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Peripherally administrated prions reach the brain at sub-infectious quantities in experimental hamsters

Baian Chen [a,b], Claudio Soto [a], Rodrigo Morales [a,*]

[a] Protein Misfolding Disorders Laboratory, Mitchell Center for Alzheimer's Disease and Related Brain Disorders, University of Texas Medical School at Houston, Houston, TX 77030, USA
[b] Department of Laboratory Animal Science, School of Basic Medical Science, Capital Medical University, Beijing 100069, China

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ABSTRACT

The mechanisms implicated in prion infection and tissue distribution are not completely understood. In this study we investigated the levels of 263K prions in brain and spleen of Syrian hamsters few days after intra-peritoneal challenge. For this purpose we utilized the PMCA technology which permits to detect as little as few PrP^{Sc} molecules. Our results show that peripherally administered prions directly reach the brain, although at levels below the minimum necessary to produce disease. PrP^{Sc} remains in the brain several days after administration suggesting inefficient clearance or early replication. Understanding the fate of the infectious agent after administration and its uptake in different organs and fluids may provide useful information to develop strategies to minimize further spreading of prion diseases.

1. Introduction

Transmissible Spongiform Encephalopathies (TSEs) are a group of proteopathies affecting several mammalian species [1]. Creutzfeldt–Jakob disease (CJD) is the most prevalent disease in humans affecting one individual per million people every year [1,2]. Despite their low incidence, TSEs have been extensively studied due to their intriguing mechanisms of propagation and their zoonotic transmission potential [3–6]. As of today, TSEs are the only group of diseases transmitted by a protein-based infectious agent [7–9].

TSEs are characterized by long incubation periods followed by a short clinical phase that invariably leads to death [4]. In order to experimentally decrease these long incubation periods, intra-cerebral inoculations are used to assess infectivity in animal models. However, peripheral routes of administration are more relevant when assessing natural mechanisms of transmission. It is well documented that PrP^{Sc} accumulates in several peripheral tissues, much before the appearance of the first clinical signs [10,11]. Spleen and lymph nodes are important contributors to peripheral prion replication as well as to the transport of the agent to the brain, either by the vascular, sympathetic or parasympathetic systems [12,13]. Replication and accumulation of prions in peripheral organs do not produce any important damage to the tissue and the clinical disease is only manifested when they extensively accumulate in the brain.

The long incubation periods and the fact that peripheral tissues, including blood, can harbor infectious quantities of prions [14–16] is a major concern for public health [3]. Reports of disease transmission by transfusion using blood from individuals silently incubating variant Creutzfeldt–Jakob disease (vCJD) urge the development of methods that can detect the infectious agent during its long and silent incubation period [3,15,17]. Animal bioassays and cell-based prion replication systems can be used to detect the infectious material. However, these assays take a long time to be completed and are often limited to only a subset of prion strains/animal species, reducing their applicability. *In vitro* assays such as Protein Misfolding Cyclic Amplification (PMCA, [8,18]) and Quaking Induced Conversion (QuIC, [19]) look promising as effective techniques for the identification of misfolded prions in different organs and fluids [20–22], even at pre-symptomatic stages of the disease [11,23].

To understand the mechanism of prion propagation within the body, it is important to study how PrP^{Sc} spreads and replicates in different tissues and organs after peripheral challenge. The purpose of this study was to analyze the tissue distribution and brain uptake of infectious prions in experimental hamsters few days after peripheral infection. Since the amount of prions in this organ is presumably very low at this stage, we estimated PrP^{Sc} levels

* Corresponding author. Address: University of Texas Medical School at Houston, 6431 Fannin St, MSE R468, Houston, TX 77030, USA. Fax: +1 (713) 500 0773.
E-mail address: Rodrigo.MoralesLoyola@uth.tmc.edu (R. Morales).

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using the PMCA technology. We compared the levels of infectious prions in this organ with the ones in spleen, where PrP^{Sc} replication appears to occur at earlier stages [24].

2. Materials and methods

2.1. Samples

4 weeks old female Syrian Golden Hamsters (Harlan®) were intra-peritoneally (i.p.) injected with 75 μL of 10% brain homogenates (prepared as previously explained [8]) from a symptomatic 263K infected hamster. Hamsters were sacrificed at 0 (30 min), 2, 4 and 9 days after injection (n = 5/group). Spleens and brains were collected and stored at −80 °C until use. All animal manipulations were carried out in accordance to NIH regulations and approved by the Animal Welfare Committee of the University of Texas Medical School at Houston.

2.2. Preparation of tissue homogenates

Brain and spleen homogenates were prepared at 10% in phosphate saline buffer (PBS, MP Biomedicals, cat. No. 1860454) with a protease inhibitor cocktail (Roche Diagnostics, cat. No. 11 697 498 001). After a brief centrifugation to eliminate debris (805 g for 45 s in a Beckman-Coulter Allegra 25R centrifuge), supernatants were used for Western blot (WB) analysis. To concentrate PrP^{Sc} and remove tissue components that may interfere with PMCA, 500 μL of sample were mixed with a sarkosyl (Fisher Bioreagents, cat. No. BP234) solution prepared in PBS (final concentration, 10%) and centrifuged at 100000×g for 1 h at 4 °C [25]. Supernatants were discarded and PBS was added to the pellets (without resuspension) in order to dilute out traces of detergent. Samples were centrifuged again at 100,000 g for 30 min at 4 °C. Final pellets were resuspended in 100 μL of normal hamster brain homogenate prepared at 10% in Conversion Buffer (PMCA substrate, 150 mM NaCl and 1% Triton X-100 in PBS) [8,26] and submitted to PMCA.

