Grand ideas floating freely

Conference on the new prion biology: basic science, diagnosis and therapy

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In the exquisite gothic Palazzo Franchetti on the bank of the Grand Canal in Venice, the International Symposium on 'New Prion Biology' took place on April 12–13, 2002. The fabulous setting and excellent organization by the Scientific Committee together with the promotion and sponsorship of the Istituto Veneto di Scienze, Lettere ed Arti resulted in a stimulating and informative gathering for participants from a wide variety of countries.

during this meeting included the structure and function of PrP, TSE pathogenesis, new efforts towards diagnostic tests, and the normal and abnormal roles of the newly described 'doppel' protein in gametogenesis and neurodegeneration.

Doppel talk

In the past several years, various laboratories have used different strategies to generate knock-out mice in which normal PrP expression is eliminated. Although the first two of these strains had no obvious neurological problems, the two that were generated subsequently developed cerebellar degeneration in the first months of life. These findings led to the discovery of a gene located downstream of *Prnp*, known as *Prnd*. This gene encodes a protein called Doppel (Dpl) (Moore *et al.*, 1999), which has partial homology to PrP at the sequence and structural levels and appears to be responsible for the cerebellar degeneration observed. Dpl is overexpressed in the brains of certain PrP-null mice in which the PrP knock-out construct places the downstream *Prnd* gene under the control of the PrP promoter (D. Westaway, Toronto, Canada). The biochemical mechanism by which Dpl overexpression in the brain leads to cerebellar degeneration remains unclear; however, there are similarities between this model and the previous findings by Shmerling *et al.* (1998) that cerebellar degeneration was exhibited in transgenic mice expressing N-terminal deletion mutants of PrP itself (PrP Δ 32–121 and PrP Δ 32–134). Interestingly, in both the Shmerling model and the PrP-null mice overexpressing Dpl, the neurological deficit was eliminated by expression of a normal PrP gene, suggesting that perhaps these molecules compete for a common target protein important in cerebellar structure and function (for a review, see Behrens and Aguzzi, 2002). Furthermore, in the Shmerling mice, the presence of a transgene expressing PrP exclusively in oligodendroglial cells can protect against death but does not prevent ataxia (A. Aguzzi, Zürich, Switzerland). This suggests that two separate pathogenic processes may be occurring: one in Purkinje cells, the large neurons of the

Introduction

Prion protein (PrP) is critically involved in the transmission and pathogenesis of the transmissible spongiform encephalopathies (TSEs), which include scrapie in sheep, variant and sporadic Creutzfeldt–Jakob disease (CJD) in humans and bovine spongiform encephalopathy (BSE) in cattle. The normal, cellular PrP^C protein encoded by the *Prnp* gene is required for susceptibility to TSE. The interconversion of this normal form into the highly protease-resistant PrP^{Sc} conformation leads to the formation of PrP aggregates and amyloid fibrils in the brain and ultimately to neurodegeneration in the affected animal. It has been proposed that this abnormal form of PrP is itself the infectious agent of transmission; however, this matter remains unsettled for a variety of reasons (Chesebro, 1998, 1999). The topics covered

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cerebellum whose perturbation results in ataxia; and a second in other neurons, leading to white matter vacuolization and death.

Transgenic mice have been generated that express PrP with or without expression of Dpl in their central nervous systems. When these animals were inoculated with a TSE agent no difference in disease phenotype was observed between the two groups of mice, suggesting that the expression of Dpl in the brain does not influence the pathogenesis of TSE (Tuzi *et al.*, 2002). Recent studies have now focused on the normal function of the Dpl protein. Interestingly, high levels of Dpl expression have been detected in human testis, in the Sertoli cells associated with spermatid maturation and the testosterone-producing Leydig cells, and also in the flagella of spermatozoa (K. Peoch, Paris, France). Furthermore, male mice lacking Dpl expression have decreased fertility, with abnormalities in the spermatozoa including decreased sperm counts, lower motility and inability to penetrate the zona pellucida (A. Behrens, London, UK).

Biochemical studies indicate that Dpl is a GPI-anchored cell surface glycoprotein with two intrachain disulfide bonds (Silverman *et al.*, 2000). Dpl shows a selectivity and affinity for binding copper ions reminiscent of PrP, but it remains unclear whether these properties are related to the aforementioned Dpl/PrP 'competition' observed *in vivo* (D. Westaway). Thermal denaturation studies indicate that Dpl is more stable and recovers its helical structure more readily than does PrP (M.C. Sorgato, Padua, Italy). Furthermore, Dpl was found to colocalize frequently with PrP on dendrites, axons and cell bodies of live differentiated human neuroblastoma cells, except that after antibody cross-linking, PrP was internalized to a greater extent than Dpl (M.C. Sorgato). Thus far, no protein that binds both Dpl and PrP has been identified.

