Sensitive Detection of Prion Protein in Human Urine

HARASH K. NARANG,*[2] AYUNA DAGDANOVA,† ZHILIANG XIE,† QIWEI YANG,† AND SHU G. CHEN†,[1]

*BioTech Global, 22-40 Brentwood Avenue, Newcastle Upon Tyne, NE2 3DH, UK; and †Institute of Pathology, Case Western Reserve University, 2085 Adelbert Road, Cleveland, Ohio 44106

Transmissible spongiform encephalopathies are a group of infectious diseases typically associated with the accumulation of a protease-resistant and β-sheet–rich prion protein, PrP^{Sc}, in affected brains. PrP^{Sc} is an altered isoform derived from the host-encoded glycoprotein, PrP^{C}. The expression of PrP^{C} is the highest in brain tissue, but it can also be detected at low levels in peripheral tissue. However, it is unclear whether a significant amount of PrP^{C} is released into body fluid and excreted into urine. We have developed a simple, rapid method for the reliable detection of PrP^{C} in urine from normal subjects by Western blotting. Our method can easily and reliably detect PrP^{C} in apparently healthy individuals using less than 1 ml of urine in which the amount of urinary PrP^{C} is estimated to be in the range of low micrograms/liter. Exp Biol Med 230:343–349, 2005

Key words: urine; prion protein; transmissible spongiform encephalopathies; bovine spongiform encephalopathy; Creutzfeldt-Jakob disease

Introduction

Bovine spongiform encephalopathy (BSE) in cattle; scrapie in sheep and goats; and Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome, and kuru in humans are all fatal neurologic disorders that are collectively known as transmissible spongiform encephalopathies (TSEs) or prion diseases (1–3). Since the appearance of BSE in 1985 in the UK (4), a number of new-variant cases of CJD have been identified and believed to be caused by the consumption of meat contaminated with BSE (5, 6). The incubation period between infection and appearance of clinical symptoms may be several decades. Following the BSE epidemic in the UK, there have been small outbreaks of BSE in several other countries. All TSEs result in the accumulation of a protease-resistant prion protein (PrP^{Sc}) that is derived from its normal counterpart (PrP^{C}; Refs. 7, 8). The prion protein PrP^{C} is expressed by a host gene that is predominantly expressed in brain tissue and detected at low levels in other types of tissue (7, 8). It is unclear what the normal physiologic function of dominantly α-helical PrP^{C} may be. It is, however, a membrane-bound, sialo-glycolipoprotein with a glycophosphatidylinositol moiety (9), many of which are known to be associated with transmembrane-signaling functions (10).

The protein sequences of PrP^{C} and PrP^{Sc} are identical (11). However, the two isoforms differ in physicochemical properties. The normal PrP^{C} isoform exists as a soluble, dominantly α-helical monomer and is almost completely degraded by a proteolytic enzyme such as proteinase K (PK). In contrast, PrP^{Sc} has a β-sheet–rich conformation, and when subjected to PK, a large C-terminal 27- to 30-kDa segment of PrP^{Sc} resists further degradation allowing detection by Western blotting (12–14). The unique property of PrP^{Sc} in affected brain tissue to PK digestion has been used in the postmortem diagnosis of TSEs.

Elevated levels of the 14-3-3 protein in the cerebrospinal fluid of patients with CJD are currently used as preliminary screen assays for TSEs, but their specificity is not assured (15–17). The development of a specific, noninvasive test is critical in assessing the prevalence of TSEs along with the source of infection and potential treatment options. Therefore, there is an essential need for a preclinical diagnostic test for TSEs.

Although PrP^{C} is predominantly expressed in brain tissue, it is unclear whether a significant amount of PrP^{C} is circulated in body fluids and eventually eliminated from the body. Shaked *et al.* (18) used an ultracentrifugation and dialysis technique to show that PrP can be detected by the mouse monoclonal antibody (mAb) 3F4 in 10 ml to 50 ml

This work was supported, in part, by grants from the U.S. National Institutes of Health, the Department of Agriculture, and the Department of Defense, and by a gift from Mr. Ken Bell. H.K.N. and A.D. contributed equally to this work.

[1] To whom correspondence should be addressed at Institute of Pathology, Case Western Reserve University, 2085 Adelbert Road, Cleveland, OH 44106. E-mail: shu.chen@case.edu
[2] Current address: West Acres, West Road, Hexham NE46 3DD, UK.

Received May 12, 2004.
Accepted February 21, 2005.

