Immunohistochemical staining of urokinase plasminogen activator-like and urokinase plasminogen activator receptor-like proteins in the urinary tract of healthy dogs

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Objective—To determine distribution of urokinase plasminogen activator-like protein and urokinase plasminogen activator receptor-like protein in urinary tract tissues of healthy dogs.

Animals—11 healthy dogs.

Procedures—Necropsy specimens from kidney, ureter, bladder, urethra, prostate, and testis were obtained from 4 sexually intact female dogs, 5 sexually intact males, and 2 castrated males; dogs ranged in age from juvenile to adult. Urokinase plasminogen activator-like protein and urokinase plasminogen activator receptor-like protein in tissue lysates from kidney, prostate, and testis were identified by use of SDS-PAGE, western blot analysis, and immunoprecipitation. Urokinase plasminogen activator-like protein and urokinase plasminogen activator receptor-like protein in kidney, ureter, urinary bladder, urethra, prostate, and testis were identified by use of immunohistochemical staining of tissue sections.

Results—Urokinase plasminogen activator-like protein and urokinase plasminogen activator receptor-like protein in the molecular-weight range published for urokinase and urokinase receptor (53 and 33 kd for urokinase and 60 to 65 kd for urokinase receptor) were identified. Distribution of the proteins identified by use of immunohistochemical staining was comparable with published information for humans and mice for the urinary tract. Staining of these proteins was detected in more tissue types than reported in healthy humans.

Conclusions and Clinical Relevance—Urokinase plasminogen activator-like protein and urokinase plasminogen activator receptor-like protein were detected in the urinary tract of healthy dogs. This information is important for further evaluation of the functions of urokinase and urokinase receptor in the canine urinary tract and the pathophysiologic features of urinary tract disease. (Am J Vet Res 2006;67:1628–1634)
examination, absence of gross pathologic abnormalities in the urinary tract, and absence of histopathologic abnormalities as determined by evaluation of H&E-stained tissue sections from the urinary tract. Dogs were euthanized for purposes other than this study by IV administration of sodium pentobarbital (120 mg/kg). Dogs were scheduled for euthanasia because of positive results of heartworm antigen tests following a terminal surgical exercises laboratory that did not involve the urinary tract. All procedures were approved by the institutional animal care and use committee.

**Specimens**—Tissue specimens were collected immediately after euthanasia from kidney, ureter, urinary bladder, urethra, prostate, and testis from 5 sexually intact male dogs; kidney, ureter, urinary bladder, urethra, and prostate from 2 castrated male dogs; and kidney, ureter, urinary bladder, and urethra from 4 sexually intact female dogs. A 2 × 1-cm full-thickness wedge was taken from the midurethra in female dogs. A 2-cm full-thickness section was obtained from the ventral portion of the urinary bladder wall. A 1 × 1-cm wedge was taken from 1 lobe of the prostate. A 2-cm full-thickness section was taken from the prostatic and penile urethra in male dogs. A 2-cm full-thickness section was taken from the midurethra in female dogs. A 2-cm full-thickness wedge was taken from 1 testis. Tissue specimens for morphologic study were immediately snap-frozen in liquid nitrogen. Specimens were stored at −80°C until preparation for formalin fixation and paraffin embedding. Sections (4 µm) were cut and stained with H&E or used for immunohistochemical staining.

**Antibodies**—Antibodies used in the study were mouse monoclonal antibody against mouse urokinase, mouse monoclonal antibody against human urokinase B-chain, mouse monoclonal antibody against human urokinase receptor CD 87, and goat polyclonal peroxidase conjugated anti-mouse IgG.