Dimers, knock-ins and transmembrane forms

Central to disease pathogenesis is the conformational transition of the α -helical PrP^C into the predominantly β -sheet PrP^{Sc} form, which is then capable of forming aggregates associated with disease. Previous NMR studies have consistently shown PrP to be monomeric in solution, but surprisingly, recent X-ray crystallographic data have revealed a dimeric structure (V. Yee, Cleveland, OH; Figure 1). The dimer results from three-dimensional domain swapping of the C-terminal helix 3 (residues 119–226) and a rearrangement of the disulfide bond to link the two polypeptide chains (Knaus *et al.*, 2001). These results suggest a mechanism for oligomerization of PrP that might provide an important intermediate in the process of conversion to protease-resistant PrP during TSE pathogenesis. In fact, non-disulfide-linked dimeric PrP has been detected in SDS polyacrylamide gels after protease digestion of TSE brain extracts and also in a protease-sensitive form in cell culture biochemical studies, but the role of these structures in the initiation or maintenance of the PrP conversion process remains to be proven. Interestingly, the amino acids involved in familial human spongiform encephalopathies are concentrated in the crystallized prion protein fragment in the swapped helix 3, the neighboring helix 2 and the switch region between these two helices. Possibly these mutations might act by influencing the equilibrium in favor of dimer or higher oligomer formation *in vivo*.

Other effects of PrP allelic variation were shown using recombinant knock-in mice expressing a variant PrP with leucine at position 101 (R. Barron, Edinburgh, UK). In human patients, this mutation is associated with a familial TSE, Gerstmann–Sträussler–Scheinker disease (GSS). In previous studies, transgenic mice expressing Leu101 developed spontaneous brain disease, but in contrast to known TSEs, this was not easily transmissible to mice lacking the mutant PrP transgene (Hsiao *et al.*, 1994). To avoid possible transgene artifacts relating to high copy number or integration site influences, recombinant knock-in mice expressing Leu101 were created. These mice more closely model the situation in humans with the GSS mutation because they have the mutant PrP gene present as a single copy per chromosome located precisely at the *Prnp* locus. Interestingly, these mice failed to develop spontaneous brain disease over several years of observation, suggesting that the Leu101 mutation alone is insufficient for disease induction in mice (Manson *et al.*, 1999). However, these mice showed enhanced susceptibility to certain TSE strains from other species including hamster 263K and SSBP/1 from sheep, and decreased susceptibility to other TSE strains including variant CJD (vCJD) from humans (Barron *et al.*, 2001). Residue 101 is located in the flexible N-terminal region of PrP. The results presented at this meeting demonstrate an important role for mutations in this region of PrP in controlling susceptibility to various TSE strains.

Although the majority of normal PrP is expressed as a GPI-linked cell surface molecule, a smaller amount is found in two types of transmembrane form. Signal peptide differences appear to influence production and distribution of these PrP conformers, which are distinguishable from one another by their membrane orientation (V. Lingappa, San Francisco, CA). Their roles in TSE remain unclear, but some data suggest that the C-terminal transmembrane form may have a role in transducing a toxic, possibly apoptotic, signal to brain cells in diseased tissues.

The brain damage that occurs due to TSE is believed to be mediated largely by the abnormal protease-resistant PrP^{Sc} and its interactions with normal PrP. Previous *in vitro* and *in vivo* studies have shown that a PrP peptide consisting of residues 106–126 can form amyloid-like fibrils and induce neurotoxicity (for a review, see Tagliavini *et al.*, 2001). However, this peptide has been difficult to detect *in vivo*. In contrast, PrP peptide 82–146 is abundant in the brains of patients with genetic TSE, forms aggregates and amyloid-like fibrils *in vitro*, and is toxic to both PrP-null and PrP-expressing neuronal cells (G. Forloni, Milan, Italy). Future studies of this peptide may unravel the mysteries of prion neurotoxicity and the role of normal PrP in this process.

Innovative methods for early diagnosis

The recent discovery of a form of protease-resistant PrP in the urine of animals and humans with TSE (Shaked *et al.*, 2001) may make it possible to develop a non-invasive test for this family of diseases (R. Gabizon, Jerusalem, Israel). So far this material has been found prior to both the observation of clinical scrapie in inoculated hamsters and the clinical onset of inherited CJD in humans with the *Prnp* E200K mutation. Although protease-resistant PrP^{Sc} has usually been associated with TSE infectivity, when excreted in urine, PrP^{Sc} appears not to be infectious. This is consistent with previous data indicating that urine is not infective

Fig. 1. Stereo view ribbon diagram of the human wild-type prion protein dimer crystal structure (adapted from Knaus *et al.*, 2001). The two peptide chains are in green and purple, with labeled termini; side chain atoms of the two disulfide bridges are shown in space-filling representation. Figure courtesy of Vivien Yee.

and could be due to the fact that urine PrP^{Sc} has a fundamentally different structure than does brain PrP^{Sc}. Detection of urine PrP^{Sc} requires a dialysis step, which is not needed for other PrP^{Sc} sources, and this process could also influence the nature of the final material obtained. In any case, a urine test has the potential to be extremely useful for diagnosis in a variety of situations.