1535-3702/05/2305-0343$15.00

of urine from normal and diseased subjects. However, the validity and reproducibility of this finding has been challenged by two recent reports (19, 20). The evidence of a cross-reactivity of the anti-mouse IgG with either contaminating bacterial proteins (19) or urinary IgG fragments (20) was used to argue that Shaked *et al.* (18) mistakenly identified nonspecific urinary proteins as PrP. Therefore, the presence of either PrP^{C} or PrP^{Sc} in urine was never reliably demonstrated in these studies (18–20). Nevertheless, it is widely hoped that the ability to demonstrate the presence of PrP in urine, as well as in blood, will provide a useful marker of preclinical TSEs.

In the present study, we describe a simple, reliable ion-capture method that can be used to concentrate PrP from small or large volumes (i.e., 1 ml to 1 liter) of urine samples. Following solid-phase extraction, normal PrP in less than 1 ml of urine collected from healthy individuals was sufficient for detection by Western blotting. We demonstrated the successful detection of normal PrP in all urine specimens with the anti-C antibody (21, 22) against the C-terminal region of PrP, but not with 3F4 mAb (23) recognizing an epitope in the N-terminal region of PrP that was also used unsuccessfully in the two recent studies (19, 20). Our findings highlight the importance of understanding the unique structural properties of urinary PrP in devising an appropriate analytic strategy.

Materials and Methods

Urine Collection. In this study, we examined urine samples from 50 apparently healthy individuals, both male and female, aged between 25 years and 60 years. The first morning urine specimens were collected from these normal individuals who were not affected by any disease conditions at the time of urine collection. From six of these individuals we collected three urine samples daily (i.e., first-morning pass, midday, evening) for 5 days. For the assessment of kidney function, creatinine levels in the urine samples were determined at the core laboratory at the University Hospitals of Cleveland using a Dimension clinical chemistry system (Dade Behring Inc., Newark, DE).

Enrichment of PrP From Urine. Proteins in urine were concentrated by ion-capture–based, solid-phase extraction using the urine concentration kit (GB98/00374 and 9601054; BioTec Global, Newcastle Upon Tyne, UK). All buffers and ion-capture resin mentioned here were supplied with the kit. The procedure was performed at room temperature. Urine samples were centrifuged at 1000 *g* for 10 mins to sediment the occasional debris. The supernatant was transferred to fresh tubes, and 1 ml of the concentrate buffer (250 m*M* sodium phosphate, pH 7.5; 68 m*M* potassium chloride; and 3 *M* sodium chloride) was added to each 50-ml urine sample. After a gentle mix, the samples were subdivided into 1-, 3-, 5-, and 10-ml aliquots. To each tube that contained 1- to 5-ml urine samples, we added 100 μl of the ion-capture resin (i.e., calcium phosphate, which was supplied with the kit), and 200 μl was added to the 10-ml urine sample. The samples were gently mixed by hand flicking and were then left on a shaking platform (Red Rotor; Hoefer Pharmacia Biotech, San Francisco, CA) at the speed setting of 3 for 60 mins and agitated by hand every 10 mins to ensure that the resin was well-dispersed in suspension. After the protein adsorption, the tubes were centrifuged at 500 *g* for 5 mins and the supernatant was discarded. The resulting pellet was resuspended in 0.75-ml of wash buffer (10 m*M* sodium phosphate, pH 7.5; 3 m*M* potassium chloride; and 137 m*M* sodium chloride), transferred to 1-ml microfuge tubes, and centrifuged at 16,000 *g* for 10 secs. The supernatant was discarded and replaced with 30 μl of the sodium dodecyl sulfate (SDS) sample buffer (63 m*M* Tris-hydrochloride, pH 6.8; 2 m*M* EDTA, 3% SDS, 10% glycerol, and 1% β-mercaptoethanol). Samples were then boiled for 10 mins and centrifuged at 16,000 *g* for 30 secs. The supernatant containing eluted proteins was used for Western blotting.

Western Blotting. Samples were applied to a 12% or 16% Tris-glycine SDS polyacrylamide gel electrophoresis (SDS-PAGE; precast gels; Invitrogen, Carlsbad, CA) in a mini-cell apparatus (Bio-Rad, Hercules, CA) and subsequently transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) at 70 V for 2 hrs at 4°C. The membranes were then blocked using blocking buffer containing 3% fat-free milk and 1% bovine serum albumin (BSA) in Tris-buffered saline supplemented with 0.1% Tween 20 (TBS-T; pH 7.6) for 1 hr. Then, the membranes were incubated with one of the following primary antibodies: (i) the rabbit anti-C antiserum (21, 22) against human PrP residues 220–231 at a dilution of 1:3000, and (ii) mouse 3F4 mAb (23) recognizing an epitope of human PrP residues 109–112 at a dilution of 1:50,000 in antibody dilution buffer (1% [v/v] normal goat serum, 0.05% [w/v] BSA, 0.1% [w/v] thimerosal in TBS) for 2 hrs at room temperature or overnight at 4°C. The membranes were then rinsed four times in TBS-T for 15 mins each, followed by incubation with an appropriate secondary antibody (donkey anti-rabbit IgG $F(ab')_2$ fragment (catalog number NA9310) and sheep anti-mouse IgG $F(ab')_2$ fragment (catalog number NA9340) conjugated with horseradish peroxidase (Amersham Biosciences, Piscataway, NJ) for 1 hr at room temperature. After being rinsed another four times in TBS-T, PrP was visualized on Kodak X-Omat films by enhanced chemiluminescence (ECL Plus kit; Amersham Biosciences).