2.3. Protein Misfolding Cyclic Amplification and Western blotting

Spleen and brain pellets resuspended in PMCA substrate were submitted to 96 PMCA cycles. Serial rounds were performed by mixing 10 μL of the resulting sample with 90 μL of fresh PMCA substrate. Tissue homogenates and PMCA products were treated with 50 μg/mL of Proteinase K (PK, Sigma–Aldrich, cat. No. P2308) for 1 h at 37 °C. PK digestion was stopped by adding LDS loading buffer (Invitrogen, cat. No. NP0007) and heating for 5 min at 100 °C. Samples were fractionated by SDS–PAGE and transferred into nitrocellulose membranes. Membranes were probed with 3F4 antibody (Covance, cat. No. SIG-39600) and signal observed by using ECL Plus detection system (GE Healthcare, cat. No. RPN2132) as recommended by the manufacturer.

3. Results

In order to analyze the distribution of PrP^{Sc} at early stages after infection we i.p. injected 263K prions in Syrian hamsters and brains and spleens were collected at several days (0, 2, 4 and 9) post-injection. Crude brain homogenates taken at these stages did not show any detectable amount of PrP^{27-30} after conventional PK digestion and Western blotting (Fig. 1a). For comparison, Fig. 1b shows the PrP^{27-30} levels typically observed in the brain of a symptomatic 263K hamster inoculated with the same amount of prions by the same route.

Previous reports showed that prions can rapidly distribute in several tissues, including the brain, after intra-venous (i.v.) injection [27,28]. In order to investigate the brain uptake of prions soon after exposure, we assessed the presence of PrP^{Sc} in this organ by PMCA [29]. This technique has been proven to detect as little as one particle of infectious prions [30]. Our results showed that we were able to detect PrP^{Sc} in brains after 5 rounds of PMCA in a fraction of animals sacrificed at 2, 4 and 9 days after treatment (Fig. 2). Only 1 out of 5 animals was positive 2 days after inoculation whereas no animals showed to be positive at day 0 (sacrificed 30 min after injection). The fraction of positive animals increased to 2/5 in 4 and 9 days post-challenge. This data suggest that after reaching the brain, PrP^{Sc} is not efficiently cleared. We analyzed the detection limit of our method by performing dilutions of 263K brain homogenates from a symptomatic hamster (Fig. 3a). According to this data we estimated that the PrP^{Sc} levels in the brain of PMCA-positive animals were equivalent to a brain dilution of 5×10^{-11} of a symptomatic hamster. It is important to highlight that control brain homogenates submitted to PMCA amplification in the absence of PrP^{Sc} seeds did not show any positive signal (Fig. 3b).

It is well documented that spleen is one of the most important peripheral organs in terms of prion replication. PrP^{Sc} generated in this tissue acts as a reservoir of infectious units that later on invade the central nervous system [31]. However, it is suggested that prions replicated in the periphery reach the brain only at later stages of their incubation periods. Using PMCA we detected the presence of PrP^{Sc} in spleen of some animals just after 3 PMCA rounds at the same day of injection (Fig. 4). PrP^{Sc} detection was lower, but still positive, 2 days after infection (2/5 animals), but decreased at 4 days. At 9 days after challenge no PrP^{Sc} was detectable at the 3rd PMCA round. At the 5th PMCA round the tendency was even clearer: at 0 and 4 days post inoculation PrP^{Sc} was detected in the spleen of 4/5 animals, whereas a lower proportion of animals was detected at 9 days post inoculation (2/5). These results suggest that prions in spleen at these early time points correspond to the original inoculum injected, which is progressively cleared over time from this organ. Altogether, this data suggest that PrP^{Sc} detected in spleen and brain at these short time points correspond to the original inoculum administered.

It is important to highlight that there appears to be a high animal-to-animal variability in PrP^{Sc} detection both in spleen and brain. This variability is not due to the PMCA reaction, since analysis of replicate samples in PMCA containing the same amount of diluted PrP^{Sc} showed little variability. This data indicate that distinct animals handle prions differently at the moment of infection.

4. Discussion

In this study we investigated the uptake of infectious prions in spleens and brains at early stages after peripheral challenge. The relatively rapid detection of prions in the brain suggests that PrP^{Sc} can directly reach this organ after inoculation. These results are in agreement with our previous observations using purified, radiolabeled PrP^{Sc}, which clearly demonstrated that prions can cross the blood–brain barrier to reach the brain parenchyma [27,28,32]. According to our amplification control shown in Fig. 3, the amount of prions we detected in brain few days after inoculation is equivalent to a 5×10^{-11} brain dilution of a symptomatic 263K infected hamster. Previous reports estimated that $1LD_{50}$ of 263K prions after i.c. infection is equivalent to a brain dilution of $\sim 1 \times 10^{-9}$ [8], although this estimation can vary between samples [33,34]. The higher infectious brain dilution for i.c inoculations has been determined in the same range for several other prion strains such as RML, ME7 and Hyper-TME as tested by *in vivo* and cell based assays [35–37]. The last infectious dilutions for peripheral routes are considerably lower than the ones described for direct i.c. inoculations [35]. Thus, our results indicate that the quantity of prions