A Protease-resistant Prion Protein Isoform Is Present in Urine of Animals and Humans Affected with Prion Diseases*

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Prion protein (PrP)Sc, the only known component of the prion, is present mostly in the brains of animals and humans affected with prion diseases. We now show that a protease-resistant PrP isoform can also be detected in the urine of hamsters, cattle, and humans suffering from transmissible spongiform encephalopathies. Most important, this PrP isoform (UPrPSc) was also found in the urine of hamsters inoculated with prions long before the appearance of clinical signs. Interestingly, intracerebrally inoculation of hamsters with UPrPSc did not cause clinical signs of prion disease even after 270 days, suggesting it differs in its pathogenic properties from brain PrPSc. We propose that the detection of UPrPSc can be used to diagnose humans and animals incubating prion diseases, as well as to increase our understanding on the metabolism of PrPSc in vivo.

Since the appearance of BSE¹ in 1985 (1, 2) the need for an in vivo diagnostic test for prion diseases has become acute. In the absence of such a method, an extensive slaughtering of cattle was required once an affected animal was identified within a herd. The need for such an in vivo test was reinforced since the first cases of variant Creutzfeldt-Jakob disease were reported in 1996 (3–5). Variant Creutzfeldt-Jakob disease is a fatal neurodegenerative disease believed to be caused by the consumption of BSE-contaminated meat, and the incubation time between infection to clinical symptoms may be as long as decades (4). As opposed to cattle, the incubating individuals (which at this point can be any of us), will be present for many years, donating blood, and in some cases other organs, to the non-affected population.

The only identified component of the prion, the agent causing prion diseases (also referred to as transmissible spongiform encephalopathies; TSEs), is PrPSc, a protease-resistant abnormal isoform of PrP². PrPSc is a glycosphatidyl inositol-anchored glycoprotein of unknown function (7, 8). Although some other markers for prion diseases have been suggested (9–11), PrPSc remains not only an obligatory prion component but also the only reliable and universally accepted marker for this family of diseases (12–14). We now show that a protease-resistant isoform of PrP, hereby denominated UPrPSc, can be detected, following a specific enrichment procedure, in the urine of scrapie-infected hamsters, BSE-infected cattle, and humans suffering from CJD. These results pave the way for the development of a simple in vivo test for prion diseases.

EXPERIMENTAL PROCEDURES

Analysis of Urine Samples—Urine samples (2 ml for hamster, 10 ml for human, and 15 ml for bovine) were sedimented for 5 min at 3000 rpm to discard occasional cell debris and then dialyzed overnight in a cellulose tubular membrane ( pore range 6000–8000 dalton; Membrane Filtration Products, Inc., Seguin, TX) against 5 liters of saline at 4 °C (saline was changed twice during dialysis). For experimental purposes, (see Fig. 1c), the dialysis step was omitted in some cases. Subsequently, the urine samples were centrifuged at high speed (100000 g, average gravitational force equivalent executed by the centrifugation process on the sample for 1 h at 4 °C). Pellets were resuspended in 100 μl of 2% sарkosyl STE buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM EDTA). Samples were divided and digested in the presence or absence of proteinase K (PK). Digestion conditions were optimized for each. For hamster urine, 40 μg/ml PK for 60 min at 37 °C; for human urine, 40 μg/ml PK for 30 min at 37 °C; for bovine urine, 20 μg/ml for 30 min at 37 °C.

Following protease digestion, the urine samples were boiled in SDS sample buffer, applied to a 12% SDS-polyacrylamide gel electrophoresis and subsequently transferred to a nitrocellulose membrane. Membranes were blocked with 3% milk fat milk except for the bovine samples, which were blocked with 5% human serum albumin; Sigma. A second blocking step was performed with a mixture of 1:3000 anti-mouse IgG and 1:3000 anti-rabbit IgG in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20; for 30 min) to avoid nonspecific binding of the secondary antibody to IgG light chain present in some urine samples. Membranes were then rinsed in TBST for 15 min and immuno-blotted with either α PrP mAb 3F4 or 6H4 (hamster and human) at 1:5000 or 6H4 (bovine) at 1:5000.

