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A Protease Resistant PrP Isoform Is Present In Urine of Animals and Humans Affected with Prion Diseases

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Summary

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 TSEs. Most important, this PrP isoform ($UPrP^{Sc}$) was also found in the urine of hamsters inoculated with prions long before the appearance of clinical signs. Interestingly, i.c. inoculation of hamsters with $UPrP^{Sc}$ did not cause clinical signs of prion disease even after 270 days, suggesting it differs in its pathogenic properties from brain PrP^{Sc}. We propose that the detection of $UPrP^{Sc}$ can be used to diagnose humans and animals incubating prion diseases, as well as to increase our understanding on the metabolism of PrP^{Sc} in-vivo.

Key words

Urine, prion, PrP, diagnosis

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Introduction

Since the appearance of BSE 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 (vCJD) were reported in 1996 (3-5). vCJD 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 (6). 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 encephalopaties or TSEs), is PrP^{Sc}, a protease resistant abnormal isoform of PrP^{C}. PrP^{C} is a GPI anchored glycoprotein of unknown function (7,8). Although some other markers for prion diseases have been suggested (9),(10,11), PrP^{Sc} 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 $UPrP^{Sc}$, 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.

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Experimental Procedures

Analysis of urine samples

Urine samples (2ml for hamster; 10ml for human; 15ml for bovine) were sedimented for 5 min at 3000 rpm to discard occasional cell debris, and then dialyzed over night in a cellulose tubular membrane (pore range 6000-8000 Dalton, FPI; Texas, USA) against 5 liters of saline at 4°C (saline was changed twice during dialysis). For experimental purposes, (figure1c), the dialysis step was omitted in some cases. Subsequently, the urine samples were centrifuged at high speed (100000g_{av}*1hr*4°c). Pellets were resuspended in 100μl 2% sarkosy/ ISTE buffer (10mM Tris HCl pH 7.5, 10mM NaCl, 1mM EDTA) . Samples were divided and digested in the presence or absence of proteinase K (PK). Digestion conditions were optimized for each species. For hamster urine; 40μg/ml PK for 60 min at 37°C; for human urine, 40μg/ml PK for 30 min at 37°C; for bovine urine, 20μg/ml for 30 min at 37°C.

Following protease digestion, the urine samples were boiled in SDS sample buffer, applied to a 12% SDS PAGE and subsequently transferred to a nitrocellulose membrane. Membranes were blocked with 3% fat milk except for the bovine samples which were blocked with 5% HAS (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 (for 30 min) to avoid non specific binding of the secondary Ab to IgG light chain present in some urine samples. Membranes were then rinsed in TBST for 15 min and immunobloted either with α PrP mAb 3F4 or 6H4 (Hamster, Human) at 1:5000 or 6H4(Bovine) at 1:5000.

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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% BSA/PBS. Brain samples from scrapie infected hamsters were diluted to contain similar concentrations of PrP^{Sc} (see figure 3) and inoculated to additional groups of hamsters. To achieve similar concentrations of protease resistant PrP in brain and urine inoculi, each animal was inoculated, depending on the appropriate experimental group, with 50 µl sample containing PrP originating from either 0.5 ml 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 3 hamsters in an equivalent stage of disease incubation, were housed each week in a metabolic cage for urine collection from 1500 to 0800 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 homogenization buffer (10mM Tris HCl pH 7.5, 300mM Sucrose). 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

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catheters, and in these cases urine was collected for a period of up to 8h 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. The VLA samples constituted 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 while some were kept chilled. No information was provided regarding time of day for sample collection.

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Results

Most of the CJD patients tested in this work (6 out 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, 4 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 England. For the bovine samples, the urine test was performed as a blind study. Urine from Syrian hamsters, either inoculated with the 263K prion strain(20), or normal controls, were collected with the use of a metabolic cage (as explained in the methods), as pools from 3 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 $UPrP^{Sc}$ as described in the methods, and subsequently immunobloted for PrP peptides. Human and hamster urine samples were immunobloted with either mAb 3F4 or 6H4 (not shown), while bovine samples were blotted only with mAb 6H4. Parallel samples were blotted only with secondary α mouse antisera and showed no interfering signals.

