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Agent-specific Shadoo Responses in Transmissible Encephalopathies

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Abstract

Transmissible spongiform encephalopathies (TSE) are neurodegenerative diseases caused by an infectious agent with viral properties. Host prion protein (PrP), a marker of late stage TSE pathology, is linked to a similar protein called Shadoo (Sho). Sho is reduced in mice infected with the RML scrapie agent, but has not been investigated in other TSEs. Although PrP is required for infection by TSE agents, it is not known if Sho is similarly required. Presumably Sho protects cells from toxic effects of misfolded PrP. We compared Sho and PrP changes after infection by very distinct TSE agents including sporadic CJD, Asiatic CJD, New Guinea kuru, vCJD (the UK epidemic bovine agent) and 22L sheep scrapie, all passaged in standard mice. We found that Sho reductions were agent-specific. Variable Sho reductions in standard mice could be partly explained by agent-specific differences in regional neuropathology. However, Sho did not follow PrP misfolding in any quantitative or consistent way. Tga20 mice with high murine PrP levels revealed additional agent-specific differences. Sho was unaffected by Asiatic CJD yet was markedly reduced by the kuru agent in Tga20 mice; in standard mice both agents induced the same Sho reductions. Analyses of neural GT1 cells demonstrated that Sho was not essential for TSE infections. Furthermore, because all infected GT1 cells appeared as healthy as uninfected controls, Sho was not needed to protect infected cells from their “toxic” burden of abundant abnormal PrP and intracellular amyloid.

Keywords

CJD; kuru; BSE; neurodegeneration; prion protein; tissue culture; toxicity; amyloid

Introduction

Transmissible spongiform encephalopathies (TSEs), such as human Creutzfeldt-Jakob Disease (CJD), endemic sheep scrapie, and epidemic bovine spongiform encephalopathy (BSE) are fatal neurodegenerative diseases caused by infectious particles of ~25 nm diameter and 10^6 – 10^7 daltons (Manuelidis 2006; Manuelidis et al. 1995). Their molecular composition remains speculative. These infectious particles separate from the majority of host-encoded prion protein (PrP) by field flow sedimentation (Silveira et al. 2005; Sklaviadis et al. 1992) as well as other purification procedures. According to the prion hypothesis, PrP spontaneously misfolds to become an infectious protein, and then propagates its “infectious” conformation by directly converting other members (Prusiner 1998). Compared with normal PrP, misfolded PrP is a form of amyloid. It shows enhanced resistance to proteolysis in a test tube and is visualized as

partially digested bands on Western blots (PrP-res or PrP^{Sc}). PrP-res is clearly a pathological marker for infection. However, misfolded PrP has not yet shown reproducible or significant infectivity. Numerous animal model (Barron et al. 2007; Manuelidis 1998; Manuelidis and Fritch 1996; Sakaguchi et al. 1993; Xi et al. 1992), tissue culture (Arjona et al. 2004; Baker et al. 2002), and subcellular fractionation (Manuelidis et al. 1995; Sun et al. 2008) experiments have also demonstrated that PrP-res levels are a poor predictor of infectivity titers.

PrP characteristics often change in ways that are unrelated to the agent-induced phenotype. Many distinct TSE agents have now been identified by their vastly different incubation times and regional pathology when analyzed in mice with a normal genotype. As with viruses, TSE agents breed true whereas PrP-res patterns do not. Hence PrP folding does not encode agent-specific information. Indeed, altering the PrP-res pattern by propagating CJD, scrapie and BSE agents in the stable GT1 monotypic cell line has shown that the PrP-res pattern is cell-type specific. These PrP changes do not have any demonstrable effect on agent characteristics as shown by re-inoculating mice (Arjona et al. 2004; Manuelidis et al. 2009b). Moreover, most TSE agents also maintain their original identity even after serial passage in foreign species with different PrP-res band patterns. Like viruses with mutable nucleic acid genomes, TSE agents can also progressively adapt or evolve during serial passages in a constant host (Manuelidis et al. 1976; Manuelidis et al. 1997). Furthermore, TSE agents appear to be exogenous rather than host derived. They have a specific geographic origin and distribution consistent with endemic exposure of isolated populations (Manuelidis 1998; Manuelidis et al. 2009a; Tateishi et al. 1979), and are not spontaneously generated without environmental exposure (Hunter and Cairns 1998). Nevertheless, TSE agents can quickly spread to many countries, as happened with the epidemic UK BSE agent. For public health reasons it has become essential to identify different TSE agent-strains, follow their progression, and identify new emerging infectious outbreaks and agent-strains. Thus additional markers of agent-specified TSE disease are of interest.

Cumulative data on the behavior of many different TSE agents and their basic requirement for host PrP are most simply explained if PrP is not the agent, but is rather an essential receptor for a TSE particle that contains mutable nucleic acid. A nucleic acid can code for different TSE agent-strains, and nucleic acid mutation provides for agent adaptations to new species as well as to monotypic cells. Although no agent-specific nucleic acid has been sequenced, infectious preparations contain protected nucleic acids of >500 nt, capsid, and nucleic acid binding proteins (Akowitz et al. 1994; Sklaviadis et al. 1993) and a small group of other selected proteins. Some of these may be agent specific (Sun et al. 2008).