**SDS-PAGE and western blot analysis**—Samples of kidney tissues from all groups of dogs were pooled. Samples of prostate tissue from all male dogs were pooled. Samples of testis tissues from all male intact dogs were pooled. Each pooled tissue sample was manually crushed with a tissue grinder in radioimmunoprecipitation buffer at a temperature of 4°C. The resulting tissue lysate was transferred to polypropylene screw-cap microtubes by use of a pipette and frozen at −20°C for later analysis. The tissue lysates were thawed at room temperature (21°C) prior to SDS-PAGE and western blot analysis. The SDS-PAGE gel and buffers were prepared as described by Laemmli. Tissue lysates were denatured by boiling at 100°C. Four microliters of 4X SDS sample buffer was added to 20 µL of denatured sample and applied to the SDS gel (10-cm resolving gels of 7.5% acrylamide and 2-cm stacking gels of 4% acrylamide). The samples were subjected to electrophoresis at room temperature at 100 mV for 2 hours or until the dye reached the bottom of the gel. Molecular-weight markers were electrophoresed in the first lane beside the samples for identification of the molecular weight of each protein band. Separated proteins were transferred from the SDS gel to polyvinylidene fluoride membranes for western blot analysis with western transfer buffer. Protein transfer was performed in a western blot apparatus over 1 hour at 100 mV. The polyvinylidene membrane was incubated with the primary antibody at a 1:1,000 dilution in a 4% nonfat skin milk powder in PBS solution for 16 hours at 4°C. For detection of uPA-like protein, the primary antibodies were mouse anti-mouse uPA and mouse anti-human uPA. For detection of uPAR-like protein, the primary antibody was mouse anti-human uPAR antibody. After incubation, the membrane was washed 3 times (30 minutes each) with the 4% nonfat skin milk solution at room temperature. Following washing, the membrane was incubated at room temperature for 2 hours with goat anti-mouse peroxidase-labeled antibody at a 1:5,000 dilution. The membrane was again washed 3 times (30 minutes each) with a 1% nonfat skin milk solution. The results were visualized by use of a chemiluminescence reagent on radiographic film.

**Immunoprecipitation**—From each tissue lysate, 200 µL was incubated at room temperature for 2 hours with and without 2 µL of anti-mouse uPA or anti-human uPA. The antibody complexes were precipitated by combination with protein A and protein G sephrose beads for 1 hour at room temperature. The beads were washed with radioimmunooassay buffer at 4°C. The antibody-antigen complexes were separated from the beads by boiling at 100°C for 3 minutes. Negative controls contained the tissue lysate alone without the antibody. All immunoprecipitated tissue lysates were then analyzed by use of the SDS-PAGE and western blot analysis protocol outlined in the previous section and compared to the samples that were not immunoprecipitated.

**Immunohistochemical analysis**—All immunohistochemical stains were performed on paraffin-embedded sections (4 µm). For uPA-like protein detection, an endogenous enzyme block was performed with 0.3% hydrogen peroxide for 10 minutes. Sections were treated with proteinase K for 2 minutes for enzyme retrieval followed by protein block with horse serum for 30 minutes. The sections were incubated for 30 minutes with primary mouse anti-mouse uPA antibody at a 1:200 dilution. The primary antibody was omitted for negative controls. The secondary antibody system used a mouse system (a biotin-free chain polymer-conjugated system with horseradish peroxidase as the chromogen) with incubation for 30 minutes. Sections were incubated for 8 minutes with a peroxidase substrate to allow visualization of the antigen-antibody complexes. Slides were counterstained with hematoxylin for 5 minutes. Intensity of staining was subjectively evaluated by 1 investigator (DP). Cells that stained were identified and described by 1 investigator (DP).

For uPAR-like protein detection, an endogenous enzyme block was performed with 0.3% hydrogen peroxide for 10 minutes. Sections were treated with a high-pH target retrieval system at 96°C for 10 minutes for enzyme retrieval, followed by protein block with horse serum for 30 minutes. Sections were incubated for 60 minutes with primary antibody mouse anti-human uPAR at a 1:100 dilution and the same secondary antibody system as used previously. Sections were incubated for 8 minutes with peroxidase substrate to allow visualization of the antigen-antibody complexes. Slides were counterstained with hematoxylin for 5 minutes. Intensity of staining was subjectively evaluated by 1 investigator (DBP). Cells that stained were identified and described by 1 investigator (DBP).

