney ($n = 10$, $P = 0.0068$, unpaired Student's *t*-test). In kidney samples with chronic pyelonephritis ($n = 6$), *RNASE6* and *RNASE7* expression increases (Figure 3a). When comparing expression in other tissues, *RNASE6* mRNA expression was significantly higher compared with *RNASE7* mRNA in human spleen and thymus (Figures 3a, $P = 0.0243$, unpaired Student's *t*-test).

Western immunoblot analysis was performed to evaluate RNase 6 and RNase 7 protein production in the human urinary tract. RNase 7 peptide was detected in human bladder lysates ($n = 4$), noninfected kidney lysates ($n = 6$), and chronic pyelonephritis kidney lysates ($n = 6$). In contrast, RNase 6 peptide was detected only in pyelonephritis kidney lysates and was absent in noninfected kidney and bladder lysates (Figure 3b). Western immunoblot analysis was also performed on noninfected and UPEC-infected human urine samples. Western immunoblot analysis routinely detected RNase 7 in sterile and infected urine samples ($n = 4$). In contrast, RNase 6 was only detected in infected urine samples. When infected urine was cleared by centrifugation, RNase 6 reactivity was found exclusively in the pellet of the urinary sediment. In contrast, RNase 7 protein was detected in both the sediment and the supernatant fraction (Figure 3c; Supplementary Figure S2 online).

Immunohistochemistry was carried out to localize RNase 6 and RNase 7 protein in the human urinary tract. As previously reported, RNase 7 production localized to the