Host-encoded proteins can be involved in defense against TSE invasion as well as in the neurodegenerative cascade. Hosts recognize TSE agents as foreign invaders. We previously identified a variety of innate immune responses to particular TSE agents, but not to PrP-res, in standard murine models (Baker et al. 2004; Baker and Manuelidis 2003; Lu et al. 2004). These recognition responses occurred well before PrP molecular pathology, and were specific for particular geographic isolates (Lu et al. 2004). Study of additional host membrane-associated molecules will facilitate the diagnosis of individual TSE agent strains to reveal new and fundamental agent-host interactions. These include proteins with neuroprotective functions as well as those that accelerate disease progression. We have now propagated a variety of TSE agents, including primary isolates of sporadic CJD (sCJD) (Arjona et al. 2004; Manuelidis et al. 1978), New Guinea kuru (Manuelidis et al. 2009a), and BSE-linked human variant CJD (vCJD) (Manuelidis et al. 2009b) in normal mice as well as in monotypic GT1 cells. We sought to find if the PrP “shadow protein” designated Shadoo (Sho), recently identified by comparative gene analysis (Premzl et al. 2003), is useful for distinguishing host responses to different agent strains. We also determined if Sho, as host PrP, is required for TSE infections.

On a structural basis both Sho and PrP proteins are abundant in the CNS, and Sho shares many characteristics with PrP. For example, Sho has a series of N-terminal charged tetrarepeats that is similar to the N-terminal octarepeats of PrP. In addition, the hydrophobic regions of Sho and PrP are conserved (Watts et al. 2007). However, Sho was markedly reduced in cerebellar granule cells, and it was suggested that Sho might have neuroprotective activity because Sho transgenes were able to counteract toxic cerebellar granule cell effects in transgenic (Tg) mice with PrP internal deletions (Watts et al. 2007). On the other hand, Sho exhibited no clear neuroprotective role in infected mice. Although infection with the RML scrapie agent induced widespread spongiform lesions and a corresponding reduction of Sho (Watts et al. 2007), Sho did not reduce the incubation time to neurological disease (Gossner et al. 2009; Lloyd et al. 2009).

We sought to find if Sho protein levels would vary in mice infected with six TSE isolates representing a broad spectrum of distinct agents. These agents had been established and serially passaged in mice with wild type (wt) murine PrP. To find if the mouse genotype has a significant influence, we studied standard CD-1 and NZW mice expressing diploid levels of wt PrP. We also used Tga20 mice expressing wt murine PrP at 8x normal levels (Fischer et al. 1996) to find if there was a clear effect of Sho on PrP or vice versa. We report here that Sho levels can distinguish different TSE agent groups in Western blots of brain homogenates. Additionally, we used monotypic GT1 cells that express wt murine PrP to determine if Sho is required for infection. All the TSE agents with the exception of sCJD maintain obvious and robust growth in GT1 neural cells. Despite high infectious titers, as well as the production of abundant PrP-res and amyloid accumulation, all infected cells grew as well as controls. Yet Sho could not be detected in these GT1 cells. Thus Sho is not required for TSE infection, nor is it required to protect GT1 cells from progressive degenerative changes assumed to be induced by misfolded PrP.

Materials and methods

TSE agents from two typical sCJD cases (SY-CJD and LU-CJD), a UK BSE agent vCJD case, a Japanese CJD isolate FU-CJD, and a kuru New Guinea isolate were serially passaged as previously described (Manuelidis et al. 1978; Manuelidis 1998; Manuelidis et al. 2009a, b) and in accord with national and institutional IACUC approval. CD-1 and NZW mice were purchased from Jackson labs and the Tga20 mice with $\times 8$ wt PrP were a gift of C. Weissmann (Fischer et al. 1996) and bred at Yale. The 22L scrapie agent (22L-Sc) is a murine adapted strain of UK sheep scrapie titrated in animals by dilution as previously described (Nishida et al. 2005). Brains from clinical animals with agent-specific neuropathology were homogenized in 9 volumes of PBS for Western blot. Protein concentrations of homogenates were determined by BCA protein assay (Thermo Fisher Scientific, Waltham, MA), and 12 μ g of protein were loaded for a 1x concentration on gels as indicated. For PrP-res detection, proteinase K (PK) digestion was done with 25 μ g/ml for 30 min at 37°C and Western blots were made with standard methods as previously described (Manuelidis and Fritch 1996). A commercial goat anti-PrP antibody (M20, Santa Cruz Biotechnology, Santa Cruz, CA) was used for the detection of PrP and PrP-res. The rabbit anti-Shadoo (Sho) antibody (06rSH-1) was kindly provided by D. Westaway (Watts et al. 2007).

To normalize of each reference lane, we re-probed the blot with mouse anti-Tubulin (clone 6-11b-1, Sigma-Aldrich St. Louis, MO) after removal of anti-Sho antibodies by Re-Blot Plus Mild Stripping Solution (Millipore, Billerica, MA). Signals were developed with chemiluminescent substrate (ChemiGlow, Alpha Innotech, San Leandro, CA) and the % PrP-res (of total PrP) and %Sho (of uninfected brain) were determined in the linear signal range with Fluorochem software. ANOVA and Fisher's least significant difference test were used for analysis.

