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The molecular pathology of CJD: old and new variants

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Abstract
The study of prion disease has become an area of intense interest since experimental evidence emerged for the transmission of phenotypic variation without the involvement of a nucleic acid component. Additional impetus has come from the widespread concern that exposure to bovine spongiform encephalopathy contaminated material poses a distinct and, conceivably, a severe threat to public health in the UK and other countries. The occurrence of new variant Creutzfeldt-Jakob disease has dramatically highlighted the need for a precise understanding of the molecular basis of prion propagation. The molecular basis of prion strain diversity, previously a major challenge to the "protein only" model, can now be reconciled with propagation of infectious protein topologies. The conformational change known to be central to prion propagation, from a predominantly α-helical fold to one predominantly comprising β-structure, can now be reproduced in vitro, and the ability of β-PrP to form fibrillar aggregates provides a plausible molecular mechanism for prion propagation. Concomitantly, advances in the fundamental biology of prion disease have done much to reinforce the protein only hypothesis of prion replication.
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Keywords: Creutzfeldt-Jakob disease; prion propagation; prion disease; protein only model

Prion diseases are fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), fatal familial insomnia (FFI), and kuru in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle. The human prion diseases are unique in that they can have a sporadic, inherited, or transmissible origin.[1]

International interest in prion disease has grown rapidly since the "protein only" hypothesis of prion replication, first proposed by Griffith in 1967,[2] was brought to the attention of a new generation by Prusiner in 1982.[3] The hypothesis argues that the infectious agent, the prion, is composed of a conformational isomer of a normal host encoded protein that is able to convert other isoforms to itself in an autocatalytic manner. Thus, infection and the inevitable onset of disease occurs without the transmission of a nucleic acid genome.

A wealth of experimental evidence now shows that the central feature of prion diseases is indeed the post-translational conversion of a normal host encoded, glycoinositol phospholipid (GPI) anchored sialoglycoprotein, the prion protein (PrP^{C}),[4] to an abnormal form, designated PrP^{Sc}. This transition appears to involve only conformational changes because amino acid sequencing and systematic analysis of known post-translational modifications show no differences between PrP^{C} and PrP^{Sc}.[5]

Little or nothing is known about the molecular state of the protein that constitutes the self replicating disease material. PrP^{Sc} is deposited as stable aggregates, which are resistant to proteolysis with proteinase K, and in infected tissue PrP^{C} can readily be degraded to leave only PrP^{Sc}.[6] When this resistant material is enriched by detergent extraction and differential centrifugation, electron microscopy shows that the material contains a proportion of fibrillar material,[7] but it is uncertain whether this ordered aggregation state constitutes the infectious agent. In several cases, there have been claims that infectivity can be separated from fibrils, and infectious brain tissue has been shown to contain no detectable protease resistant material.[8]

Although tremendous advances have been made in our understanding of prion diseases, many key questions remain unanswered. For example, we still know little about the normal cellular function of PrP^{C}, the molecular events underlying the conversion of PrP^{C} to PrP^{Sc}, or the mechanism of neurodegeneration that characterises prion disease. Answers to these questions will be crucial to the development of rational therapeutic strategies for treating prion diseases, the urgency for which is emphasised by recent evidence showing that new variant CJD (vCJD) and BSE are caused by the same prion strain.[9–11]

Models of prion replication

Despite the doubts about the structural characteristics of the infectious form of PrP, it is well established that prion diseases arise by one of three processes. In outline, all three aetiological routes can be described with reference to a single, general model. In this model, the native PrP^{C} molecule is in equilibrium with the rare PrP^{Sc} conformational isoform. PrP^{Sc} can then be stabilised by complimentary association with a like molecule or can actively convert PrP chains to a like conformation. Assembly then continues until a stable seed is formed. Such structures can continue to grow by accretion and can divide by breakage into smaller, infectious units (fig 1). A mechanism involving linear polymerisation has been modelled mathematically and predicts many of the observed aspects of the disease.[12] This gross