In Vivo Experiments—Syrian hamsters were inoculated with samples containing urine PrP from normal or scrapie-sick hamsters. For inoculation, urine samples were prepared as described above (including PK digestion but not SDS boiling) and diluted as required in 1% bovine serum albumin/phosphate-buffered saline. Brain samples from scrapie-infected hamsters were diluted to contain similar concentrations of PrP⁰ (see Fig. 3) and inoculated to additional groups of hamsters. To achieve similar concentrations of protease-resistant PrP in brain and urine inoculate, each animal was inoculated, depending on the appropriate experimental group, with 50 μl of sample containing PrP originating from either 0.5 ml of urine or from 1.25 μl of 10% scrapie hamster brain homogenate.

Collection of Urine from Hamsters—Following inoculation, animals were examined daily for scrapie-associated symptoms. For time course experiments, groups of three hamsters in an equivalent stage of disease incubation were housed each week in a metabolic cage for urine collection from 15:00 to 08:00 of the next day. Urine was collected in the morning and immediately frozen at −80 °C. Food and water were supplied ad libitum. A similar procedure was applied to scrapie-sick hamsters.

Tissue Homogenates—Whole brain or kidney were homogenized in 10 volumes of homogenization buffer (10 mM Tris-HCl, pH 7.5, 500 mM sucrose/phosphate-buffered saline). Following centrifugation (2000 rpm, 15 min, 4 °C), the supernatant was frozen (−80 °C).

Human Urine Samples—Whenever possible, the human samples from CJD patients and controls were the first morning urine. Some CJD and post-stroke patients were bearing catheters, and in these cases

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² The abbreviations used are: BSE, bovine spongiform encephalopathy; TSE, transmissible spongiform encephalopathy; PrP, prion protein; CJD, Creutzfeldt-Jakob disease; PK, proteinase K; mAb, monoclonal antibody; VLA, Veterinary Laboratory Agency; i.c., intracerebrally; i.p., intraperitoneally.

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urine was collected for a period of up to 8 h in a urine collecting bag. All samples were frozen until further use.

**Bovine Urine Samples**—All BSE and most control bovine urine samples were obtained from the Veterinary Laboratory Agency (VLA) in London, United Kingdom. The VLA samples consisted of 51 samples of 24 cows, all coded for blind testing. Additional freshly frozen control samples were obtained from the Hebrew University Veterinary School. According to VLA records, most samples were frozen following collection whereas some were kept chilled. No information was provided regarding time of day for sample collection.

**RESULTS**

Most of the CJD patients tested in this work (6 of 8) were genetic patients carrying the E200K mutation (15–18). One of the patients was a 52-year-old individual homozygous for this mutation (19). Among the other genetic patients, four were MM at codon 129 and one was MV. The E200K mutation is located at a Methionine 129 allele (17). The human controls (n = 15), were either healthy individuals (n = 7) or patients suffering from diverse neurological disorders, such as Alzheimer’s disease (n = 3), multiple sclerosis (n = 2), and stroke (n = 3). Urine from BSE-infected cattle, as well as most of the bovine controls used in this work, were obtained from the VLA laboratory in the United Kingdom. For the bovine samples, the urine test was performed as a blind study. Urine from Syrian hamsters, inoculated with either the 263K prion strain (20) or normal hamster urine samples were collected with the use of a metabolic cage (as explained under “Experimental Procedures”), as pools from three animals.

Urine samples from scrapie-infected hamsters, CJD patients, and BSE-infected cattle, as well as from their appropriate controls, were processed for enrichment of UPrPSc as described under “Experimental Procedures” and subsequently immunoblotted for PrP peptides. Human and hamster urine samples were immunoblotted with either mAb 3F4 or 6H4 (not shown), whereas bovine samples were blotted only with mAb 6H4. Parallel samples were blotted only with secondary a mouse antiserum and showed no interfering signals.

Fig. 1 demonstrates the results of such an experiment. Although a precipitable and protease-resistant form of PrP could be detected in the dialyzed urine of prion disease-affected humans and animals, this was not the case for the urine of the appropriate controls. The PrP urine assay was negative for all controls humans (n = 15), hamsters (n = 10), when each sample represents a pool from three hamsters, and cattle (n = 15), positive for all CJD patients tested (n = 8), for a large number of hamster groups (n = 20), and for 10 of 12 BSE-infected cattle, whereas the other two BSE-positive cows showed a positive but poor signal. As can be seen in Fig. 1c dialysis was essential for the detection of the protease-resistant UPrPSc in urine from scrapie-infected hamsters. The fact that the PrP signal in urine could be blocked by the 3F4 peptide and did not react with the secondary antibody provides strong evidence that this signal belongs to a PrP peptide (Fig. 1d).