Results

Sho expression in CD-1 mouse brains infected with six TSE isolates

We first analyzed western blots of PrP, PrP-res and Sho in CD-1 mouse brains after infection by a variety of TSE agents. The origin and major features of each of the six isolates analyzed here in CD-1 mice are summarized in Table 1. Several important agent-specific features deserve further mention. First, incubation times to terminal disease at the maximal intracerebral dose differed by as much as 300 days. The Japanese FU-CJD agent, like another independent Japanese isolate (Manuelidis et al 2009a,b), had a relatively short incubation times of ~140 days to terminal disease. In contrast, CJD agents, e.g., SY-CJD and LU-CJD, with only long ~360-day incubations, were isolated from patients in the USA and Europe. Second, this incubation time difference was not a consequence of the 102L PrP mutation because GSS patients in the Western hemisphere yielded the same slow sCJD agent (Manuelidis et al 2009a,b). Third, despite the marked differences in the incubation times, severity of brain lesions, and lymphoid tissue pathology of sCJD and Asiatic agents, both displayed indistinguishable PrP-res band patterns. Fourth, other agents, such as kuru, with obvious neuropathological and virulence differences (Manuelidis 2010), also had the same PrP-res band pattern, again indicating that PrP folding did not encode these agent properties. Fifth, even though the vCJD agent had replicated in a human brain for over 5 years, it produced identical neuropathology as the agent isolated from UK cows. Remarkably, the vCJD isolate was easier to grow in wt mice with non-homologous PrP than in Tg mice expressing only human PrP (Manuelidis et al 2009b), another finding not in accord with the prion concept.

To ensure that there was no bias toward early or late pathology that might skew results, we sampled half mouse brains from murine passages 2–3 that were verified for agent-specific pathology. These specimens (three mice per agent) included the full range of incubation times for each of these agents. Figure 1 shows a representative Western blot of CD-1 mouse brain homogenates analyzed for tubulin (tubl), PrP, PrP-res (+PK lanes) and Sho, with the days incubation to disease for each brain sample inoculated. Tubulin (~50 kd, Tubl) was used for normalization. There was a wide range of PrP-res (% of total undigested PrP) induced by these different TSE agents, from 7.9% to 56.1%, as noted below each PK+lane. Sho differences induced by each of these agents are also obvious on inspection, with corresponding numbers indicated (%Sho). Uninfected controls (Mock lane) showed no PrP-res, and had the highest Sho levels (taken as the 100% standard). It is striking that the two sCJD isolates provoked similar low levels of PrP-res, but had very different reductions in Sho (99.8% versus 40.9% for SY-CJD and LU-CJD, respectively).

Table 2 summarizes the %PrP-res ($\pm$ SEM) and the %Sho determined on the complete range of infected samples. The two sCJD isolates (SY-CJD and LU-CJD) gave low PrP-res levels of 9–12%, consistent with previous estimates (Manuelidis et al. 2009a; Manuelidis and Lu 2000). BSE-linked vCJD gave an intermediate level of 23.6%, and the unique geographic human isolate kuru yielded 58% the highest PrP-res value. Asiatic CJD (FU-CJD) and the sheep 22L-Sc agent from the UK gave similar high PrP-res values of 50–52%. No uninfected brains displayed PrP-res. The variable PrP-res levels did not have a simple predictive value for the Sho reductions found. All TSE infected CD-1 brains displayed significant reductions in Sho protein compared to uninfected controls ($P < 0.0005$), with the notable exception of the sCJD isolate SY-CJD; SY-CJD brains had Sho levels that were insignificantly reduced from normal (96%, $P > 0.05$) in the full range of brain samples. Although LU-CJD brains had similarly low levels of PrP-res, they displayed much lower levels of Sho than SY-CJD brains (57% versus 96%, $P < 0.0005$). This indicated subtle sCJD agent differences were brought out by analysis of Sho. These sCJD differences are probably based on each agent's history of species passages, and the minor mutations acquired during their adaptations; only SY-CJD was first serially passaged in Guinea Pigs (Manuelidis et al. 1976). Sho levels in 22L-Sc, FU-CJD

and kuru infected brains displayed more marked reductions, to less than 40% of controls. Overall these data demonstrate no simple inverse relationship between PrP-res and Sho. Instead, they indicate complex cell processing mechanisms rather than direct effects of PrP-res on Sho.

Because there was a range of Sho brain values for each agent group, consistent with sampling early as well as late clinical animals, we calculated the angle ($\emptyset$) between Sho and PrP-res levels of each individual animal ($n=3$ per group). The value of Sho (% of uninfected levels) is connected to %PrP-res (of total undigested PrP) for each mouse inoculated with the six different TSE isolates as plotted in Fig. 2. These graphed angles reveal obvious differences generated by the various agents, as well as the remarkable statistical power of this analysis. The mean positive or negative angle ($\emptyset$ or $\emptyset'$ respectively, $\pm$ SEM) is shown at the top of each agent group. TSE agents clearly separated into at least two groups, with $\emptyset$ attaining an opposite negative value ($\emptyset'$) for three of the agents. Based on these determinations, distinct TSE agent groups became prominent. The 22L-Sc and Asiatic FU-CJD agents were statistically indistinguishable from each other ($P>0.05$). The kuru agent showed a minor but significant difference from those two, with a tight value of $+75^{\circ}\pm 0.6^{\circ}$ ($P<0.05$). A more dramatic difference was found between the sporadic CJD isolates SY-CJD and LU-CJD, as well as with the BSE-linked vCJD isolate. These samples showed negative angles ($\emptyset'$) of -78° to -84° . This $\emptyset$ analysis confirms yet another obvious distinction of kuru from sCJD by $\emptyset$, a distinction previously determined by major differences in incubation time, neuropathology and splenic involvement (Manuelidis et al. 2009a). Although the two sCJD isolates SY-CJD and LU-CJD could again be differentiated statistically by $\emptyset$ ($P<0.05$), this difference is probably not of great biological significance as compared to the major phenotypes of incubation time and tissue pathology.