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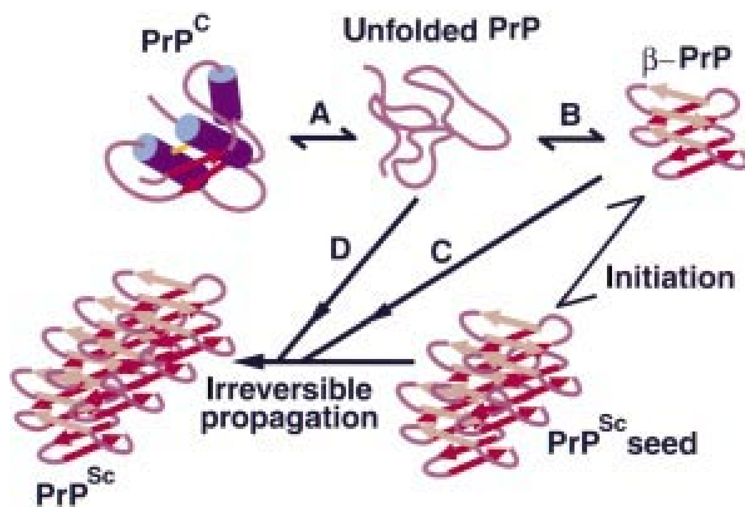

Figure 1 Schematic representation of the possible molecular events underlying infection with prions. PrP^C is shown in dynamic equilibrium with the unfolded PrP (A), from which β-PrP can form upon reduction of the native disulphide bond (B). β-PrP is prone to aggregation and may form a stable seed of PrP^{Sc}. Recruitment of either more β-PrP (C) or unfolded PrP (D) leads to irreversible propagation of insoluble PrP^{Sc}. Pathogenic mutations may destabilise PrP^C and move the equilibrium A to the right. Infection with a preformed seed of PrP^{Sc} will lead to recruitment and irreversible propagation directly.

mechanism explains the observation that prion diseases occur by inherited mutations, which destabilise the cellular form and therefore predispose it to conversion to PrP^{Sc}, or by iatrogenic or dietary infection with PrP^{Sc}. Sporadic cases are also described where the cause is unknown, but within the above paradigm they can be explained either by somatic mutation or by a rare, spontaneous conversion of the wild-type protein to the PrP^{Sc} conformation. There have been recent studies suggesting that sporadic CJD may be linked to a past history of surgery or residency on a farm.[13] Although strong statistical associations can be found in a proportion of cases there remain a considerable number of patients for whom the aetiology of the disease is unknown.

The molecular basis of prion strains

A major problem for the protein only hypothesis of prion propagation has been how to explain the existence of multiple isolates, or strains, of prions. Such strains are distinguished by their biological properties: they produce distinct incubation periods and patterns of neuropathological targeting in inbred mouse lines. Because they can be serially propagated in inbred mice with the same Prnp genotype, they cannot be encoded by differences in PrP primary structure. Furthermore, strains can be re-isolated in mice after passage in intermediate species with different PrP primary structures.[14] Understanding how a protein only infectious agent could encode such phenotypic information has been of considerable biological interest.

Support for the contention that strain specificity is encoded by PrP alone was provided by the study of two distinct strains of transmissible mink encephalopathy prions, which can be serially propagated in hamsters, designated hyper (HY) and drowsy (DY). These strains can be distinguished by differing physiochemical properties of the accumulated PrP^{Sc} in the brains of affected hamsters.[15] After limited proteolysis, strain specific migration patterns of PrP^{Sc} on polyacrylamide gels can be seen that relate to different N-terminal ends of HY and DY PrP^{Sc} following protease treatment, implying differing conformations of HY and DY PrP^{Sc}.[16]

Recently, several human PrP^{Sc} types have been identified that are associated with different phenotypes of CJD.[9,17] The different fragment sizes seen on western blots after treatment with proteinase K suggest that there are several different human PrP^{Sc} conformations (fig 2). However, to fulfil the criteria of strains, these biochemical properties must be retained after transmission to experimental animals of both the same and different species. This has been demonstrated in studies with CJD isolates, with both PrP^{Sc} fragment sizes and the ratios of the three PrP glycoforms (diglycosylated, monoglycosylated, and unglycosylated PrP) maintained on passage in transgenic mice expressing human PrP.[9] Furthermore, transmission of human prions and bovine prions to wild-type mice results in murine PrP^{Sc} with fragment sizes and glycoform ratios that correspond to the original inoculum.[9] New variant CJD is associated with PrP^{Sc} glycoform ratios that are distinct from those seen in classic CJD. Similar ratios are seen in BSE and BSE when transmitted to several other species.[9] These data strongly support the protein only hypothesis of infectivity and suggest that strain variation is encoded by a combination of PrP conformation and glycosylation. Furthermore, polymorphism in the PrP sequence can influence the generation of particular PrP^{Sc} conformers.[9] Transmission of PrP^{Sc} fragment sizes from two different subtypes of inherited prion disease to transgenic mice expressing a chimaeric human mouse PrP has also been reported.[18]

