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Differential effects of prion particle size on infectivity *in vivo* and *in vitro*

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ABSTRACT

The conversion of cellular prion protein to the disease-associated isoform (PrP^{Sc}) has been suggested to follow a mechanism of seeded aggregation. Here, we show that fragmentation of PrP^{Sc} aggregates by sonication increases converting activity in cell culture in a way similar to *in vitro* conversion assays. In contrast, under the same conditions the infectious titer of sonicated samples *in vivo* was reduced. We modified the size distribution of PrP^{Sc} by adsorption to nitrocellulose, which resulted in a reduction of the infectious titer in non-sonicated samples and an increase in sonicated samples. Our results indicate that NC-adsorption can (i) block some active sites of PrP^{Sc} aggregates and (ii) reduce the rate of clearance from the brain. For large particles with low clearance the effect of NC-particles on the number of available active sites may dominate, whereas for smaller particles (i.e. sonicated samples) the effect of NC-adsorption on clearance dominates resulting in increased infectivity.

Prion diseases are infectious neurodegenerative diseases of mammals that include bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, chronic wasting disease of deer and elk, and Creutzfeldt-Jakob disease (CJD) in humans. According to the "protein-only" hypothesis, the misfolded, partially proteinase K-resistant PrP isoform (PrP^{Sc}) is the infectious agent that propagates in infected hosts by inducing the structural conversion of the normal host-encoded cellular prion protein (PrP^{C}) to PrP^{Sc} [1–3]. The mechanistic details of the conversion process are not understood in detail, but appear to involve direct interaction between PrP^{Sc} and PrP^{C}. In this context, the protein misfolding cyclic amplification reaction (PMCA) that was originally developed by Saborio et al. [4] provides a unique opportunity to convert large quantities of PrP^{C} into PK-resistant PrP (PrPres) and to model prion propagation *in vitro*.

During PMCA, sonication induces aggregate fragmentation and leads to a shift in the size distribution of PrPres aggregates towards smaller particles [5,6]. On the molecular level, sonication enhances the converting activity of prion aggregates by increasing the availability of catalytically active surfaces for PrP^{C}–PrP^{Sc} conversion and is essential for an efficient *in vitro* amplification of misfolded PrP [7,8]. Since smaller oligomers have been predicted to have greater converting activity per unit mass [9,10] the infectious titer per unit mass should increase upon sonication-induced aggregate fragmentation. However, by subjecting PMCA samples to varying numbers of sonication pulses per amplification cycle we could recently demonstrate that the amplification efficiency is dependent on the ultrasonic power applied to the assay and follows a bell-shaped curve with an optimum between 5 and 20 pulses à 1 s [11].

Here, we investigated the effect of sonication-induced changes of prion particle size distribution on specific infectivity *in vivo* and in cell culture. As in our PMCA experiments, we found a bell-shaped-like dependence of infectivity on particle size in an infectivity assay using N2a cells. In contrast, sonication-induced fragmentation of prion aggregates was associated with a dose-dependent prolongation of incubation times *in vivo* in a murine bioassay. Interestingly, by combining sonication-induced aggregate fragmentation with prion delivery on suitable inert carriers we found that the stabilizing effect nitrocellulose particles exert on prion aggregates has a stronger impact on the specific infectivity of smaller rather than on larger aggregates. The relationship between particle size, converting activity *in vitro* and specific infectivity *in vivo* is discussed.

Materials and methods

Evaluation of the size distribution of scrapie prion aggregates in brain homogenates. To obtain a robust measure of sonication-induced changes in the size distribution of PrP^{Sc} aggregates in brain homogenates, the centrifugation assay described in Weber et al. [5] was used. Briefly, a 10% (wt/vol) brain homogenate of mice infected with scrapie strain RML [12] in phosphate buffered saline (PBS) containing 0.2% (wt/vol) SDS was digested with proteinase K (PK; 100 μg/ml, total protein: PK ≈ 1:60) for 1 h at 37 °C. The reaction was terminated with 5 mM PMSF and the reaction mixture was subjected to 13,400*g* for 15 min in a minifuge (Eppendorf, Hamburg, Germany). The resulting pellet (P1) was resuspended in PBS/0.2% SDS and either left untreated or subjected to sonication, respectively, using a microtip sonicator (Bandelin, Berlin, Germany) with power setting at 40%. Subsequently, each sample was split and either left unmodified or incubated with NC-particles for 2 h at room temperature

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(see below). NC-particles were prepared as described [5]. To analyze the size distribution of PrP^{Sc} aggregates in the untreated and sonicated P1 samples used for inoculation into wild-type mice (see below), 200 μl-aliquots of these samples were subjected to centrifugation for 15 min at 13,400g. The supernatant (S2) was collected and the pellet (P2) was resuspended in PBS/0.2% SDS. The recovery of PK-resistant PrP in the pellet (P2) and supernatant (S2) fractions was determined by Western blot analysis.