**Results**

**uPA-like protein**—Western blot analysis resulted in 2 distinct 53-kd- and 33-kd-molecular-weight protein bands (Figure 1). Immunoprecipitation enhanced visualization of the protein bands in the prostate and testis samples. The intensity of the band obtained by use of the kidney lysate was not visually different than that obtained without immunoprecipitation. Without immunoprecipitation, a much stronger band was observed in the kidney than the testis and prostate. Both antibodies were used successfully precipitated the urokinase-like antigen.
**uPAR-like protein**—Western blot analysis with and without immunoprecipitation of kidney, prostate, and testis lysates with human monoclonal uPAR antibody resulted in a distinct protein band at 65 kd. Immunoprecipitation enhanced the visualization of the protein bands in the prostate, kidney, and testis (Figure 2). The mouse anti-human antibody successfully precipitated the uPAR-like antigen.

**Immunohistochemical staining in kidney**—Staining for uPA-like protein in the kidney was consistent across all dogs. Staining was red to red-brown and uniform in the tubular epithelial cells throughout the cortex and medulla (Figure 3). In some sections, cells in the medullary tubules stained more intensely than cells in the proximal convoluted tubules. There was staining in the endothelial cells of the glomeruli in some kidneys. Infrequent macrophages were stained. Endothelial cells of arteries and veins also stained. Staining was observed in the mucosa of the renal pelvis in the cytoplasm of the luminal cells in the transitional epithelium; this occurred in distinct segments. Within the renal pelvis, the transitional epithelium had an undulating appearance. The segments of transitional epithelium that appeared recessed, compared with the open cavity of the renal pelvis, stained throughout all cell layers, extending to but not including the lamina propria. The segments that were not recessed stained only along the membrane of the luminal cell layer or, in some segments, did not stain at all.

**Immunohistochemical staining in ureter**—Intense and uniform staining for uPA-like protein was detected along the luminal cell membranes of the surface epithelial cells and in the cytoplasm of all layers of transitional epithelial cells of the mucosal lining. The cytoplasm of infrequent macrophages in the connective tissue of the ureter wall and vascular endothelial cells stained as well.

Granular staining for uPAR-like protein was in the cytoplasm of the luminal and, sometimes, second layer of transitional epithelium. No staining was detected in the deeper cells of the transitional epithelium. There was staining of infrequent macrophages in the connective tissue of the ureter wall (Figure 3). There was also staining of the cytoplasm of smooth muscle cells and skeletal muscle cells, endothelial cells of vessels, and infrequent macrophages in the urinary bladder wall. Two of the urinary bladder sections had inflammation consistent with chronic cystitis and had intense uPA-like protein staining of the mucosa in the same segmental pattern as tissue samples with no evidence of inflammation. In the inflamed tissue, staining was more intense than in normal tissue. Staining was present in all cell layers of the transitional epithelium and was more intense in the invaginated segments.

**Urethra**—Consistent staining for uPA-like protein was evident in the transitional epithelium along the luminal border and in the cytoplasm of the cells extending all the way through the mucosa. This pattern of staining was similar to that observed in the urinary bladder mucosa in areas that had high numbers of cells with vacuolated cytoplasm and were invaginated. In other areas of the urethral mucosa, the staining was only at the luminal surface, and some sections did not stain. There was staining in infrequent macrophages in the connective tissue of the urethral wall. There was no difference in the pattern of staining observed between the transitional epithelium of the prostatic and penile urethra of male dogs.

There was granular staining for uPAR-like protein of transitional epithelium of the ureter on the luminal surface of the cells and in the cytoplasm of the cells closest to the lumen. There was staining in the infrequent macrophages in the connective tissue of the urethral wall. In some sections, the staining was more intense in the cells of the transitional epithelium of the prostatic urethra than in the cells of the transitional epithelium of the penile urethra.