Because PrP glycosylation occurs before conversion to PrP^{Sc}, the different glycoform ratios may represent selection of particular PrP^C glycoforms by PrP^{Sc} of different conformations. According to such a hypothesis, PrP

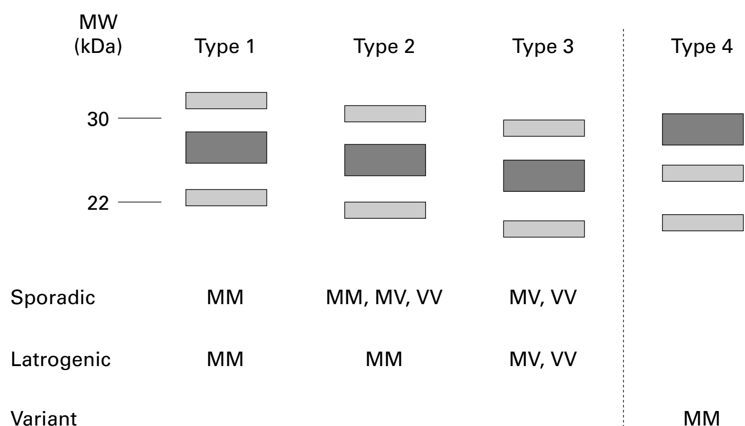

Figure 2 Molecular strain types of Creutzfeldt-Jakob disease (CJD). Schematic representation of western blot detection of proteinase K digestion products generated from distinct PrP^{Sc} conformers associated with human prion diseases. PrP polymorphism at residue 129 (methionine (M) or valine (V)) contributes to genetic susceptibility to both sporadic and acquired forms of CJD and to generation of particular PrP^{Sc} conformers. Box sizes represent the relative difference in intensity of the three PrP glycoforms.

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conformation would be the primary determinant of strain type, with glycosylation being involved as a secondary process. However, because it is known that different cell types may glycosylate proteins differently, PrP^{Sc} glycosylation patterns may provide a substrate for the neuropathological targeting that distinguishes different prion strains.[9] Particular PrP^{Sc} glycoforms may replicate most favourably in neuronal populations with a similar PrP glycoform expressed on the cell surface. Such targeting could also explain the different incubation periods that also discriminate strains, the targeting of more crucial brain regions, or regions with higher degrees of PrP expression, producing shorter incubation periods. Further supportive evidence for the involvement of PrP glycosylation in prion strain propagation has come from the study of transgenic mice expressing PrP with mutations interfering with *N*-linked glycosylation.[19]

Molecular strain typing of prion isolates can now be applied to the molecular diagnosis of vCJD,[9 10] and to produce a new classification of human prion diseases, with implications for epidemiological studies investigating the aetiology of sporadic CJD. Such methods allow strain typing to be performed in days rather than the one to two years often required for classic biological strain typing. This technique might also be applicable to determining whether BSE has been transmitted to other species[9] (for example, sheep[20 21]), and thereby poses a threat to human health. A novel conformation dependent immunoassay has been reported[22] and shown to differentiate several classic rodent adapted scrapie strains in the hamster model. It has not yet been shown to differentiate strains of naturally occurring prion diseases where PrP^{Sc} values vary enormously between cases and different brain regions.

Function and trafficking of PrP^{C}

In view of the peculiar status of the prion as an infectious protein devoid of nucleic acids it seems reasonable to ask whether some aspect of its normal role may predispose it to this unique behaviour. Mice with an ablated Prnp gene show only weak phenotypic effects. Among these are altered electrophysiology, consistent with an abnormal architecture of the synaptic cleft,[23] a lower level of exploratory behaviour, and altered electroencephalogram patterns during sleep. Although none of these observations gets close to defining a molecular role for PrP^{C}, it has been argued that its transport properties make it likely that the protein acts as a receptor for an, as yet, unidentified extracellular ligand. Newly synthesised PrP^{C} is transported to the cell surface and then cycles rapidly via a clathrin mediated mechanism, with a transit time of around one hour, between the surface and early endosomes. This type of behaviour is associated with other cell surface receptors, such as those for transferrin and low density lipoprotein.