Bioassay of infectivity in C57Bl/6 mice. For *in vivo* infectivity assays P1 fractions of RML brain homogenate following sonication and corresponding untreated control samples were either diluted 1:10 in PBS (unmodified samples) or adsorbed to NC-particles, which also resulted in a 1:10 dilution. Prior to inoculation, the samples were diluted again 1:10 in PBS (final dilution 1:100) and 30 μl each were inoculated *intracerebrally* (*i.c.*) into seven week-old C57Bl/6 mice (6 animals per experimental group).

Western blotting and quantification of proteinase K-resistant PrP. For quantification of PrP^{Sc}, samples were digested with PK (100 μg/ml) for 1 h at 37 °C and subjected to Western blot analysis using 4H11 antibody at a dilution of 1:2000 [13]. PrP was visualized by enhanced chemiluminescence reaction (GE Healthcare, Freiburg, Germany). For quantification a Diana II luminescence imaging system along with the AIDA software package (Raytest, Straubenhardt, Germany) was used.

Cell culture and infection of N2a cells. Prion susceptible N2a subclone I13 A7 was grown in minimum essential Eagle's medium supplemented with penicillin/streptomycin and 10% fetal calf serum at 37 °C and 5% CO_2 using 24 well-plates. Mouse-adapted scrapie strain 22L, kindly provided by the TSE Resource Center, Institute of Animal Health (Compton, UK), was propagated in C57Bl/6 mice (Charles River, Sulzfeld, Germany). The brains of mice at the terminal stage of disease were homogenized in phosphate buffered saline (PBS) at 10% (w/v). The brain homogenate was PK digested (100 μg/ml) for 1 h at 37 °C and subjected to 13,400g for 15 min in the presence of 0.2% SDS in a microfuge (Eppendorf, Germany). The supernatant (S1) was collected and the pellet (P1) was resuspended in PBS with 0.2% (wt/vol) SDS. Aliquots of PK-digested P1 (300 μl each) were either left untreated or subjected to sonication for 1 × 25 s and 1 × 20 min, respectively, using a water bath sonicator (Bandelin, Berlin, Germany). Subsequently, dilution series (10^{-2} to 10^{-9}) of untreated and sonicated P1 samples in culture medium were added to growing N2a cells in 24 well-plates containing 1.5 ml medium per well. After 24 h, the medium was refreshed, and cells were serially passaged, first at a 1:4 dilution in dishes containing 4 ml medium each (first passage) and subsequently at a 1:9 dilution in dishes containing 10 ml medium each (second to eighth passage). After the sixth and eighth serial passage of cells, aliquots were lysed in buffer containing 100 mM NaCl, 10 mM Tris–HCl (pH 7.5), 0.5% Triton X-100 and 0.5% sodium deoxycholate, and the samples were then digested with PK (10 μg/ml) for 30 min at 37 °C. The reaction was terminated with 2.5 μl of PEFA-block SC (Calbiochem, Schwalbach, Germany) and the samples were subjected to centrifugation at 100,000g for 60 min at 4 °C. Subsequently, the amount of PK-resistant PrP was analyzed by Western blotting using antibody 4H11 [13].