To evaluate the amount of PrP in urine sample, protein concentrates were prepared from 0.5- to 10-ml urine samples as previously described. Protein concentrates were run in parallel with 1 ng to 8 ng of the recombinant human PrP 23–231 (Abcam, Cambridge, MA). Western blotting was performed as previously described. The amount of PrP was quantified by densitometry according to the intensity of PrP bands using the UN-SCAN-IT software (Silk Scientific, Orem, UT).

Peptide: N-Glycosidase F (PNGase F) Treatment. Deglycosylation was performed using PNGase F and other reagents provided by the supplier (New England BioLabs, Beverly, MA). Proteins were concentrated from 10 ml of urine as previously described. The final pellet was suspended in 30 μl of denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) and boiled for 10 mins. The supernatant containing eluted and denatured proteins was supplemented with G7 buffer (50 m*M* sodium phosphate, pH 7.5) and 1% NP-40 and digested with 3 μl of PNGase F (500,000 U/ml) for 60 mins at 37°C. Digestion was stopped by the addition of SDS sample buffer followed by boiling for 10 mins. Samples were applied to a 10%- to 20%-gradient Tris-tricine SDS-PAGE using precast gels (Invitrogen) and were, subsequently, subjected to Western blotting as previously described.

Protease Digestion. Proteins were concentrated from 10-ml aliquots of urine samples as previously described. After the pellet was resuspended in 200 μl of wash buffer, 20 μl of trypsin (2 mg/ml) or PK (2 mg/ml) was added for on-resin digestion. Control was made in which no enzyme was added. All tubes were incubated for 60 mins at 37°C. Following enzyme digestion, the samples were centrifuged for 30 secs at 16,000 *g* in microfuge tubes, and the supernatant was removed. The pellet was washed with 0.75 ml of wash buffer and resuspended in 30 μl of SDS sample buffer followed by boiling for 10 mins. Samples were run on 10%- to 20%-gradient Tris-tricine SDS-PAGE gels and were subjected to Western blotting as previously described.

Spiking of Brain Homogenate Into Urine. To model the possibility of our method being used for the PrP^{Sc} detection in urine, brain homogenate of both normal and scrapie-adapted (i.e., 263K prion) hamsters was spiked into the urine samples. The total brain homogenate (10% [w/v]) of normal and 263K scrapie hamsters was made in phosphate-buffered saline (pH 7.5) followed by brief centrifugation. Urine (1 ml) was mixed with 20 μl of the concentrate buffer and 100 μl of the ion-capture resin. The clarified brain homogenate (10 μl) was spiked into these urine samples. The samples were left for incubation on a shaking platform (Red Rotor; Hoefer Pharmacia Biotech) at the speed setting of 3 for 60 mins at room temperature. After adsorption of proteins onto the resin, samples were centrifuged at 16,000 *g* for 10 secs and the supernatant was discarded. The resulting pellet was resuspended in 0.75 ml of wash buffer. After centrifugation again at 16,000 *g* for 10 secs, the supernatant was discarded and 0.3 ml of fresh wash buffer was added. Each sample was then divided into two groups: 0.1 ml for the control sample without the addition of PK and 0.2 ml for PK digestion at final enzyme concentration of 50 μg/ml. All samples were incubated for 60 mins at 37°C. Reaction was stopped by adding 1 μl of 100 m*M* Pefa block (Roche Molecular Biochemicals, Indianapolis, IN). Samples were centrifuged for 20 secs at 16,000 *g*, and the supernatant was removed. The pellet was resuspended in 30 μl of SDS sample buffer and processed as previously described for Western blotting.

Results

The apparently healthy individuals that we tested had creatinine levels ranging from 60 mg/dl to 300 mg/dl in morning urine samples, with no medical conditions indicative of kidney malfunction. We intended to use these samples in developing our techniques for the detection of PrP in human urine.