A surprising result depicted above is that a protease-sensitive PrP isoform is present in the precipitable fraction of the normal urine samples, as opposed to what is expected for PrPSc. It is to be noticed, however, that no detergent was added to the urine before ultracentrifugation as performed in membrane extractions that result in a soluble PrPSc (21, 22). It is also possible that all PrP molecules are present in urine in a partially denatured state because of the presence of urea. Also dialysis of normal urine may induce the aggregation of the PrPSc isoform, which, as opposed to UPrPSc, is protease-sensitive. Although the exact chemical nature of UPrPSc is yet to be determined, its molecular weight seems to be slightly higher than full-length and fully glycosylated PrP or PrPSc. In addition, the pattern of UPrPSc in the immunoblots suggests it may be composed mostly of the higher molecular band of PrP and not of the less glycosylated species. This may indicate that partially or non glycosylated PrP is less resistant to the conditions encountered by PrPSc until it is excreted in urine as UPrPSc. That normal PrP is excreted in urine is not entirely surprising, because this is also the case of other GPI-anchored proteins (23–25).

UPrPSc did not originate directly from the kidneys, because no PrPSc could be identified in the kidney tissue of the scrapie-infected hamsters (Fig. 1b). This suggests UPrPSc originates from other organs and arrives to the urine from blood. The detection of PrPSc at the end stages of prion disease may result from some degree of blood brain-barrier disruption by brain degeneration (26). Contrarily, the presence of PrPSc in prion-infected urine early in the incubation time would suggest a clearance pathway for the aberrant PrP protein either from brain or from a peripheral organ, through its excretion into urine. To address this question, we inoculated Syrian hamsters either intracerebrally (i.c.) or intraperitoneally (i.p.) with hamster prions and collected urine samples, as described under “Experimental Procedures,” every week during the incubation period. Each sample was frozen immediately after collection.
samples were thawed, enriched for PK-resistant UPrPSc as described above, and subsequently immunoblotted with a PrP mAb 3F4.

The results of such an experiment can be seen in Fig. 2. A light signal of prion-specific PrP was detected in the urine samples of the i.c. inoculated hamsters after only 17 days (Fig. 2a), following by the disappearance of the PrP signal until day 35. Subsequently, the signal for protease-resistant PrP increased until the appearance of clinical signs. Similar results were obtained for the i.p. inoculated hamsters. A PrP signal was detected in the first weeks following the inoculation, disappeared at later dates, and reappeared at about 60 days. These results may infer that some of the prion inoculum is immediately secreted following inoculation. Thereafter, no PrP signal appeared in urine until the first stages of prion protein accumulation in brain. Indeed, whereas scrapie incubation time for i.c. or i.p. inoculated hamsters with the 263K strain is about 75 and 120 days, respectively, PrPSc can be identified in enriched brain samples of these hamsters at about 40 (i.c.) or 70 (i.p.) days (27–29). Our experiments therefore suggest that UPrPSc is excreted in urine in parallel to its accumulation in brain.

The results of the experiments described in both Figs. 1 and 2 indicate therefore that urine testing for protease-resistant PrP can be used not only to diagnose prion diseases in animals and humans at terminal stages of the disease but also to diagnose these diseases in the subclinical stages of infection. If indeed the PrP signal detected at the first weeks-post infection is because of clearance of the inoculum, the PrP urine test may serve to diagnose a potential new occurrence of infection. This will allow in the future, providing an effective anti-prion therapy becomes available, to treat individuals at risk of a new prion exposure.

The detection of UPrPSc raises the alarming possibility that urine from prion-infected individuals, either ill or as yet incubating the disease can somehow transmit prion diseases. This prospect is especially disturbing in the case of BSE-infected cattle, as well as in natural scrapie in sheep. Because the mechanism by which these diseases are transmitted among animals within the herd was never elucidated (30, 31), it is conceivable that urine can contaminate the living areas of these animals.