**Prostate**—Staining for uPA-like protein in the prostate was highly variable. Males 1 through 5 were sexually intact, and males 6 and 7 were neutered. Male 1 had a mature prostate and had variable faint staining in the cytoplasm of the connective tissue cells of glandular epithelium, but most glandular tissue did not stain. Epithelial cells lining the prostatic ducts did not stain. There was staining of infrequent macrophages in the connective tissue of the prostate. Male 2 had a mature prostate with some evidence of hyperplasia. Some faint staining of the cytoplasm of glandular cells was seen, but most of the glandular cells did not stain. Male 3 had a mature, hyperplastic prostate. Fixation of the tissue was not optimal, but there was faint staining.
of some glandular cells. Male 4 was a young dog, and the prostate had little acinar development. There was intense staining in the epithelial cells lining the developing ducts (Figure 3). Staining was less intense in epithelial cells of more differentiated ducts. There was staining of infrequent macrophages in the glandular connective tissue. Male 5 had a mature prostate with multiple hyperplastic and cystic regions. Epithelium in the ducts and glands of the cystic regions had moderate to intense staining. There was faint to no staining in the glandular cells of the hyperplastic regions. Male 6 had a normal prostate, and no staining was detected in glands or ducts, except for faint staining in the cytoplasm of a few loamy glandular prostatic cells. Male 7 had a hypoplastic prostate. There was staining in epithelial cells of the prostate glands and ducts, infrequent macrophages in the connective tissue, and endothelial cells of vessels. There was inconsistent but intense staining in the cytoplasm of epithelial cells lining primitive or poorly differentiated ducts.

There was no uPAR-like protein staining in most prostatic sections. There was staining of infrequent macrophages in the connective tissue, which acted as an internal control. Male 4 was young but sexually intact and had granular staining in the epithelial cells of the developing ducts at the apical surface and the cytoplasm (Figure 3). Staining was also detected in the epithelial cells of the more mature ducts at the apical borders. Staining intensity was equal to that in the less mature ducts. Intense staining was evident in interstitial resident macrophages. Male 7 was neutered and had a hypoplastic prostate. There was granular staining in the apical region of epithelial cells lining primitive or poorly developed ducts. There was staining of infrequent macrophages in the glandular connective tissue.

Testes—There was intense staining for uPA-like protein in all interstitial cells and seminiferous tubules, including all phases of spermatogonia. There was faint staining for uPAR-like protein in the cytoplasm of interstitial cells but no staining of seminiferous tubules or spermatogonia. There was staining of infrequent macrophages in the connective tissue.

Discussion

High–molecular-weight uPA and low–molecular-weight uPA are 53 and 33 kd, respectively. The molecular weight of uPAR is more variable but usually is from 65 to 60 kd. These molecular weights were used as references for western blots to positively identify uPA-like and uPAR-like proteins in the study reported here. Immunoprecipitation concentrated the proteins and enhanced visualization of the protein bands in prostate and testis tissue lysates.

The canine kidneys had staining for uPA-like protein in the epithelium of all renal tubules and staining along the epithelial cells of the glomeruli in most specimens. Detection of uPA-like protein in the kidneys of the dogs studied was consistent with that reported for humans and mice. Immunohistochemical staining cannot be used to differentiate whether the antigen is present because of synthesis in the cells or because of binding to a receptor in or on the cell membrane. However, in vitro and in vivo studies of cell lines from human mesangial and tubule cells reveal that uPA is synthesized and secreted by glomerular visceral epithelial cells and kidney tubular epithelial cells. Given these findings in other mammalian species, the uPA-like protein in the renal tubules and glomeruli of the dogs reported here was likely synthesized there also. To verify this, cell culture studies would be required to verify secretion of uPA-like protein into the cell medium.

In the kidney, intense uPA-like protein staining was also detected along the transitional epithelium of the renal pelvis. Detection of uPA and associated fibri-
nolistic activity has been reported in epithelial cells of the renal calyces of humans. Urokinase is produced in the transitional epithelial cells in cattle but has not been investigated in other species. In the tubular structures of the urinary tract, uPA is important for maintaining patency during hemorrhage and fibrin deposition.