On Western blots probed by the rabbit anti-C antiserum (21, 22) recognizing human PrP residues 220–231, detectable amounts of PrP^C migrating at 28 kDa to 30 kDa were observed in urine samples of all healthy individuals ($N = 50$). When urine samples were subdivided into 1-, 3-, and 5-ml aliquots and all proteins concentrated from the samples were applied, a proportional increase in the amount of PrP^C was demonstrated (Fig. 1, Lanes 1–3). The total protein loading in these urine samples displayed a similar trend of increase as judged by silver staining (Fig. 1, Lanes 4–6), which demonstrates a quantitative recovery of urinary proteins by the solid-phase extraction. However, no bands were observed after immunoblotting with 3F4 mAb (Fig. 1, Lanes 7–9). To test if the binding of the secondary IgG to nonspecific proteins such as bacterial outer membrane proteins (19) or urinary human IgG fragments (20) accounted for the false positive detection of PrP as reported previously (18), control experiments were performed with the use of secondary antibodies (i.e., donkey anti-rabbit IgG, sheep anti-mouse IgG, $F(ab')_2$ fragment) in the absence of the respective primary antibodies (i.e., rabbit anti-C antibody, mouse 3F4 mAb). No immunoreactive bands were observed for either secondary anti-rabbit IgG (Fig. 1, Lanes 10–12) or secondary anti-mouse IgG (Fig. 1, Lanes 13–15) under our experimental conditions, confirming that our method reliably detected PrP^C in human urine, not any other nonspecific proteins. The detection of PrP^C could be achieved in as little as 1 ml of urine (Fig. 1) or less (Fig. 2) from normal individuals ($N = 50$).

Quantitation of PrP^C present in the urine samples was accomplished through comparison with known amounts of human recombinant PrP run in parallel on SDS-PAGE gels. Variations, ranging from 2 ng/ml to 25 ng/ml of the first-morning urine specimens (with an average of 7.2 ± 6.8 ng/ml) were observed among normal individuals who were not affected by any medical conditions (mean ± SD; $N = 50$). As shown in Figure 2, the amount of urinary PrP^C was approximately 10 ng/ml in the urine of one healthy individual. To clarify whether PrP^C is excreted into urine throughout the day, urine samples from six individuals were collected in the morning, midday, and evening for 5 days. Regardless of the time of specimen collection, PrP^C was detectable by Western blotting with the anti-C antibody in all urine specimens (data not shown).

Because PrP^C is a glycoprotein that contains two

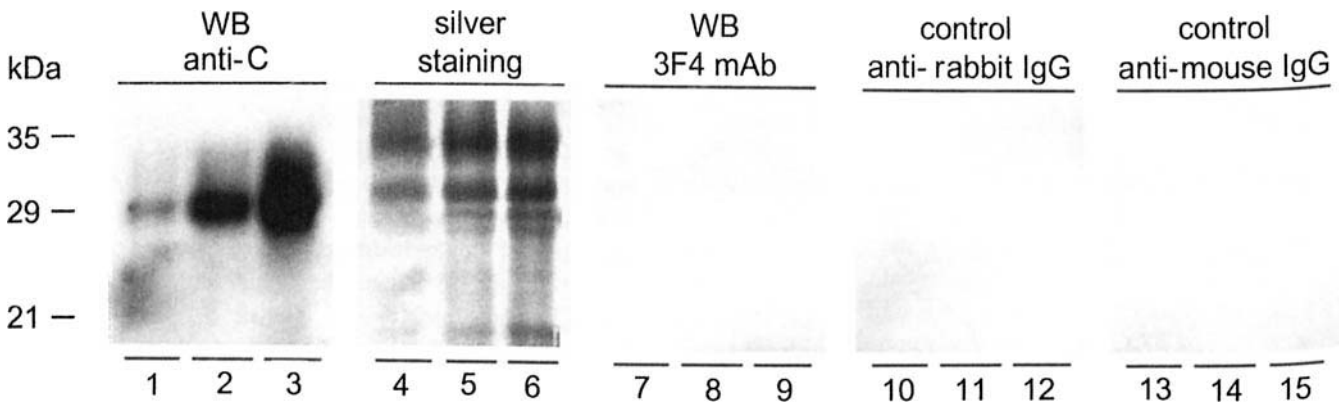

Figure 1. Detection of prion protein (PrP) in human urine. A urine sample from a normal individual was subdivided into 1-, 3-, and 5-ml aliquots. Proteins were concentrated by solid-phase extraction as described in the Materials and Methods section. Samples equivalent to 1 ml (first lanes in each panel), 3 ml (middle lanes in each panel), and 5 ml (last lanes in each panel) of urine were analyzed on Western blots. Rabbit anti-C antibody was used to detect PrP (Lanes 1–3; note the gradient increase of PrP from 1- to 5-ml urine samples). Total proteins captured by solid-phase extraction from 1 ml to 5 ml of urine showed a similar increase as judged by silver staining (Lanes 4–6). No detectable bands were recognized by using 3F4 monoclonal antibody (mAb) (Lanes 7–9). Nonspecific immunoreactivity was not found in the control experiments in which the secondary antibodies (i.e., anti-rabbit IgG, Lanes 10–12; anti-mouse IgG, Lanes 13–15) were used without the respective primary antibodies. The position (in kDa) of molecular-weight markers run in parallel on each blot is indicated on the left.