Cyclic amplification of misfolded PrP (the PMCA test) is also a potentially useful approach for sensitive TSE diagnosis (Saborio *et al.*, 2001). In this case, the cell-free conversion of normal PrP to the protease-resistant form *in vitro* is repeated cyclically with sonication after each cycle. The test is very sensitive, and has been successfully applied for amplification of PrP^{Sc} from different species (C. Soto, Geneva, Switzerland). So far, the sensitivity appears to depend on technical details, which have been difficult to transfer effectively to new laboratories.

Another possibility for a diagnostic test is to use western blot analysis of PrP^{Sc} after concentration by precipitation with sodium phosphotungstate, which has been used in humans with variant and sporadic CJD (Wadsworth *et al.*, 2001). This method reveals differences in the deposition of PrP^{Sc} in different tissues in different TSE disease types. For example, in sporadic CJD, the tonsils of affected patients were not positive for PrP^{Sc}, whereas in vCJD, high levels of PrP^{Sc} were uniformly seen in this tissue. Tonsil remains the most promising tissue for diagnosis of vCJD outside the brain; to date, 22 confirmed or suspected vCJD individuals have tested positive for PrP^{Sc} using this method (J. Wadsworth, London, UK).

Efforts have continued to generate an antibody capable of distinguishing between protease-resistant PrP^{Sc} and normal PrP (Williamson *et al.*, 1998). A new approach that may have succeeded takes advantage of the binding of PrP^{Sc} to a specific region of normal PrP (R.A. Williamson, La Jolla, CA). The transfer of the sequence for this region to the CDR3 region of an antibody resulted in a reagent that binds PrP^{Sc}. Affinity tests are in progress and if binding strength is high, this should provide a useful tool for new diagnostic tests. In related experiments, antibodies reactive with the normal form of PrP have been found to reduce expression of abnormal PrP in scrapie-infected cell cultures, raising the hope that passive immunotherapy for these diseases may be feasible *in vivo* (C. Weissmann, London, UK and R.A. Williamson). This possibility was also raised in a surprising experiment where expression of an anti-PrP antibody in transgenic mice (Heppner *et al.*, 2001) resulted in high resistance to scrapie infection (F. Heppner, Zürich, Switzerland).

Neuroinvasion and persistence during subclinical TSE disease

The role of follicular dendritic cells (FDCs) in TSE infection and neuroinvasion continues to be quite complex. FDCs appear to be the main site of deposition of abnormal PrP after intraperitoneal infection, and may serve as a replication site for amplification of the infectivity prior to neuroinvasion. Functional B lymphocytes are required for successful FDC maturation, but

these B cells do not need to express PrP for this function, which explains some of the earlier data using knock-out mice. Recent studies indicate that the anatomic location of FDC relative to central arteries and nerves might also play a role in the efficiency of neuroinvasion following FDC infection (A. Aguzzi). In mice deficient in the CXCR5 chemokine receptor, FDC and B cells are located closer to the central artery and nerves than in wild-type mice. These knock-out mice have shorter incubation periods after peripheral scrapie infection, and it is possible that this might be due to increased efficiency of neuroinvasion based on the proximity of FDC to splenic nerves.

There is continuing concern about the possibility of subclinical BSE infection in non-bovine species including humans. Subclinical infection after cross-species infection in a hamster–mouse system showed that the hamster scrapie agent could be re-isolated from asymptomatic mice even after two serial mouse passages over more than 1400 days (B. Chesebro, Hamilton, MT). These results indicate that species-specific properties of TSE agents can be maintained during replication in a relatively resistant foreign species in the absence of a PrP molecule from the homologous species. The implication is that feeding of TSE-contaminated material to a resistant species might pose dangers of agent propagation in spite of the lack of overt clinical disease.

Future directions

In the past several years, research on TSE has grown enormously, largely due to the interest in the BSE problem at many levels. The pressing issue of developing a non-invasive diagnostic test for large-scale use in humans and animals remains a top priority. However, it also appears likely that there will be renewed interest in developing new strategies for therapy for this disease family. Although the goal seems to be a daunting one, there are several compounds that can prolong the lifespan of infected animals. Perhaps with the combination of multiple approaches more effective results can be achieved. It is worth recalling that four or five decades ago cancer chemotherapy was also only marginally effective, but as a result of persistent attempts certain malignancies can now be cured with drugs alone.

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