Potential partner proteins identified by two hybrid screening include the laminin receptor.[24] However, these data are of questionable relevance owing to the methodology used for identification—the prion protein does not fold in the cytoplasm of cells and thus candidates identified so far are unlikely to interact specifically with PrP.

The conformation and stability of PrP^{C}

The conformation of the cellular isoform was established three years ago by nuclear magnetic resonance (NMR) measurements made on the recombinant mouse protein.[25] Since then, NMR measurements on recombinant hamster[26] and human PrP[27] show that they have essentially the same conformation; however, despite strenuous efforts, no group has yet determined the three dimensional structure of PrP^{C} by crystallographic methods. A schematic representation of the C-terminal globular domain of human PrP^{C} is shown in fig 3, with the GPI anchor and sugar trees attached. Recent work on the folding kinetics of mouse PrP^{C} shows that there are no populated intermediates in the folding reaction and that the protein displays unusually rapid rates of folding and unfolding.[28] These findings have been reinforced by hydrogen/deuterium exchange measurements on the human protein, which demonstrate that the overall equilibrium constant describing the distribution of folded and unfolded states is the same as the protection factor.[29] This shows that there are no partially unfolded forms or intermediates that have a population greater than the unfolded state. The data suggest that PrP^{Sc} is unlikely to be formed from a kinetic folding intermediate, as has been hypothesised in the case of amyloid formation in other systems. In fact, on the basis of population it would be more likely that PrP^{Sc} were formed from the unfolded state of the molecule.

Inherited prion diseases may produce disease by destabilising PrP^{C}, which would predispose the molecule to aggregate. Alternatively, a mutation could facilitate the interaction between PrP^{C} and PrP^{Sc}, or affect the binding of a ligand or co-protein. To relate the folding stability of PrP^{C} to its propensity for forming PrP^{Sc}, several of the human mutations have been copied into the recombinant mouse protein.[30] Although this work broadly concluded that there is no absolute correlation between stability and disease, all of the fully penetrant mutations show significant destabilisation, whereas polymorphisms have little effect.

Studies on transgenic animals have been widely used in the field and have provided several key insights. $Prnp^{0/0}$ mice, devoid of normal PrP^{C}, are resistant to infection with prions and do not replicate the infectious agent.[31] The introduction of prion genes from different species into these mice restores susceptibility to prions in a species dependent manner. Thus, there is an absolute requirement for PrP in disease pathogenesis.

From the introduction of prion transgenes with various N-terminal truncations it was observed that deletions up to residue 106 gave a normal phenotype. Deletion beyond this led