consensus sites for asparagine-linked glycosylation, PNGase F digestion was performed to remove glycans and reveal the protein backbone of urinary PrP^C. Following deglycosylation (Fig. 3), the heterogeneous 28 kDa to 30 kDa urinary PrP^C bands (Lane 1) shifted mainly to a lower molecular weight band of 18 kDa (Lane 2). This is consistent with the similar, approximately 10-kDa shift expected from the removal of two asparagine-linked complex glycans from PrP, as shown in cultured cells and brain tissue (21). Therefore, our detection of urinary PrP is highly specific, without artifacts associated with the unrelated proteins (18–20). As expected, PrP^C in the urine of normal individuals was sensitive to digestion by both trypsin and PK, as exogenous protease digestion almost completely degraded PrP into small peptides of less than 7 kDa (Fig. 3).

It is essential to test whether our method is capable of capturing PrP^{Sc} as effectively as PrP^C because PrP^{Sc} associated with TSEs has an altered protein conformation that is different from normal PrP^C. This possibility was tested in experiments in which small quantities of scrapie brain homogenate containing PrP^{Sc} was spiked into urine. As shown on Western blots probed by the anti-C antibody, PrP bands were detected in all PK-untreated samples (i.e., control urine, urine spiked with normal or scrapie brain homogenate). Following PK digestion, PK-resistant PrP derived from PrP^{Sc} was only found in the urine sample spiked with scrapie hamster brain homogenate (Fig. 4, upper panel). When probed by 3F4 mAb (Fig. 4, lower panel), no immunoreactive PrP bands were found in nonspiked control urine, a finding that is consistent with the results from the previous experiments (Fig. 1). However, strong PrP bands were detected by 3F4 mAb in urine spiked with either normal or scrapie brain homogenate before PK digestion. This result, as expected, suggests that 3F4 mAb readily recognizes PrP from the brain, (23, 24) but not from urine. As with anti-C antibody, PK-resistant PrP^{Sc} was detected on Western blots probed with 3F4 in the urine sample spiked with scrapie brain homogenate (Fig. 4, lower panel). Taken together, we have shown that the present method was able to recover and detect both PrP^C and PrP^{Sc} from urine samples spiked with normal and scrapie brain homogenate.

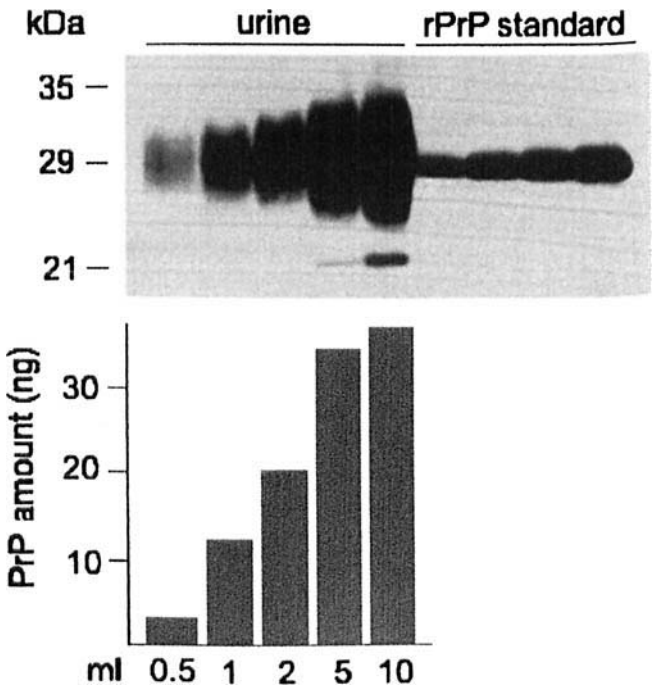

Figure 2. Estimation of amounts of urinary prion protein (PrP). Samples equivalent to 0.5 ml, 1 ml, 2 ml, 5 ml, and 10 ml of urine (Lanes 1–5) were loaded along with known quantity (i.e., 1 ng, 2 ng, 4 ng, and 8 ng) of recombinant human PrP23-231 (Lanes 6–9). Western blotting was performed using the anti-C antibody as described in the Materials and Methods section. The position (in kDa) of molecular-weight markers is indicated on the left.