Results

To obtain a robust measure of sonication-induced changes in the size distribution of PrP^{Sc} aggregates in brain homogenates, we developed a centrifugation assay using PK-digested brain homogenates extracted from prion infected mice [6]. Based on this method, we first investigated the effect of the sonication energy on the size distribution of PrP^{Sc} aggregates by subjecting RML brain homogenate to pulses of constant power but increasing duration. Regarding the untreated starting material, the bulk of PrP^{Sc} was recovered in the pellet fraction P1 as evidenced by the minimal amount of PrP^{Sc} in the supernatant fraction S1 (Fig. 1A, lanes 2 and 3). The P1 pellet fraction was subsequently subjected to different sonication protocols followed by a second analytical centrifugation step. Sonication induced a dose-dependent shift towards smaller aggregate size, as evidenced by the increased recovery of PrP^{Sc} in supernatant (S2) fractions (Fig. 1A, lanes 9–14). Noteworthy, when we compared the infectious titer of differentially sonicated P1 samples that contained the same amount of protease-resistant PrP, albeit with a different size distribution of PrP^{Sc} aggregates, we found that sonication-induced aggregate fragmentation was associated with a dose-dependent prolongation of incubation times (Fig. 1B). Whereas animals receiving the untreated starting material showed an attack rate of 100% and a mean incubation time to terminal disease of 167 ± 1 day (mean ± standard deviation), mice inoculated with RML brain homogenate sonicated under conditions resembling one sonication step in PMCA reactions (5 × 1 s) succumbed to scrapie disease with a mean incubation time of 185 ± 7 days. The difference in incubation times was statistically significant ($p < 0.002$, two-tailed *t*-test). Notably, only two of six animals challenged with RML brain homogenate sonicated for 1 min became ill after 223 and 328 days, respectively. These results are in line with our previous finding that showed a decrease in infectivity with increasing fragmentation of PrP^{Sc} aggregates [5,6].

To analyze the infectious properties of PrPres with a modified size distribution and/or stability, we then employed adsorption to nitrocellulose (NC-) particles which are known to firmly retain proteins including PrP^{Sc} [5]. Following adsorption to NC-particles, the incubation times of wild-type mice inoculated with 5 × 1 s sonicated RML brain homogenate (183 ± 17 days) and those inoculated with the untreated starting material (182 ± 5 days) converged (Fig. 1C). Notably, only one of six mice challenged with NC-adsorbed RML homogenate sonicated for 1 min showed signs of scrapie after 236 days.

When prion infectivity is analyzed in the murine bioassay, the apparent specific infectivity also depends on biological clearance from the brain [5,6]. Therefore, we also investigated prion infectivity in a cell culture assay in a second set of experiments. For these experiments we used 22L brain homogenate with a sonication-induced modified size distribution and prion susceptible N2a cells. It has been described previously that wild-type N2a cells are relatively resistant to infection with many other mouse-adapted scrapie strains including 87 V, 139A, ME7 [14], whereas scrapie strain 22L has been shown to be especially suited for cell culture studies [15]. Using the same centrifugation assay as described above we thus investigated the effect of the sonication time on the size distribution of 22L prions. In line with the findings obtained with RML brain homogenate (see above), sonication induced a dose-dependent shift towards smaller aggregate size as evidenced by the increased recovery of PrP^{Sc} in supernatant (S2) fractions (Fig. 2, lanes 4–6). As these samples were used for cell culture studies, we used sealed sample containers and a water bath sonicator for these experiments. We could show previously, that indirect sonication in a water bath can be used for prion fragmentation and PMCA amplification similar to sonication using a microtip sonicator [11].

Exposure of prion susceptible N2a cells to dilution series (10^{-2} to 10^{-6}) of untreated and differentially sonicated 22L brain homogenate resulted in a dose-dependent stable infection of this cell line. Following exposure, cells were serially passaged and screened for misfolded PK-resistant PrP after the sixth and eighth passage, respectively. The amount of PrP^{Sc} increased from the sixth to the eighth passage, which corroborates that contamination of the passaged cells with residual inoculum-derived 22L prions can be ruled out. In fact, with increasing dilution factor, PrP^{Sc} levels in N2a cells decreased as reflected in reduced signal intensities in corresponding Western blots (Fig. 3A and B). Thus, the signal intensity provides a direct measure of the infectious dose of the inoculum.

Remarkably, cells exposed to similar dilutions of untreated and sonicated 22L brain homogenate differed in the amount of *de novo* generated PrP^{Sc} indicating different infectious titers of the inocula (Fig. 3A and B). In contrast to the dose-dependent prolongation of incubation times found in the murine bioassay, the amount of PK-resistant PrP accumulated in prion susceptible N2a cells increased with decreasing particle size generated under mild (25 s) sonication conditions, and decreased following intense (20 min) sonication conditions (Fig. 3A and B). After the sixth passage, cells incubated with 22L brain homogenate sonicated for 25 s showed the highest PrP^{Sc} level as compared to the untreated samples and samples sonicated for 20 min, respectively (Fig. 3A). After the eighth passage, this bell-shaped-like dependence between sonication time and the infectious titer of the inoculum was even more pronounced (Fig. 3B).