Comparison of Sho levels in three genotypes of uninfected mice

To further assess host differences that might affect Sho protein we evaluated two representative normal genotypes of mice (CD-1 and NZW) and compared them to Tga20 mice with 8x levels of wt murine PrP. This study demonstrated that Sho levels were independent of PrP production. Sho levels were not significantly different in Tga20 mice as compared to mice with normal PrP levels ($P>0.05$, Fig. 3). CD-1 mice appeared to have slightly lower Sho levels than the other two genotypes but this difference was not significant ($P>0.05$). Thus any Sho changes found after infection must relate to the agent induced disease, rather than to direct effects or relative levels of host PrP.

Agent-specific effects on Sho in wt and Tga20 mouse brains

In contrast to uninfected mice, Sho protein levels were quite different in infected CD-1 and Tga20 mouse brains. This change was dependent on the specific agent inoculated. We compared FU-CJD and kuru because both of these TSE agents elicit high levels of PrP-res as well as widespread neuropathologic changes. Sho levels in FU-CJD infected Tga20 brains were much higher than in CD-1 brains (Fig. 4a). Indeed, Sho was maintained at normal levels in individual Tga20 mice infected with FU-CJD (Fig 4b). The mean value in Tga20 mice ($n=4$) was $102\pm 10.8\%$ of that found in normal uninfected mice ($100\pm 4.7\%$). The pattern was very different in FU-CJD infected CD-1 brains, as shown in the blot (Fig. 4a) and in the graph (Fig. 4b). The reduction of Sho was obvious and significant in these CD-1 brains ($P<0.001$). The kuru agent did not induce a similar Sho discrepancy in the two mouse genotypes: Tga20 mice revealed the same reduction in Sho as CD-1 mice infected with kuru (Fig. 4a, kuru lanes). Sho was $26\pm 2.6\%$ in CD-1 mice and $27\pm 4.9\%$ in Tga20 mice, an insignificant difference (Fig. 4b, $P>0.05$).

To find if these differences might be related quantitatively to abnormal PrP-res changes we analyzed both Sho and PrP-res in the every brain sample. In kuru infected brains (Fig. 4c), both mouse genotypes showed the same high range of the PrP-res for CD-1 (53.4–60.1%) and Tga20 (45.7–66.7%, $P>0.05$) and were not informative in this context. However, FU-CJD infected Tga20 mice accumulated much less PrP-res as compared with CD-1 mice (16% versus 56%), and these values were non-overlapping and clearly significant. PrP-res in individual CD-1 mice ranged from 42.3% to 64.1%, while in Tga20 brains it ranged from 9.0% to 20.8%. Thus the reduction of Sho was inversely proportional to PrP-res in FU-CJD. While the reduction of Sho protein in TSE-infected brains could be caused by the accumulation of pathologic PrP, albeit not by normal or PK sensitive PrP levels, other host-membrane responses are probably involved in the Sho differences noted between FU-CJD and kuru agents in Tga20 mice.

Sho is not required for infection or for neuroprotection of GT1 cells

The Sho antibody was used to evaluate uninfected GT1 cells as well as chronically infected GT1 cells with kuru, BSE-vCJD, and FU-CJD agents. The PrP and PrP-res band patterns in infected cells were very different than the patterns seen in brain. For example, the most glycosylated PrP band was 37–38 kd in GT1 cells, and in brain was 34–35 kd. Additionally, a variety of new PrP-res bands were also found in GT1 cells (Manuelidis et al 2009a, b). Continuous production of all these agents were verified using the surrogate pathologic marker PrP-res. These infected cells, unlike mock controls, also show a high burden of PrP amyloid in the cytoplasm by immunocytochemistry (Arjona et al. 2004; Manuelidis et al. 2009b) as well as by electron microscopy (Manuelidis et al. 2007). Since these cells produced abundant abnormal PrP, but remained healthy in appearance and grew comparably to uninfected cells, we thought they might have high levels of Sho, in accord with Sho's proposed neuroprotective effect (Watts et al. 2007). In fact, only a non-specific band of ~15 kd was seen on blots of both uninfected and infected GT1 cells. This band was of the same intensity in both normal and infected cells (data not shown) and was comparable to a non-specific band previously depicted using the same antibody on other neural cells (Watts et al. 2007). High gel loads failed to show any detectable Sho in GT1 cells. Thus in the GT1 neural cell model Sho was not required for the healthy growth of cells carrying a high intracellular burden of abnormal PrP.

Discussion

Previous studies reported that Sho protein levels are reduced in Chandler (RML) scrapie infected mouse brains during clinical disease (Watts et al. 2007). Sho was not reduced at the mRNA level (Lloyd et al. 2009). This indicates functional nuclear transcription with either nonfunctional Sho synthesis at the ER level, or the post-synthetic destruction of Sho in cellular compartments such as lysosomes. We here demonstrated that Sho responses are agent-specific. Only some TSE agents induced a reduction in Sho protein. Incubation time was not a major factor in these differences. Both the kuru agent with a prolonged incubation time (440 days), as well as FU-CJD agent with a much shorter incubation time (120 days), displayed a clear reduction of Sho protein. Moreover, vCJD infected mice with an intermediate incubation time of 185 days maintained significantly higher levels of Sho.