To investigate whether urine from TSE-infected animals can be infectious, we inoculated 20 hamsters with UPrPSc pooled and enriched from urine of 10 hamsters terminally ill with scrapie. 20 hamsters were inoculated with similarly prepared samples from 10 normal hamsters. Brain samples from scrapie-infected hamsters, which were diluted to contain similar concentrations of PrPSc (1.25 μl of 10% homogenate) to the enriched UPrPSc (from 0.5 ml of urine), were inoculated to additional groups of hamsters (Fig. 3a). Hamsters were observed daily for symptoms of scrapie infection. Urine was collected periodically from animals inoculated with UPrPSc. At different time points during the experiment, some of the hamsters inoculated with UPrPSc were sacrificed and tested for the presence of PrPSc in their brains.

As expected, the animals inoculated with scrapie-infected brain samples suffered from fatal disease symptoms at about 80 days post-inoculation. Contrarily, none of the animals inoculated with urine samples (normal or scrapie-infected) developed clinical symptoms of prion disease to date (270 days post-infection). 12 hamsters (four groups of three), inoculated with scrapie urine samples, were tested for the presence of UPrPSc, and all were found positive from about 60 days post-inoculation (Fig. 3, b, 2). In addition, low concentrations of PrPSc could be identified in the brain of one of three hamsters sacrificed at about 120 days (Fig. 3, b, 3). All other hamsters in this experiment are still under observation to determine whether they will develop a fatal prion disease at a later date. These results suggest that UPrPSc inoculation can result in a subclinical or carrier state prion infection. The clinical and epidemiological implications of this finding are yet to be determined.

**DISCUSSION**

Why is UPrPSc excreted into urine? Because most urine proteins originate from blood, we speculate that some PrPSc, either from brain or from a peripheral organ, is released during the disease incubation into the blood serum in a non-aggregated form, although at a low and undetectable concentrations. Because of its protease resistance, this PrPSc is not digested by blood proteases. However, and because the MW of PrP is below the cut off size for filtering through kidney cells (about 40 kDa) (32), PrP may subsequently be secreted into the urine and thereby be concentrated, as other proteins, at about 120 times over its concentration in blood (32). The concentration by the kidney makes it possible to detect PrPSc in urine much easier than in blood. Because dialysis of the urine seems to be a necessary step in our detection procedure, we propose that UPrPSc is present in a semi-denatured form, probably because of the relative high concentrations of urine-denaturing agents, and is subsequently renatured during the dialysis step. This denaturation/renaturation effect may also happen in the field because of absorption of the urea by the soil.

UPrPSc may differ in its conformation from brain PrPSc, thereby explaining the fact that inoculation of comparable amounts of both protease-resistant PrP isoforms produced such different results. It is to be remembered however that not all protease-resistant PrP molecules carry prion infectivity. Indeed, *in vitro* conversion experiments of PrPSc to PrPSc, in which protease resistance was achieved by a denaturation/renaturation procedure, resulted in a protease-resistant but not infectious PrP isoform (33, 34). Contrarily, UPrPSc may resemble the new protease-resistant soluble isoform we have identified lately, which is associated with very low levels of infectivity, if any (35, 36). The latest possibility is consistent with the fact that UPrPSc is found in urine, because an aggregated molecule could not filter through the kidney.

It can also be speculated that UPrPSc is a component of a new prion strain, less virulent than the original 263K strain, which may produce not a fatal but a subclinical or a carrier state prion disease. Recent publications indicate the presence of low levels of PrPSc in the brains of animals that did not succumb to prion disease (6, 37), suggesting a subclinical state of prion disease may exist. In some cases, the brains of these animals transmitted a fatal prion disease to other rodents, suggesting appar-
ently healthy carriers of prions disease can transmit disease.

To summarize, we have identified a prion-specific protease-resistant PrP isoform in the urine of prion-infected animals and humans (UPrPSc) that may be used for the in vivo early diagnosis of ill, as well as seemingly healthy but prion-infected, individuals. Our findings, in addition to their practical aspects, may also open new avenues to investigate the metabolism and clearance mechanism of PrPSc during prion infection and disease.

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