Urokinase receptor staining is not detected in the kidneys of mice. This is consistent with findings in humans in which uPAR staining is not detected in the tubules of the kidneys of healthy donors. In another human study, glomerular cells in healthy kidney samples were not stained. In the dog kidneys of the present study, uPAR-like protein was detected along the basement membrane of renal tubular cells. This could indicate that in healthy dogs, kidneys produce uPAR-like protein, which would be different from humans. Many of the dogs in the present study had heartworm disease, indicated by microfilariae in glomeruli. This may have resulted in mild glomerulonephritis and increased production of uPAR-like protein. However, histopathologic evidence of glomerulonephritis was not detected. On the basis of our histologic findings, we believe that uPAR-like protein is present in the kidneys of healthy dogs. The role of uPAR-like protein and its sites of production cannot be determined without additional studies such as cell culture.

Transitional epithelium in the renal pelvis had staining for uPAR-like protein along the luminal surface and in the cytoplasm of luminal cells in the same regions as uPA-like protein staining. This may indicate that uPA-like protein is bound to uPAR at the cell surface or that uPA-like protein is produced in this tissue.

The pattern of uPA-like protein staining along the mucosal surfaces of the urinary tract was consistent among sections and sites. The ureter stained intensely, whereas staining in the urinary bladder and urethra was variable. The segments of transitional epithelium commonly stained were areas that appeared to be invaginated or depressed with respect to the lumen and had vacuolization in the cytoplasm of the stained cells. The importance of this finding was unknown. The pattern of staining for uPA-like protein in the testes in the seminiferous tubules and interstitial tissue sections examined, it was masked in the testes. The uPAR-like protein in the testes may be so heavy that the monoclonal human uPAR antibody was directly labeled, or the epitope on uPAR-like protein of dogs may have been unaccessible. The importance of uPAR-like protein in the testes is believed to play a role during spermatogenesis and in spermatozoid motility. It has been suggested that infertility in some men may be attributable to low concentrations of uPA in the testes. Urokinase plasminogen activator receptor-like protein was not detected via staining in the seminiferous tubules of the dogs reported here. Staining was very weak in the cytoplasm of the interstitial cells. This observation differs from published data in rhesus monkeys and mice, in which uPAR was identified at high concentrations. Results of the western blot assays revealed that a uPAR-like protein was present in the canine testes. The low-degree staining of uPAR-like protein in the testes may have been caused by unavailability of the epitope on uPAR-like protein of dogs to which the monoclonal human uPAR antibody was directed.

The variability of staining for uPA-like protein in the prostate tissue suggests the interesting possibility that production of uPA-like protein and uPAR-like protein may depend greatly on stage of development of the prostate. In rats, uPA production is influenced by hormones and is evident immunohistochemically in scattered cells at the surface of the epithelium facing the lumen of the glandular ducts. Staining is greater in castrated rats, which suggests a role for uPA in the involution of the prostate and normal turnover of prostate epithelium. Increased activity of plasminogen activators is detected via plasminogen activator assays in developing prostate tissue. This activity decreases when development is complete. This is consistent with the role of uPA and other plasminogen activators in tissue remodeling. This is supported by the findings of the present study in which staining of uPA-like protein and uPAR-like protein appeared to be limited to only developing ductile tissue. More specimens obtained at certain developmental stages are required to adequately characterize uPA-like protein and uPAR-like protein in the prostate of dogs.