Figure 1 demonstrates the results of such an experiment. While 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

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negative for all controls humans (n=15), hamsters (n=10), when each sample represents a pool from 3 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 out of 12 BSE infected cattle, while the other two BSE positive cows showed a positive but poor signal. As can be seen in figure 1c dialysis was essential for the detection of the protease resistant $UPrP^{Sc}$ 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 (figure 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 PrP^{C}. 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 PrP^{C} (21,22). It is also possible that all PrP molecules are present in urine in a partially denatured state due to the presence of urea, which may encourage protein aggregation. Also dialysis of normal urine may induce the aggregation of the PrP^{C} isoform which, as opposed to $UPrP^{Sc}$, is protease sensitive. Although the exact chemical nature of $UPrP^{Sc}$ is yet to be determined, its molecular weight seems to be slightly higher than full length and fully glycosilated PrP^{C} or PrP^{Sc}. In addition, the pattern of $UPrP^{Sc}$ in the immunoblots suggest it may be composed mostly of the higher molecular band of PrP, and not of the less glycosilated species. This may indicate that partially or non glycosilated PrP is less resistant to the conditions encountered by PrP^{Sc} until it is excreted in

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urine as UPrP^{Sc}. That normal PrP is excreted in urine is not entirely surprising since this is also the case of other GPI anchored proteins (23-25).

UPrP^{Sc} did not originate directly from the kidneys, since no PrP^{Sc} could be identified in the kidney tissue of the scrapie infected hamsters (figure 1b). This suggests UPrP^{Sc} originates from other organs and arrives to the urine from blood.

The detection of PrP^{Sc} 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 PrP^{Sc} 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 intra-cerebrally (i. c.), or intraperitoneally (i.p.) with hamster prions and collected urine samples, as described in the methods, every week during the incubation period. Each sample was frozen immediately after collection. At the end of the experiment, similar volumes of these urine samples were thawed, enriched for PK resistant UPrP^{Sc} as described above and subsequently immunobloted with α PrP mAb 3F4.

The results of such an experiment can be seen in figure 2. A light signal of prion specific PrP was detected in the urine samples of the i.c. inoculated hamsters after only 17 days (figure 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

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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, while scrapie incubation time for i.c. or i.p. inoculated hamsters with the 263 strain is about 75 and 120 days respectively, PrP^{Sc} 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 $UPrP^{Sc}$ is excreted in urine in parallel to its accumulation in brain.

The results of the experiments described in both figures 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 due to 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 $UPrP^{Sc}$ 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. Since 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.

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To investigate if urine from TSE infected animals can be infectious, we inoculated 20 hamsters with $UPrP^{Sc}$ pooled and enriched from urine of 10 hamsters terminally ill with scrapie. Twenty 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 PrP^{Sc} (1.25μl of 10% homogenate) than the enriched $UPrP^{Sc}$ (from 0.5 ml urine), were inoculated to additional groups of hamsters (figure 3a). Hamsters were observed daily for symptoms of scrapie infection. Urine was collected periodically from animals inoculated with $UPrP^{Sc}$. At different time points during the experiment, some of the hamsters inoculated with $UPrP^{Sc}$ were sacrificed and tested for the presence of PrP^{Sc} 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 dpi). Twelve hamsters (4 groups of 3) were tested for the presence of $UPrP^{Sc}$ and all were found possitive from about 60 days post inoculation (figure 3b,2). In addition, low concentrations of PrP^{Sc} could be identified in the brain of one out of three hamsters sacrificed at about 120 days (figure 3b,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 $UPrP^{Sc}$ inoculation can result in a subclinical or carrier state prion infection. The clinical and epidemiological implications of this finding are yet to be determined.

