See related article on page 1006

COMMENTARY

Synthetic Prions Provide Clues for Understanding Prion Diseases

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Prion diseases are fatal neurodegenerative disorders that can affect both animals and humans. The prion protein (PrP), which is at the root of these diseases, is found in two different conformational versions: the normal isoform, called PrP^C (for cellular PrP), and the infectious isoform, called PrP^{Sc} (for scrapie PrP).¹ One of the chief characteristics of these diseases is that they can be transmitted from one individual to another, a feature that depends on the ability of PrP^{Sc} to template conversion of PrP^C into additional molecules of PrP^{Sc}. A major effort in the prion field has been to reproduce this molecular transformation *in vitro* by generating synthetic prions in the test tube with the use of recombinant forms of PrP and demonstrating that these molecules are infectious in animal bioassays. In this issue of the *American Journal of Pathology*, Makarava et al² make use of such a system to investigate the formation of synthetic prions and their ability to evolve into pathogenic entities during propagation *in vivo*.

History of Synthetic Prions

Several different systems have been used to generate infectious prions in a test tube. One, dubbed protein misfolding cyclic amplification (PMCA), is a PCR-like system in which a small amount of infectious PrP^{Sc} is used to seed conversion of PrP^C substrate in a sample of brain homogenate, with repeated cycles of sonication applied to facilitate the reaction.³ Another method, the one used in the present paper, involves partially denaturing purified, recombinant PrP^C with a chaotropic agent such as urea or guanidine. Under appropriate conditions of pH, the protein refolds into β -sheet-rich amyloid fibrils that share some biochemical characteristics of PrP^{Sc} but are structurally distinct in terms of the length of the protease-resistant core of the fibril.^{4,5} The first report of inducing prion disease in animals with the use of fibrils formed *in vitro* from recombinant PrP

appeared in 2004.⁶ Interestingly, it was shown that different disease phenotypes (defined by incubation times and patterns of neuropathology) were produced by recombinant mouse PrP fibrils that were prepared under different conditions and that displayed distinct morphologic and biophysical properties.⁷ This observation was interpreted to mean that different strains of synthetic prions had been generated. The initial transmission studies of these synthetic prions used as hosts transgenic mice that over-expressed PrP^C, with the idea that higher levels of substrate would facilitate the conversion process. However, a proportion of these animals spontaneously developed neurologic symptoms without inoculation,⁸ raising the possible criticism that the injected fibrils had simply accelerated an ongoing disease process, rather than initiating a neurodegenerative syndrome *de novo*.

In 2010, Baskakov and colleagues⁹ succeed in producing disease in nongenetically engineered hosts (hamsters) that had been inoculated with recombinant hamster PrP fibrils. Notably, however, clinical symptoms did not appear until the second or subsequent passages. In the first passages, Makarava et al² detected in the brains of the asymptomatic animals a form of misfolded PrP—atypical PrP^{Pres}—that was distinct from authentic PrP^{Sc}.^{4,5} Authentic PrP^{Sc} gradually appeared in subsequent passages and eventually became the predominant species, correlating with the development of clinical symptoms. The two forms of PrP could be distinguished biochemically by the characteristic size of the fragments produced after proteolytic digestion. This evolutionary process, in which atypical PrP^{Pres} was gradually transformed

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Figure 1 Makarava et al² test whether over-expression of hamster PrP^C in Tg7 transgenic mice enhances the formation of atypical PrPres or PrP^{Sc}. After inoculation of Tg7 mice with recombinant hamster PrP fibrils, production of atypical PrPres increases, but PrP^{Sc} does not appear and the mice remain healthy. During subsequent passages, PrP^{Sc} gradually replaces atypical PrPres, correlating with the development of clinical symptoms and neuropathology. This latter process is not enhanced by increased levels of hamster PrP^C substrate. The schematic shows Western blot analysis of protease-resistant fragments of PrP from the brains of the mice, which allows distinction of atypical PrPres from PrP^{Sc}. PrP, prion protein; PrP^C, cellular prion protein; PrPres, alternative self-replicating state of prion protein; PrP^{Sc}, prion protein scrapie isoform; Tg, transgenic.

into PrP^{Sc} with continued propagation,² led them to put forward a deformed templating model.^{4,5}

Theories of Prion Evolution

The concept that prions can evolve or adapt dates to the earliest, classical studies of prion transmission, in which it was observed that the biological properties of different strains could change on repeated passage, particularly during transfer from one host species to another.¹⁰ In the modern era, two kinds of molecular explanations have been offered to explain prion evolution. One (the cloud hypothesis^{11,12}) postulates that even cloned prion strains consist of a spectrum of different PrP conformers, with one of the conformers being predominant. On exposure to an altered set of conditions either *in vitro* or *in vivo* (eg, a new host), propagation of a different conformer is favored, and this one becomes predominant. This process was likened to Darwinian evolution (selection of pre-existing mutants). A second proposed mechanism is the deformed templating hypothesis of Baskakov and colleagues.^{4,5} In this model, the templating process is imperfect and will sometimes result in generation of altered PrP conformers, a process that can be enhanced by changing propagation conditions. This hypothesis could be said to be Lamarckian as opposed to Darwinian (environmentally induced changes that are subsequently propagated). It was pointed out that these two kinds of explanations are not necessarily mutually exclusive.¹³