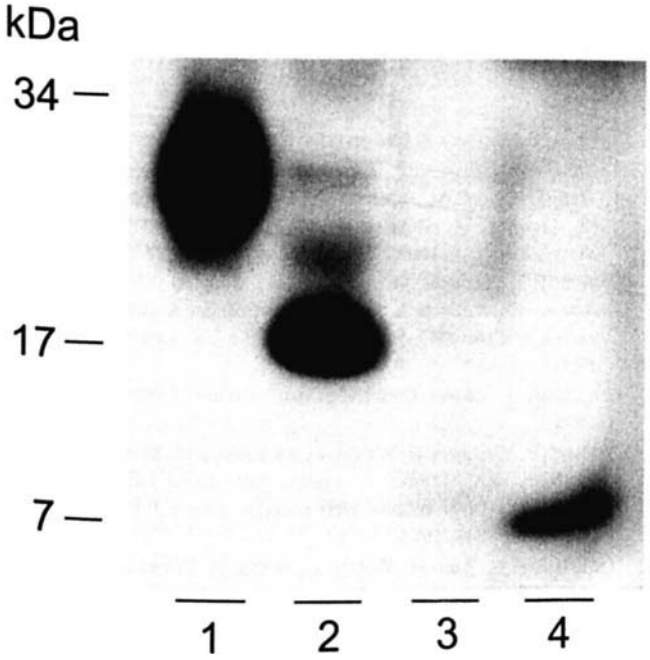

Figure 3. Digestion of urinary prion protein (PrP) by PNGase F and proteases. A urine sample from a normal individual was subdivided into 10-ml aliquots. Proteins were concentrated and treated with either PNGase F or proteases (i.e., trypsin, proteinase K [PK]), followed by Western blotting with the anti-C antibody as described in the Materials and Methods section, except that 10% to 20% Tris-tricine SDS-PAGE gels were used to reveal the presence of small peptides. Following PNGase F digestion, the diffused bands of approximately 28-kDa PrP in urine (Lane 1) was deglycosylated and shifted to a sharper band at approximately 18-kDa PrP (Lane 2). Urinary PrP was sensitive to degradation by trypsin (Lane 3) and PK (Lane 4). The position (in kDa) of molecular-weight markers is indicated on the left.

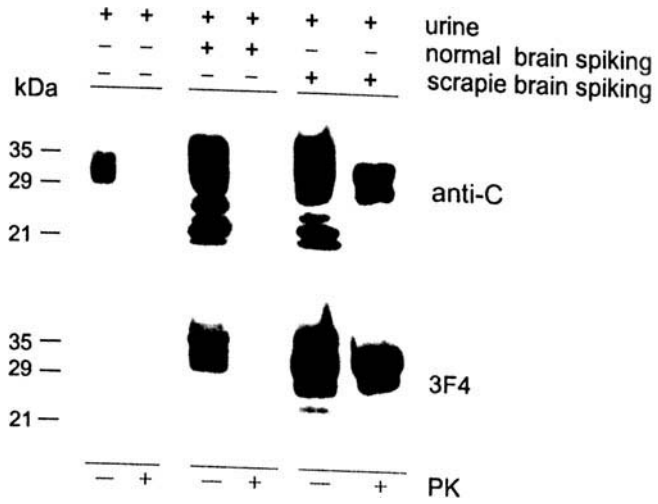

Figure 4. Detection of hamster brain prion protein, PrP^{Sc}, spiked into human urine. Aliquots of urine (1 ml each) from a normal individual were taken as controls and as samples to which brain homogenate from normal and scrapie-affected hamsters was spiked. Following the solid-phase extraction of proteins, samples were either untreated with proteinase K (PK–) or treated with PK (PK+) at a final concentration of 50 μg/ml for 1 h at 37°C as described in the Materials and Methods section. The detection of PrP on Western blots was achieved by using either the anti-C antibody (Upper panel) or 3F4 monoclonal antibody (Lower panel).

Discussion

The simple ion-capture method presented in the present study effectively extracted proteins from urine onto small quantities of resin, thus providing a convenient way for the enrichment of excreted protein from large volumes of urine. We have shown that normal PrP was detectable in less than 1 ml of urine in all healthy individuals examined. The PrP that was concentrated from the urine was detected using the anti-C antibody, an antibody that is immunoreactive to the C-terminus of human PrP (21, 22). No detectable urinary PrP was recognized by 3F4 mAb, which is contrary to an earlier report by Shaked *et al.* (18) whose findings were challenged in more recent studies (19, 20). Attempts to detect PrP^C in either human or hamster urine using the 3F4 mAb by two other groups have failed (19, 20). Instead, a nonspecific cross-reactivity to other contaminating proteins was reported (19, 20). In the present study, no bands were detected when a secondary antibody was used alone. Therefore, the detection of PrP^C using the anti-C antibody is not due to contamination with bacterial outer-membrane proteins (19) or endogenous IgG (20) as observed by other groups when using 3F4 mAb. Because 3F4 mAb is well known for its ability to recognize brain PrP (23, 24), its failure to detect urinary PrP^C suggests that the 3F4 epitope located in the N-terminal region is absent in urinary PrP^C. This conclusion is consistent with the fact that the anti-C antibody against the C-terminus of PrP readily detects urinary PrP^C. Our findings not only highlight the unique structural properties of urinary PrP that are apparently different from brain PrP, but also help clarify the recent controversy involving the attempts to detect urinary PrP using 3F4 (18–20).