The vastly different TSE agents evaluated in standard CD-1 mice could be classified into at least two groups by determining the angle between the %PrP-res and the %Sho protein. The first group, 22L-Sc and FU-CJD, had similar high PrP-res accumulations of 50–52% with reduced Sho protein and positive $\emptyset$ angles. The kuru agent had only insignificantly more PrP-res (58%), yet it induced significantly lower and non-overlapping Sho levels, again with a positive $\emptyset$. In contrast, the BSE-linked vCJD agent induced substantial amounts of PrP (23%), yet had a very different negative $\emptyset$. Additionally, even though the PrP-res levels differed 2 fold between vCJD and LU-CJD, their Sho levels and negative $\emptyset$ angles were the same (-78°

and -79° , respectively). These results show a less than persuasive correspondence between Sho and PrP-res. Reductions in Sho are not a direct and/or simple consequence of PrP-res accumulation. Instead, Sho protein levels are specific for the inoculated TSE agent, and not an intrinsic and invariant host process.

Part of these differences may rest on the targeting of certain brain regions by specific agents. Signals for both Sho protein and its cognate mRNA are widespread, but most prominent in pyramidal neurons of the hippocampus and Purkinje cells of the cerebellum (Watts et al. 2007). Thus the sCJD isolates SY-CJD and LU-CJD, that induce very limited medial thalamic vacuolar and PrP-res changes, should maintain relatively high levels of Sho protein. Despite their same limited thalamic pathology, however, Sho reductions in these two sCJD isolates were significantly different, making regional neuropathology a limited explanation for Sho effects. Conversely, in vCJD-infected mice, deposits of pathological PrP, and spongiform change are observed in the hypothalamus, thalamus and molecular layer of the cerebellum (Manuelidis et al. 2009b). Thus the few lesions in the cerebral cortex and hippocampus during vCJD infection might account for the relative preservation of Sho in this model. Yet kuru with a similar level of cortical disease (Manuelidis et al. 2009a) had much lower Sho levels than did vCJD.

Although the molecular and cell-specific pathways responsible for Sho changes are not yet clear, on a diagnostic level Sho can be useful for discriminating murine adapted agents, as demonstrated here. Sho may also be of value for identifying old as well as newly emerging TSE agents in human material. Because TSE agents maintain their unique identity in different species (Manuelidis et al. 2009a), kuru-like agents in human and primate brains may similarly display relatively low Sho levels compared to higher Sho levels in vCJD. One might also be able to distinguish sCJD agents with minor differences that define particular geographic isolates. Indeed, Sho determinations could also solidify the suggested links between sCJD and generally unrecognized but common TSE agents in cows; these are different from the new UK epidemic BSE agent (Comoy et al. 2008).

Sho brain levels did not change in response to the overproduction of normal PrP in uninfected mice. Sho levels were the same in normal PrP diploid CD-1 and NZW mice, and in Tga20 mice with $\times 8$ diploid PrP levels. Similarly, there was no significant change of Sho expression in PrP knockout mouse brain (Watts et al. 2007), further demonstrating this shadow protein neither follows nor is dependent on host PrP. Sho also bears no clear relationship with other common neurodegenerative amyloids, and was not reduced during the clinical phase of disease in a mouse amyloid model of Alzheimer's disease (Watts et al. 2007). This is additional evidence that the β -pleated amyloid structure itself is unable to directly or indirectly reduce Sho protein.

The potential function of Sho in disease progression is not narrowly confined to an accumulation of pathological PrP. Additional agent-specific capacities were brought out during infection of normal CD-1 and high PrP Tga20 mice. Because only the FU-CJD infected mice accumulated a very low percent of PrP-res in Tga20 compared with CD-1 mice, some appreciable percent of PrP-res might be necessary, at least indirectly, to induce a reduction in Sho in this agent-host combination. It is also important to recognize that TSE infection induces a variety of innate immune responses well before functional or pathological disease is apparent (Baker et al. 2004; Lu et al. 2004; Manuelidis and Fritch 1996; Manuelidis et al. 1997). Moreover, these agents disrupt cell membranes that carry many different proteins and functions besides PrP and Sho. Indeed, host PrP itself may have a neuroprotective function that helps to preserve Sho in the FU-CJD Tga20 model with artificially high PrP. The premise that high PrP may be neuroprotective is supported by the observation that the vacuolization in Tga20 mice with FU-CJD is often very subtle compared to the extensive vacuolization induced by FU-CJD in mice with diploid PrP levels (LM, unpublished data). Again, this protection appears to be

agent-specific because in kuru the vacuolization is the same in both of these two mouse genotypes. In standard animals PrP amyloid aggregates with other cell proteins, such as ApoJ (Manuelidis et al. 1997), and such aggregates may trap infectious particles as part of an attempted, but ultimately failing, neuroprotective mechanism (Manuelidis 1994).

Relevant data concerning a proposed role for Sho in neuroprotection, as well as the presumed toxicity of pathologic PrP, were obtained in our cell culture studies. A previous report showed that cells in culture could be engineered to produce Sho and confirm its identification (Watts et al. 2007). The susceptibility of these engineered cells to infection by TSE agents was not reported, and we are unaware of any experiments showing the necessity of Sho for infection. We found that Sho is not required for infection by a variety of TSE agents. GT1 cells, derived from SV40 immortalized mouse hypothalamic cells, have displayed continuous production of eight independent CJD and scrapie isolates (Arjona et al. 2004; Manuelidis et al. 2009a, b). Yet we found that both uninfected and infected GT1 cells do not produce detectable levels of Sho. This means additionally that Sho is not required for protection of these neural cells from “PrP-res toxicity”. Infected GT1 cells remained as healthy appearing as controls, and PrP-res and PrP amyloid in infected GT1 cells can be as high as 40%. With agents such as FU-CJD and vCJD it was also clear that PrP-res was carried by almost every cell as determined by in-situ labeling (Arjona et al. 2004; Manuelidis et al. 2009b). Thus the presumed inherent toxicity of PrP amyloid, demonstrated ultrastructurally (Manuelidis et al. 2007), was not evident in these living cells. These observations contrast with the toxicity observed from added PrP peptide fragments (Forloni et al. 1993) and artificial PrP constructs (Massignan et al. 2009) in other studies. The interaction and specificity of different TSE agents with the host animal, and in monotypic living cells that reproduce infectious particles, will hasten fundamental insights into the progression of these diseases. Cell cultures provide accessible models for targeting specific genes and critical processing pathways in these neuro-degenerative diseases. Monotypic cultures can also be used to illuminate new strategies to prevent and arrest the infectious cycle.