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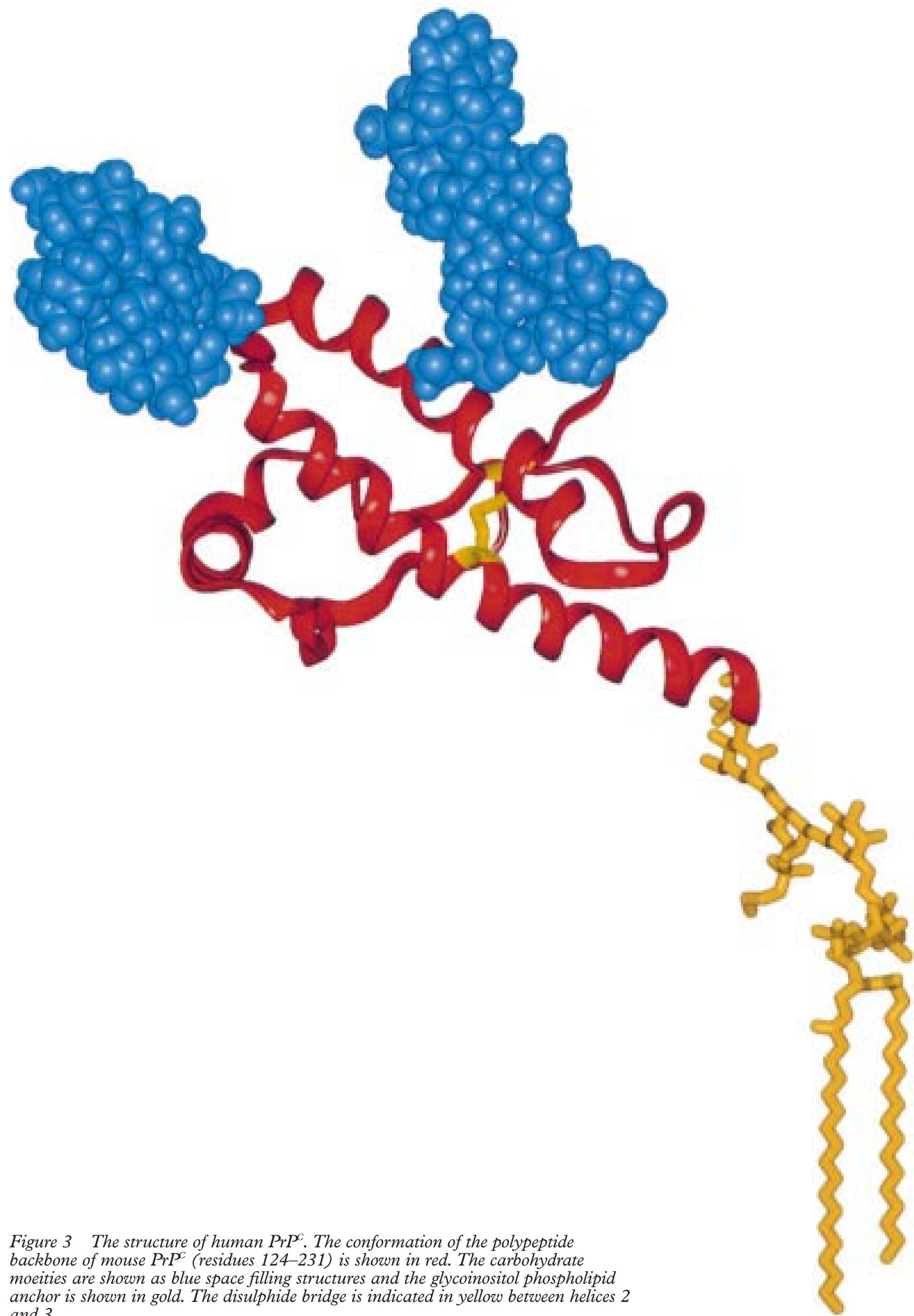

Figure 3 The structure of human PrP^{C}. The conformation of the polypeptide backbone of mouse PrP^{C} (residues 124–231) is shown in red. The carbohydrate moeities are shown as blue space filling structures and the glycoinositol phospholipid anchor is shown in gold. The disulphide bridge is indicated in yellow between helices 2 and 3.

to severe ataxia and neuronal loss in the granular cell layer of the cerebellum,[32] symptoms and pathology that were completely absent in the ablated mice. This has led to the hypothesis that PrP and a structural homologue compete for the same receptor or ligand. An additional gene (Prnd) has recently been discovered within the Prnp locus. It encodes a 179 residue protein with between 20% and 24% identity to PrP (depending upon the species),[33] which is a plausible candidate for the putative competitor.

PrP in its entirety is unnecessary for prion propagation. Not only can the unstructured N-terminal 90 amino acids be deleted, but also the first α-helix, the second β-strand, and part of helix 2. In transgenic animals, a 106 amino acid fragment of the protein comprising PrPΔ23–88Δ141–176 was all that was required to confer susceptibility to and propagation of prions.[34 35]

Conversion of PrP^{C} to other conformations

It has long been known that the adage "one sequence, one conformation" is not strictly true. Depending on solvent conditions, probably any protein chain can adopt a variety of

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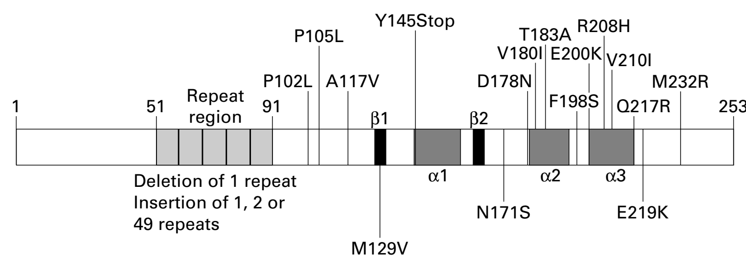

Figure 4 Pathogenic mutations and polymorphisms in the human prion protein. Pathogenic point mutations are shown uppermost, polymorphic residues below. The locations of secondary structure elements are indicated in relation to mutations. The deletion of one copy of the repeat is polymorphic, as is the insertion of one or two copies. The insertion of four or up to nine copies of the octapeptide repeat is pathogenic.

conformations in which there is a degree of periodic order; that is, extensive regions of secondary structure. For instance, a recent systematic study of the conformations adopted by the glycolytic enzyme, phosphoglycerate kinase, show that there are five distinct states that the chain can adopt in different media.[36] However such alternative states do not have precisely and tightly packed side chains, which are the hallmark of the native state of orthodox globular proteins.