Semiquantitative studies in which recombinant PrP was applied along with urinary protein concentrates from 0.5- to 10-ml samples revealed that the lower detection limit on Western blots is better than 1 ng of PrP^C in urine. On average, each healthy individual excreted approximately 7 ng of PrP^C per ml of urine. If a healthy person produces 1 liter of urine a day, the daily excretion of a microgram amount of normal PrP^C into the urine is expected. Variations in the amounts of PrP^C per ml of urine were observed among normal individuals. The reason for the observed variations is unclear, but they are not unexpected because protein excretion per ml of urine is likely to fluctuate among different individuals in untimed-morning urine used in the present study. A more accurate estimation of the rate of PrP^C excretion and its normalization among healthy individuals may require a correlation analysis of baseline parameters such as total urine protein levels, creatinine clearance, and the use of accurately timed–urine collection (e.g., 24-hr urine specimens). Moreover, the exact origin of urinary PrP^C is yet to be determined. Nevertheless, consistent detection of PrP in the urine of normal, healthy individuals raises the question of whether the protein

generated in the body is efficiently reutilized. It is possible that most proteins are, indeed, reutilized. However, if even a small proportion of PrP^C is not reutilized, it could filter through the kidney.

Deglycosylation by PNGase F is often used to reduce the heterogeneity of PrP molecules. Treatment with PNGase F revealed that PrP^C in urine was glycosylated, but the protein was truncated. Following deglycosylation, the size of urinary PrP^C shifted from 28–30 kDa to 18 kDa. As the full-length PrP^C following deglycosylation has an electrophoretic mobility of about 27 kDa on SDS-PAGE gels (20), our data suggest that PrP^C in human urine is mostly truncated with a size much smaller than that expected from the full-length protein (25). Such a truncation is unlikely an artifact generated during our experimental procedures because the same results were obtained from fresh urine samples containing the added protease inhibitors following concentration at a lower temperature (10°C) or when proteins were precipitated in cold methanol at –20°C. Therefore, the truncated PrP^C in human urine is likely the result of proteolytic processing that occurred *in vivo* before the excretion, a normal metabolic event previously shown in human neuroblastoma cells and the brain (21), possibly by a calpain-dependent proteolytic process (26).

It would be desirable to study a large number of individuals from whom 24-hr urine samples were obtained. Furthermore, it would be essential to investigate whether there is any difference in the amounts of PrP^C excreted into the urine between normal individuals and those with TSEs. A previous report (18) on the detection of the urinary PrP^{Sc} in TSEs using 3F4 mAb has recently been challenged as an artifact (19, 20), possibly due to lack of the 3F4 epitope in urinary PrP as demonstrated in the present study. Therefore, it is unclear whether TSEs lead to the excretion of urinary PrP with the PrP^{Sc}-like conformation. Assuming this is the case, the ability to detect PrP^C may lay the foundation for a future technique to be used in PrP^{Sc} detection. Furthermore, the positive data obtained in our experiments in which brain PrP^{Sc} was spiked into urine suggest the potential of our method for identifying urinary PrP^{Sc} in TSEs which may have, so far, evaded detection due its extremely low concentration. Equally important is a further analysis of other biochemical properties of urinary PrP between normal individuals and those affected by CJD. In the present study, we present a simple, convenient, and efficient means to concentrate and detect trace amounts of PrP in urine. Our assay may be further developed to determine whether any unique characteristics of urinary PrP, or its interaction with other molecules, might be associated with a preclinical state of TSEs. Finally, it is our hope that the technique presented here will evoke further interests and improvements in diagnostic strategies for TSEs.

We thank Fang Zhang for technical assistance and Dr. Robert Petersen for helpful discussion.