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References

- Akowitz A, Sklaviadis T, Manuelidis L. Endogenous viral complexes with long RNA cosediment with the agent of Creutzfeldt-Jakob disease. *Nucleic Acids Res* 1994;22:1101–1107. [PubMed: 8152913]
- Arjona A, Simarro L, Islinger F, Nishida N, Manuelidis L. Two Creutzfeldt-Jakob disease agents reproduce prion protein-independent identities in cell cultures. *Proc Natl Acad Sci U S A* 2004;101:8768–8773. [PubMed: 15161970]
- Baker CA, Manuelidis L. Unique inflammatory RNA profiles of microglia in Creutzfeldt-Jakob disease. *Proc Natl Acad Sci U S A* 2003;100:675–679. [PubMed: 12525699]
- Baker CA, Martin D, Manuelidis L. Microglia from Creutzfeldt-Jakob disease-infected brains are infectious and show specific mRNA activation profiles. *J Virol* 2002;76:10905–10913. [PubMed: 12368333]
- Baker CA, Lu ZY, Manuelidis L. Early induction of interferon-responsive mRNAs in Creutzfeldt-Jakob disease. *J Neurovirol* 2004;10:29–40. [PubMed: 14982726]
- Barron RM, Campbell SL, King D, Bellon A, Chapman KE, Williamson RA, Manson JC. High titers of transmissible spongiform encephalopathy infectivity associated with extremely low levels of PrPSc in vivo. *J Biol Chem* 2007;282:35878–35886. [PubMed: 17923484]
- Comoy EE, Casalone C, Lescoutra-Etcheagaray N, Zanusso G, Freire S, Marce D, Auvre F, Ruchoux MM, Ferrari S, Monaco S, Sales N, Caramelli M, Leboulch P, Brown P, Lasmezas CI, Deslys JP. Atypical

- BSE (BASE) transmitted from asymptomatic aging cattle to a primate. *PLoS ONE* 2008;3:e3017. [PubMed: 18714385]
- Fischer M, Rulicke T, Raeber A, Sailer A, Moser M, Oesch B, Brandner S, Aguzzi A, Weissmann C. Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. *EMBO J* 1996;15:1255–1264. [PubMed: 8635458]
- Forloni G, Angeretti N, Chiesa R, Monzani E, Salmons M, Bugiani O, Tagliavini F. Neurotoxicity of a prion protein fragment. *Nature* 1993;362:543–546. [PubMed: 8464494]
- Gossner AG, Bennet N, Hunter N, Hopkins J. Differential expression of Prnp and Sprn in scrapie infected sheep also reveals Prnp genotype specific differences. *Biochem Biophys Res Commun* 2009;378:862–866. [PubMed: 19070601]
- Hunter N, Cairns D. Scrapie-free Merino and Poll Dorset sheep from Australia and New Zealand have normal frequencies of scrapie-susceptible PrP genotypes. *J Gen Virol* 1998;79(Pt 8):2079–2082. [PubMed: 9714260]
- Lloyd SE, Grizenkova J, Pota H, Collinge J. Shadoo (Sprn) and prion disease incubation time in mice. *Mamm Genome* 2009;20:367–374. [PubMed: 19513788]
- Lu ZY, Baker CA, Manuelidis L. New molecular markers of early and progressive CJD brain infection. *J Cell Biochem* 2004;93:644–652. [PubMed: 15660413]
- Manuelidis L. The dimensions of Creutzfeldt-Jakob disease. *Transfusion* 1994;34:915–928. [PubMed: 7940667]
- Manuelidis L. Vaccination with an attenuated Creutzfeldt-Jakob disease strain prevents expression of a virulent agent. *Proc Natl Acad Sci U S A* 1998;95:2520–2525. [PubMed: 9482918]
- Manuelidis L. A 25 nm virion is the likely cause of transmissible spongiform encephalopathies. *J Cell Biochem* 2006;100:897–915. [PubMed: 17044041]
- Manuelidis L. Transmissible encephalopathy agents: virulence, geography and clockwork. *Virulence* 2010;1:1–4.
- Manuelidis L, Fritch W. Infectivity and host responses in Creutzfeldt-Jakob disease. *Virology* 1996;216:46–59. [PubMed: 8615006]
- Manuelidis L, Lu ZY. Attenuated Creutzfeldt-Jakob disease agents can hide more virulent infections. *Neurosci Lett* 2000;293:163–166. [PubMed: 11036186]
- Manuelidis EE, Kim J, Angelo JN, Manuelidis L. Serial propagation of Creutzfeldt-Jakob disease in guinea pigs. *Proc Natl Acad Sci U S A* 1976;73:223–227. [PubMed: 1108016]
- Manuelidis EE, Gorgacz EJ, Manuelidis L. Transmission of Creutzfeldt-Jakob disease with scrapie-like syndromes to mice. *Nature* 1978;271:778–779. [PubMed: 342977]
- Manuelidis L, Sklaviadis T, Akowitz A, Fritch W. Viral particles are required for infection in neurodegenerative Creutzfeldt-Jakob disease. *Proc Natl Acad Sci U S A* 1995;92:5124–5128. [PubMed: 7761460]
- Manuelidis L, Fritch W, Xi YG. Evolution of a strain of CJD that induces BSE-like plaques. *Science* 1997;277:94–98. [PubMed: 9204907]
- Manuelidis L, Yu ZX, Banquero N, Mullins B. Cells infected with scrapie and Creutzfeldt-Jakob disease agents produce intracellular 25-nm virus-like particles. *Proc Natl Acad Sci U S A* 2007;104:1965–1970. [PubMed: 17267596]
- Manuelidis L, Chakrabarty T, Miyazawa K, Nduom NA, Emmerling K. The kuru infectious agent is a unique geographic isolate distinct from Creutzfeldt-Jakob disease and scrapie agents. *Proc Natl Acad Sci U S A* 2009a;106:13529–13534. [PubMed: 19633190]
- Manuelidis L, Liu Y, Mullins B. Strain-specific viral properties of variant Creutzfeldt-Jakob disease (vCJD) are encoded by the agent and not by host prion protein. *J Cell Biochem* 2009b;106:220–231. [PubMed: 19097123]
- Massignan T, Stewart RS, Biasini E, Solomon IH, Bonetto V, Chiesa R, Harris DA. A novel, drug-based, cellular assay for the activity of neurotoxic mutants of the prion protein. *J Biol Chem*. 2009 doi: 10.1074/jbc.M109.064949.
- Nishida N, Katamine S, Manuelidis L. Reciprocal interference between specific CJD and scrapie agents in neural cell cultures. *Science* 2005;310:493–496. [PubMed: 16239476]