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Discussion

Why is $UPrP^{Sc}$ excreted into urine? Since most urine proteins originate from blood, we speculate that some PrP^{Sc}, 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. Due to its protease resistance, this PrP^{Sc} is not digested by blood proteases. However, and since the MW of PrP is below the cut off size for filtering through kidney cells (about 40kDA)(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 possible to detect PrP^{Sc} in urine much easily than in blood. Since dialysis of the urine seems to be a necessary step in our detection procedure, we propose that $UPrP^{Sc}$ is present in a semi denatured form, probably due to 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 due to absorption of the urea by the soil.

$UPrP^{Sc}$ may differ in its conformation from brain PrP^{Sc}, 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 PrP^{C} to PrP^{Sc}, in which protease resistance was achieved by a denaturation/renaturation procedure, resulted in a protease resistant but not infectious PrP isoform(33,34). Contrarily, $UPrP^{Sc}$ may resemble the new protease resistant soluble isoform we have

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identified lately, which is associated with very low levels of infectivity, if any(35,36). The latest possibility is consistent with the fact that $UPrP^{Sc}$ is found in urine since an aggregated molecule could not filter through the kidney.

It can also be speculated that $UPrP^{Sc}$ is a component of a new prion strain, less virulent than the original 263K strain, which may produce not a fatal but a sub clinical or a carrier state prion disease. Recent publications indicate the presence of low levels of PrP^{Sc} in the brains of animals which did not succumb to prion disease (37,38), 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 apparently 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 ($UPrP^{Sc}$), which 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 PrP^{Sc} during prion infection and disease.

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Legends

Figure 1: Protease resistant PrP in urine of TSE affected humans and animals.

a. Freshly frozen urine samples from hamsters, humans, and cattle, were enriched for protease resistant PrP as described in the methods. All samples were digested in the presence or absence of Proteinase K , as described in the methods, and immunoblotted with either αPrP mAb 3F4 (hamster and human samples) or 6H4 (bovine samples). (1) Homozygous E200K CJD patient (2) Heterozygous E200K CJD patient (3) Human control (4) Scrapie sick hamster (5) Normal hamster (6) BSE sick cattle (7) Normal cattle. The volumes of urine used are described in the manuscript and methods.

b. 5µl of a 10% brain samples were analyzed for PrP (1) Homozygous CJD patient. (2) Heterozygous CJD patient. (3) Human control (4) Scrapie sick hamster. (5) Normal hamster. (6) kidney sample from scrapie sick hamster.

c. Scrapie hamster urine samples were enriched for $UPrP^{Sc}$ with and withought the dialysis step. Samples were digested in the presence or absence of PK as described in methods.

d. Human brain sample (b) and Human urine sample (U) were immunoblotted with mAb 3F4 in the absence (1) or the presence (2) of 10µg/ml of the peptide comprising the 3F4 epitope. Molecular Weight markers (top to bottom); 36 kDa, 30 kDa.

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Figure 2: Prion specific PrP can be detected during scrapie incubation time

Urine samples were collected weekly from Syrian hamsters inoculated either i. c. **(a),** or i.p **(b)** with hamster 263K prions, and enriched for $UPrP^{Sc}$ as described. Samples were immunobloted with αPrP mAb 3F4. The arrows in the figure represent the onset of clinical signs. Molecular Weight marker; 30 kDa.

Figure 3: I.C inoculation of Syrian hamsters with $UPrP^{Sc}$

Syrian hamsters were inoculated with equivalent amounts of PK resistant PrP from brain or urine of scrapie infected hamsters. All samples were immunoblotted with 1:5000 mAb 3F4.

(a) PK resistant PrP^{Sc} equivalents originating from 5μl of 10% hamster brain homogenate (1) as compared to 2ml scrapie hamster urine (2).

(b) (1) Brain sample from a scrapie infected hamster. (2) Urine samples collected (at 60 dpi) from hamsters inoculated with $UPrP^{Sc}$. (3) Brain sample of one of the animals inoculated with $UPrP^{Sc}$. All samples were digested in the presence or absence of PK. Molecular Weight markers (top to bottom); 36 kDa, 30 kDa.