1. Prusiner SB. Prion diseases and the BSE crisis. Science 278:245–251, 1997.
2. Weber T, Aguzzi A. The spectrum of transmissible spongiform encephalopathies. Intervirology 40:198–212, 1997.
3. Parchi P, Gambetti P. Human prion diseases. Curr Opin Neurol 8:286–293, 1995.
4. Wells GA, Scott AC, Johnson CT, Gunning RF, Hancock RD, Jeffrey M, Dawson M, Bradley R. A novel progressive spongiform encephalopathy in cattle. Vet Rec 121:419–420, 1987.
5. Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch A, Poser S, Pocchiari M, Hofman A, Smith PG. A new variant of Creutzfeldt-Jakob disease in the UK. Lancet 347:921–925, 1996.
6. Collinge J. Variant Creutzfeldt-Jakob disease. Lancet 354:317–323, 1999.
7. Oesch B, Westaway D, Walchli M, McKinley MP, Kent SB, Aebersold R, Barry RA, Tempst P, Teplow DB, Hood LE, Prusiner SB, Weissmann C. A cellular gene encodes scrapie PrP 27-30 protein. Cell 40:735–746, 1985.
8. Chesebro B, Race R, Wehrly K, Nishio J, Bloom M, Lechner D, Bergstrom S, Robbins K, Mayer L, Keith JM, Garon C, Haase A. Identification of scrapie prion protein-specific mRNA in scrapie-infected and uninfected brain. Nature 315:331–333, 1985.
9. Stahl N, Baldwin MA, Hecker R, Pan KM, Burlingame AL, Prusiner SB. Glycosylinositol phospholipid anchors of the scrapie and cellular prion proteins contain sialic acid. Biochemistry 31:5043–5053, 1992.
10. Sharom FJ, Lehto MT. Glycosylphosphatidylinositol-anchored proteins: structure, function, and cleavage by phosphatidylinositol-specific phospholipase C. Biochem Cell Biol 80:535–549, 2002.
11. Stahl N, Baldwin MA, Teplow DB, Hood L, Gibson BW, Burlingame AL, Prusiner SB. Structural studies of the scrapie prion protein using mass spectrometry and amino acid sequencing. Biochemistry 32:1991–2002, 1993.
12. Prusiner SB, Bolton DC, Groth DF, Bowman KA, Cochran SP, McKinley MP. Further purification and characterization of scrapie prions. Biochemistry 21:6942–6950, 1982.
13. Bendheim PE, Berry RA, DeArmond SJ, Stites DP, Prusiner SB. Antibodies to a scrapie prion protein. Nature 310:418–421, 1984.
14. McKinley MP, Bolton DC, Prusiner SB. A protease-resistant protein is a structural component of scrapie prion. Cell 35:57–62, 1983.
15. Hsich G, Kenney K, Gibbs CJ, Lee KH, Harrington MG. The 14-3-3 brain protein in cerebrospinal fluid as a marker for transmissible spongiform encephalopathies. N Engl J Med 335:924–930, 1996.
16. Giraud P, Biacabe AG, Chazot G, Later R, Joyeux O, Moene Y, Perret-Liaudet A. Increased detection of 14-3-3 protein in cerebrospinal fluid in sporadic Creutzfeldt-Jakob disease during the disease course. Eur Neurol 48:218–221, 2002.
17. Chapman T, McKeel DW Jr, Morris JC. Misleading results with the 14-3-3 assay for the diagnosis of Creutzfeldt-Jakob disease. Neurology 14:1396–1397, 2000.
18. Shaked GM, Shaked Y, Kariv-Inbal Z, Halimi M, Avraham I, Gabizon R. A protease-resistant prion protein isoform is present in urine of animals and humans affected with prion diseases. J Biol Chem 276:31479–31482, 2001.
19. Furukawa H, Doh-Ura K, Okuwaki R, Shirabe S, Yamamoto K, Udono H, Ito T, Katamine S, Niwa M. A pitfall in diagnosis of human prion diseases using detection of protease-resistant prion protein in urine: contamination with bacterial outer membrane proteins. J Biol Chem 279:23661–23667, 2004.
20. Serban A, Legname G, Hansen K, Kovaleva N, Prusiner SB. Immunoglobulins in urine of hamsters with scrapie. J Biol Chem 279:48817–48820, 2004.
21. Chen SG, Teplow DB, Parchi P, Teller JK, Gambetti P, Autilio-

Gambetti L. Truncated forms of the human prion protein in normal and in prion diseases. J Biol Chem 270:19173–19180, 1995.

22. Zou WQ, Capellari S, Parchi P, Sy MS, Gambetti P, Chen SG. Identification of novel proteinase K-resistant C-terminal fragments of PrP in Creutzfeldt-Jakob disease. J Biol Chem 278:40429–40436, 2003.
23. Kascsak RJ, Rubenstein R, Merz PA, Tonna-DeMasi M, Fersko R, Carp RI, Wisniewski HM, Diringer H. Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. J Virol 61:3688–3693, 1987.
24. Pan K-M, Stahl N, Prusiner SB. Purification and properties of the cellular prion protein from Syrian hamster brain. Protein Sci 1:1343–1352, 1992.
25. Turk E, Teplow DB, Hood LE, Prusiner SB. Purification and properties of the cellular and scrapie hamster prion proteins. Eur J Biochem 176:21–30, 1988.
26. Yadavalli R, Guttmann RP, Seward T, Centers AP, Williamson RA, Telling GC. Calpain-dependent endoproteolytic cleavage of PrPSc modulates scrapie prion propagation. J Biol Chem 279:21948–21956, 2004.