- Premzl M, Sangiorgio L, Strumbo B, Marshall Graves JA, Simonic T, Gready JE. Shadoo, a new protein highly conserved from fish to mammals and with similarity to prion protein. *Gene* 2003;314:89–102. [PubMed: 14527721]
- Prusiner SB. Prions. *Proc Natl Acad Sci U S A* 1998;95:13363–13383. [PubMed: 9811807]
- Sakaguchi S, Katamine S, Yamanouchi K, Kishikawa M, Moriuchi R, Yasukawa N, Doi T, Miyamoto T. Kinetics of infectivity are dissociated from PrP accumulation in salivary glands of Creutzfeldt-Jakob disease agent-inoculated mice. *J Gen Virol* 1993;74(Pt 10):2117–2123. [PubMed: 8409936]
- Silveira JR, Raymond GJ, Hughson AG, Race RE, Sim VL, Hayes SF, Caughey B. The most infectious prion protein particles. *Nature* 2005;437:257–261. [PubMed: 16148934]
- Sklaviadis T, Dreyer R, Manuelidis L. Analysis of Creutzfeldt-Jakob disease infectious fractions by gel permeation chromatography and sedimentation field flow fractionation. *Virus Res* 1992;26:241–254. [PubMed: 1492497]
- Sklaviadis T, Akowitz A, Manuelidis EE, Manuelidis L. Nucleic acid binding proteins in highly purified Creutzfeldt-Jakob disease preparations. *Proc Natl Acad Sci U S A* 1993;90:5713–5717. [PubMed: 8516321]
- Sun R, Liu Y, Zhang H, Manuelidis L. Quantitative recovery of scrapie agent with minimal protein from highly infectious cultures. *Viral Immunol* 2008;21:293–302. [PubMed: 18788938]
- Tateishi J, Ohta M, Koga M, Sato Y, Kuroiwa Y. Transmission of chronic spongiform encephalopathy with kuru plaques from humans to small rodents. *Ann Neurol* 1979;5:581–584. [PubMed: 382976]
- Watts JC, Drisaldi B, Ng V, Yang J, Strome B, Horne P, Sy MS, Yoong L, Young R, Mastrangelo P, Bergeron C, Fraser PE, Carlson GA, Mount HT, Schmitt-Ulms G, Westaway D. The CNS glycoprotein Shadoo has PrP(C)-like protective properties and displays reduced levels in prion infections. *EMBO J* 2007;26:4038–4050. [PubMed: 17703189]
- Xi YG, Ingrosso L, Ladogana A, Masullo C, Pocchiari M. Amphotericin B treatment dissociates in vivo replication of the scrapie agent from PrP accumulation. *Nature* 1992;356:598–601. [PubMed: 1348570]

Fig. 1.

Western blots of CD-1 mouse brains infected by different agents. Incubation time for each mouse (days) is indicated at top. Tubulin signals (TubI, ~50 kd) as shown were used for normalization. The PrP and PrP-res (PK+lanes) band patterns from the same blot are shown as the middle panel. Uninfected brain (*Mock lane*) displays no PrP-res. Sho protein in the same blot is shown in the bottom panel, and the %PrP-res and Sho levels for these samples indicated numerically. Sho levels are reduced in TSE infected brains by amounts that differ according to the TSE strain inoculated

Fig. 2.

Percent of Shw and PrP-res in wt CD-1 mouse brains infected with different agents. Light grey bars are mean %Shw and dark grey bars are mean %PrP-res. Circles, triangles and diamonds represent individual mice in each group. The value $\emptyset$ or $\emptyset'$ at the top of each pair of agent bars is the mean angle between the Shw and PrP-res plotted at a constant x axis distance. Note the dramatic reversed angle found with the 22L-Sc, FU-CJD and kuru as compared to the vCJD, SY-CJD and LU-CJD isolates. The kuru agent can also be distinguished from 22L-Sc and FU-CJD (* P <0.05). SY-CJD is also significantly different from other negative angle samples, vCJD and LU-CJD (** P <0.05)

Fig. 3.