Background to the Current Study

As a substrate for their *in vitro*, prion-generating system, Makarava et al² used purified, recombinant, hamster PrP^C

folded into its native α -helical form, which is not infectious and does not induce a neurologic disease when injected intracerebrally into hamsters. They showed previously that they could produce two different kinds of recombinant hamster PrP fibrils, depending on whether they used 0.5 or 2.0 mol/L guanidine to refold the protein.⁵ The two kinds of fibrils differed morphologically and biophysically and, importantly, displayed different biological properties when inoculated into recipient hamsters.⁵ The 0.5 mol/L fibrils eventually produced clinical symptoms of scrapie after secondary passage (but not after primary passage), whereas the 2.0 mol/L fibrils never produced disease even after repeated passage. During the first passage of the 0.5 mol/L fibrils, the brains of inoculated animals accumulated primarily atypical PrPres. During the second passage, the atypical PrPres was replaced by conventional PrP^{Sc}, correlating with the appearance of symptoms. Makarava et al² interpreted their results by postulating a two-step model for the evolution of synthetic prions: first, formation of atypical PrPres from endogenous PrP^C in a process catalyzed by the inoculated hamster PrP fibrils; and second, a deformed templating step in which continued propagation of atypical PrPres sometimes resulted in formation of PrP^{Sc} molecules, which gradually became the predominant species and caused clinical symptoms.

Description of the Current Study

In the discussed study,² the investigators asked whether increasing the amount of PrP^C substrate enhanced the efficiency of each of these two steps (Figure 1). As an inoculum, they used the PrP fibrils formed in 2 mol/L guanidine, which they previously showed did not result in

formation of either atypical PrPres, PrP^{Sc}, or the appearance of clinical disease after inoculation into hamsters.⁵ To increase the level of PrP^C, they turned to transgenic (Tg7) mice that overexpress hamster PrP^C by 3.5-fold in the absence of endogenous mouse PrP^C. They found that inoculation of Tg7 mice with 2.0 mol/L fibrils resulted in some animals that accumulated atypical PrPres, indicating that the increased expression level of hamster PrP^C in these mice enhanced the formation of atypical PrPres. However, no PrP^{Sc} (which could be detected independently on the basis of the sizes of its protease-resistant fragments) was found in animals after the first passage, and the amount of PrP^{Sc} that appeared during secondary passage was not higher in Tg7 mice than in hamsters, even though the amount of atypical PrPres was greatly increased. These latter results indicate that the second step, conversion of atypical PrPres to PrP^{Sc}, was not enhanced by higher expression of PrP^C. Makarava et al² concluded that the rate of deformed templating does not depend on the expression level of PrP^C but is likely to be controlled by other factors, including the nature of the template, the intrinsic rate of conformational errors in templating, and, possibly, the biochemical environment.

In a second set of experiments, Makarava et al² asked whether atypical PrPres and PrP^{Sc} compete with each other during the propagation process, as might be expected because they presumably use the same PrP^C substrate. They found that such competition is, in fact, observed during propagation with the use of a PMCA system but, surprisingly, not after inoculation into hamsters. They offer several explanations for this discrepancy between the *in vitro* and *in vivo* results, including differences between atypical PrPres and PrP^{Sc} in clearance rates, neurotropism, cellular replication sites and cofactors, or ability to convert sialylated PrP^C substrate.

Remaining Questions

The study of Makarava et al² raises several intriguing questions. First, what are the factors that control deformed templating? The results presented in the study make it clear that the amount of PrP^C substrate is not rate-limiting for this step. In some ways, this is surprising, because it is well known that mice with supraphysiologic levels of PrP^C have shorter incubation times after prion inoculation.¹⁴ Moreover, the accumulation of atypical PrPres is significantly accelerated by increased expression of PrP^C. Why the difference? One possibility is that the switch from atypical PrPres to PrP^{Sc} via deformed templating is stochastic, depending, eg, on small, random fluctuations in the conformation of the PrP^C template. Another possible explanation could be a role for cofactors such as lipid molecules or RNA, which could favor generation of one product versus the other by binding to critical regions of PrP^C. In fact, there is ample evidence for this idea. In a

particularly clear example, Supattapone and colleagues¹⁵ have produced two autocatalytic recombinant PrP conformers derived from the same original PrP^C template, which differ by >10⁵-fold in specific infectivity, depending on whether a lipid cofactor was included in the *in vitro* amplification reaction. One could hypothesize that, in the discussed study,² the original PrP fibrils (which were produced without cofactors) gradually produce PrP^{Sc} when propagated *in vivo* in the presence of cofactor molecules such as RNA and lipids. A third potential mechanism to explain deformed templating concerns the nature of the PrP^C substrate molecule, in particular the kinds of post-translational modifications present on the protein. It is possible that atypical PrPres and PrP^{Sc} preferentially convert PrP^C molecules with distinct arrays of post-translational modifications, and that the transition from atypical PrPres to PrP^{Sc} involves a rare switch in substrate selection during the conversion process. In fact, recent work from the Baskakov laboratory has highlighted the potential importance of the sialic acid residues on the N-linked glycans of PrP^C as a factor in conversion efficiency.¹⁶

Another important question raised by the present work is the relation between PrP conformation and pathogenic potential. In particular, why is PrP^{Sc} pathogenic, whereas atypical PrPres is not, even when it accumulates to high levels, as in Tg7 mice? There is considerable evidence that neurotoxicity and infectivity are distinct properties, and that only some misfolded conformers of PrP possess both.^{17,18} To address this question further, it will be necessary to develop experimental systems that can directly assay prion toxicity.

Finally, this study raises the question of the structural relation between PrP^{Sc}, atypical PrPres, and other misfolded forms of PrP that were described, including naturally occurring forms found in human brain and those generated *in vitro*. The structural differences among these forms have been probed by proteolytic digestion and in some cases by biophysical techniques.^{15,19–21} Importantly, however, a high-resolution atomic structure for infectious PrP^{Sc} has yet to be determined.

There is little doubt that the answers to these questions will help move the field forward and shed light on the myriad ways that PrP can misfold, propagate its conformation, and ultimately cause disease.

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