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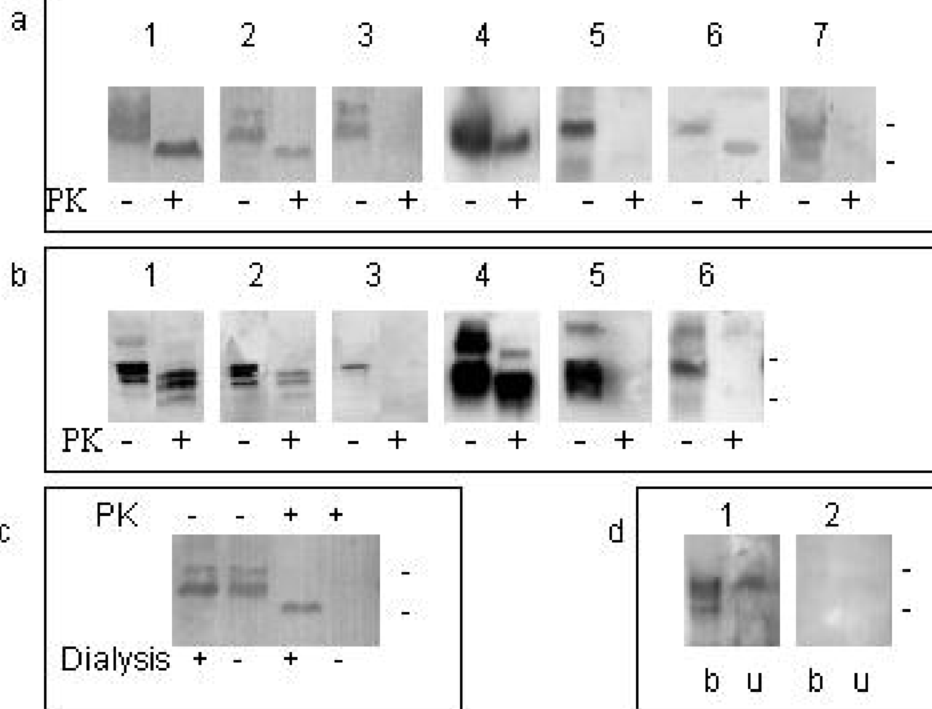

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Figure 2

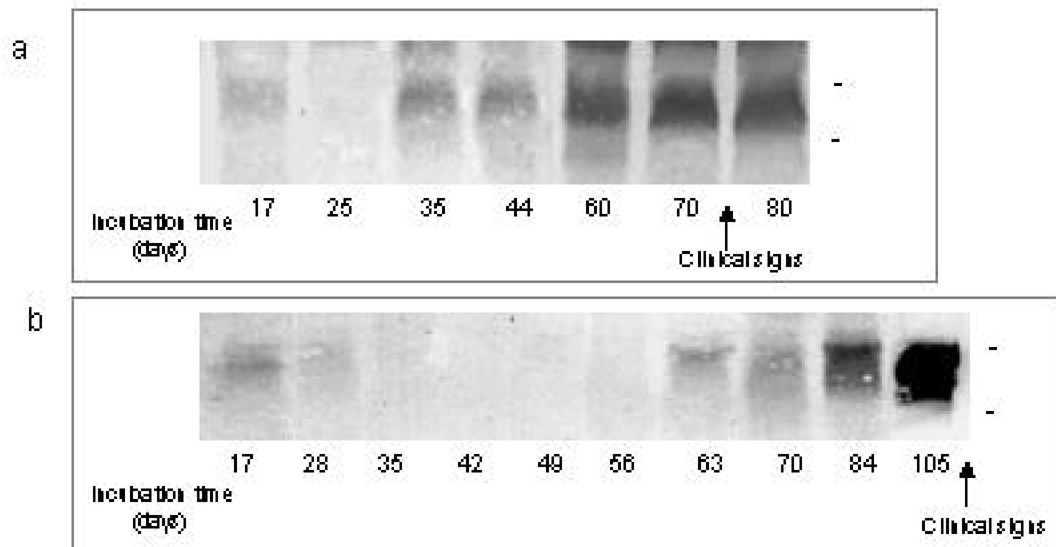

Figure 3