Comparative analysis of Sho protein expression in uninfected CD-1, NZW and Tga20 mouse brains. Panel **a** shows a representative Western blot of uninfected brain homogenates analyzed for tubulin (tubI), PrP, and Sho in each mouse genotype. Tubulin levels used for normalization are shown at the top. PrP levels of uninfected brains from three mouse genotypes are shown in the middle panel and notably Tga20 mice expresses the expected high $\times 8$ normal levels of PrP. Sho protein in these brains are shown in the bottom panel. The graph (at **b**) shows the amount of Sho in brains from three mouse genotypes. There is no significant difference ($P > 0.05$) in Sho expression in these three mouse strains, including the Tga20 mice with abnormally high wt PrP

Fig. 4.

Comparison of Sho levels in CD-1 and Tga20 mouse brains infected with FU-CJD and kuru. Panel **a** shows a blot detected with tubulin antibody (Tub1, ~55 kd) for normalization and with Sho antibody (at 20kd) in mice infected with two human agents that elicit robust pathology in mice. Each Tga20 mouse infected with FU-CJD displays much higher Sho levels than the counterpart CD-1 mice (FU-CJD lanes). In contrast, the kuru agent causes equivalent and severe Sho reductions in both Tga20 and CD-1 mice (kuru lanes). The graph **b** shows %Sho of normal ($n=4$, $\pm$ SEM) and Sho is only significantly different in the Tga20-FU infected brains ($P<0.001$). Panel **c** shows another blot probed for tubulin, PrP and Sho. The PrP and PrP-res band patterns (PK+lanes with calculated % PrP-res shown beneath these lanes) after FU-CJD

or kuru infection. Representative CD-1 and Tga20 brains are compared. Individual TSE strains show the same PrP-res band patterns in both of CD-1 and Tga20 mice. Sho levels of FU-CJD infected Tga20 mouse brains are clearly different between these two mouse genotypes in this representative blot even though Tga20 brains showed substantial amounts of accumulated PrP-res in many different brain samples (data not shown). In contrast, with kuru infection, both CD-1 and Tga20 mice show the same markedly decreased levels of Sho

Table 1
TSE agents in CD-1 (Swiss) mice investigated. Key features of different TSE agents propagated in mice with wt PrP

Isolate-origin	Diagnosis	Agent name	Incubation (days)	Mouse brain pathology	Mouse brain PrP-res
USA-human	sCJD	SY-CJD	334–405	Restricted	Type 1
Italy-human	sCJD	LU-CJD	377–435	Restricted	Type 1
Japan-human	GSS (102L)	FU-CJD	134–158	Widespread	Type 1
UK-human	vCJD	vCJD	171–270	BSE pattern+plaque	Type 2
New Guinea-human	Kuru	kCJD	403–494	Kuru specific	Type 1
UK-sheep	Scrapie	22L-Sc	154–163	Widespread	Type 1

The geographic origin and diagnosis are listed with the agent name. A very prolonged incubation time as well as the very restricted medial thalamic lesion was diagnostic for the sporadic sCJD agents SY-CJD and LU-CJD. In contrast, Asiatic isolates (Manuelidis and Lu 2000) exemplified by FU-CJD, were markedly different, with a rapid incubation time and widespread spongiform lesion, yet had the same Type 1 PrP-res pattern as sCJD, i.e., the PrP-res folding pattern in FU-CJD did not encipher its markedly different characteristic (Manuelidis et al. 2009a; Manuelidis and Lu 2000). The human variant CJD (vCJD) agent is derived from the epidemic BSE agent in the UK and produced type 2 PrP-res with 19kd diagnostic marker and a unique BSE-specific lesion pattern (Manuelidis et al. 2009b). The kuru agent (kCJD) revealed properties that were also clearly different from those induced by sCJD, vCJD, FU-CJD as well as by a representative scrapie samples (22L-Sc), by the criteria of incubation time, brain pathology and lymphoreticular involvement (Manuelidis et al. 2009a). All mice were inoculated with 1% brain homogenates as detailed in our previous reports (Manuelidis et al. 1978; Manuelidis et al. 1976)

Table 2

Sho and PrP-res percents in CD-1 mice infected with different TSE agents

Country-origin	Agent name	Incubation (days)	%PrP-res $\pm$ SEM	%Sho $\pm$ SEM
U.S.A.-human	SY-CJD	334–405	9.4% $\pm$ 0.7	96% $\pm$ 2.0
Italy-human	LU-CJD	377–435	12% $\pm$ 0.8	57% $\pm$ 8.6
U.K.-human	vCJD	171–270	23% $\pm$ 0.8	63% $\pm$ 6.8
Japan-human	FU-CJD	134–158	51% $\pm$ 4.3	36% $\pm$ 3.1
New Guinea-human	kCJD	403–494	58% $\pm$ 2.1	27% $\pm$ 2.3
U.K.-sheep	22L-Sc	154–163	50% $\pm$ 4.2	34% $\pm$ 3.4
Uninfected	–	Young to old	0%	100% $\pm$ 4.7

Agent-strains as in Table 1 with the composite %PrP-res and % Sho determined in this study (mean $\pm$ SEM); $n=3$ mouse brains including the full range of incubation times from early clinical to late stage clinical disease