Most prostate samples evaluated did not stain for uPAR-like protein. Male 4 was sexually intact, and staining was evident in the epithelial cells of the developing prostatic ducts and mature prostatic ducts at the apical surface and in the cytoplasm. Male 7 was neutered, but the prostate appeared hypoplastic or atrophied, suggesting that castration had been performed at a young age. Granular staining was detected around primitive or poorly developed ducts in the apical region. Staining for uPA-like protein and uPAR-like protein in the same regions indicated that uPA-like protein was either produced in these tissues or bound to the cells. It is possible, given the abundance of uPA-like protein in the other tissues of the urinary tract, that urokinase is truly bound at this site but produced elsewhere. Urokinase plasminogen activator-like protein and uPAR-like protein may contribute to tissue remodeling in the prostate of dogs. The importance of uPA and uPAR in development of the gland and involution after castration is evident in rat models of prostate development.
All urinary tract tissues examined had low numbers of macrophages that stained for uPA-like protein and uPAR-like protein. Macrophages secrete uPA and have uPAR on the cell membranes.\textsuperscript{14-16} Macrophages were interpreted as an internal control to verify that the staining protocol was working in tissues that otherwise had low levels of staining. Urokinase and uPAR play important roles in macrophage differentiation during inflammation and neoplasia and contribute to fibrosis, angiogenesis, and basement membrane degradation in affected tissues.\textsuperscript{17,18,19} The tissue sections from the urinary tract of all dogs in the present study had normal histologic features. The numbers of macrophages were typical for healthy urinary tract tissues, as judged on the basis of the experience of the pathologist (DBP) who evaluated the tissues. In the kidneys of healthy mice and mice with experimentally induced urinary tract obstruction, macrophages are in low numbers (< 5 cells/12 hpfs). Within 1 day of urinary obstruction, this number increases to a mean of 112.7 ± 20 cells/12 hpfs, which is a rapid increase in macrophage numbers in a short period.\textsuperscript{20} One would expect that in dogs with inflammation, infection, or neoplastic disease affecting the kidneys, much higher numbers of macrophages would have been present in the tissue sections examined. Because the kidney is not unique in its response to these types of diseases, it is expected that other tissues of the urinary tract would respond in similar ways with respect to migration of macrophages to sites of injury. The 2 sections of urinary bladder wall that had evidence of chronic cystitis had characteristic increases in neutrophils and macrophages, as would be expected with inflammation.

We hypothesized that urokinase would be present in the canine urinary tract on the basis of evidence of its presence in human studies; however, identification of urokinase in the canine urinary tract has not been reported. Cell types that stained for uPA-like protein and uPAR-like protein in the canine tissues were consistent with those described in humans, rats, monkeys, and mice in most instances.

Presently, there are no specific antibodies against canine urokinase. Selection of an antibody for identification of uPA-like protein in the canine urinary tract required consideration of any documentation of cross-species use of that antibody. Various antigens have greater or lesser degrees of species specificity. At least 2 investigations\textsuperscript{21,22} have used a mouse anti-human monoclonal antibody to successfully detect urokinase in bovine urothelium and rat mammary tissue. A third investigation\textsuperscript{23} used a polyclonal goat anti-human uPA antibody to detect uPA in bovine endothelial cells. This cross-species reactivity suggests that at least some segment of the uPA protein structure is retained across some species, and our results suggest this includes dogs.

The use of an antibody directed against another species to identify uPA and uPAR in dogs could result in difficulty with background staining or target selectivity. However, because we were able to successfully use these antibodies in the immunoprecipitation, SDS-PAGE, and western blot procedures, we believe that detection of uPA-like and uPAR-like proteins in the urinary tract of the healthy dogs was specific with the human- and mouse-targeted antibodies used.

Results of the study reported here indicated that uPA-like protein and uPAR-like protein were detectable in the tissues of the urinary tract of healthy dogs, but their role in maintenance of the healthy urinary tract is unknown. Many disease processes of the urinary tract have some degree of inflammation or infection associated with them. In humans, increased presence of uPAR occurs in such inflammatory processes. Increased presence of receptors would theoretically lead to increased binding of uPA and possibly trigger increased production of uPA that might enter the urine. Further studies are required to elucidate the role of uPA and uPAR in the pathophysiological processes associated with urinary tract disease.

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