Although at physiological pH recombinant fragments of PrP unfold via a two state mechanism, an alternative folding pathway is observed at acidic pH. Studies on recombinant human PrP residues 90–231[37] and mouse PrP encompassing residues 121–231 identified a distinct equilibrium folding intermediate at pH 4.0. Circular dichroism spectroscopy indicated that this intermediate was structured with predominantly β-sheet topology,[38] and it has been proposed that this may be an intermediate on the pathway to PrP^{Sc} formation.

Recent studies on a large fragment of the human prion protein ($PrP^{91–231}$) have shown that at acidic pH PrP can fold to a soluble monomer composed almost entirely of β-sheet.[39] The absence of the native disulphide bond was a prerequisite for β-sheet formation, and these observations of alternative folding pathways dependent upon solvent pH and redox potential could have important implications for the mechanism of conversion to PrP^{Sc}. Indeed, this monomeric β-sheet state was prone to aggregation into fibrils with partial resistance to proteinase K digestion, characteristic markers of PrP^{Sc}. It remains to be demonstrated whether such alternative conformational states of the protein are sufficient to cause prion disease in an experimental host.

The ability of PrP^{Sc} to imprint an abnormal conformation upon recombinant PrP^{C} has been demonstrated in vitro.[40] Cell free conversion reactions in which protease sensitive, metabolically labelled [^{35}S]-PrP^{C} is incubated with an excess of PrP^{Sc} produce de novo proteinase K resistant material, designated PrP^{RES}. Upon digestion with protease, the mobility of PrP^{RES} on sodium dodecyl sulphate polyacrylamide gel electrophoresis is increased, corresponding to a reduction in molecular weight of 6–7 kDa. It has been argued that this is evidence for an obligate change in conformation of the starting material during conversion.

It addition to converting PrP^{C} to PrP^{RES}, cell free conversion reactions have also been shown to propagate strain dependent conformations, as reflected in different patterns of cleavage with proteinase K.[41]

There are limitations to cell free conversion reactions in that conversion requires an excess of PrP^{Sc} to seed the reaction and the PrP^{RES} material generated has been shown to be non-infectious.[42]

Inherited human prion diseases

Further pathogenic PRNP mutations continue to be identified and the currently recognised list is summarised in fig 4. These inherited forms account for ~ 15% of all human prion diseases.[43] Although traditionally classified into GSS, CJD, or FFI, the degree of phenotypic overlap observed between different mutations and even in family members with the same mutation indicates that future classification is likely to be based upon mutation alone.[44 45] How pathogenic mutations in PRNP cause prion disease has yet to be resolved. In most cases, the mutation is thought to lead to an increased tendency of PrP^{C} to form PrP^{Sc}, although recent studies suggest that this may not be solely attributable to the decreased thermodynamic stability of mutated PrP^{C}.[46 47]

In addition, evidence has emerged indicating that experimentally manipulated mutations of the prion gene can lead to spontaneous neurodegeneration without the formation of detectable protease resistant PrP.[48 49] These findings raise the question of whether all inherited forms of human prion disease elicit disease through the same mechanism, and in this regard it is currently unknown whether all are transmissible by inoculation.

Peripheral pathogenesis of prion disease

Peripheral inoculation of experimental animals with prions is typically followed by a prolonged, clinically silent phase before detectable neuroinvasion and the subsequent appearance of neurological deficits. During this preclinical period, prions can be isolated from lymphoreticular tissues, where they may replicate to high titres.

Elucidating the cell types in which prions replicate in the periphery and, crucially, how prions are transported to the central nervous system (CNS) is of considerable interest and represents a plausible target for therapeutic and prophylactic regimens.[50]

The lack of effect of whole body ionising radiation on prion pathogenesis argues against appreciable involvement of proliferating cells in the lymphoreticular phase of prion propagation.[48] Follicular dendritic cells (FDCs), which do not proliferate, have been the favoured candidate for some time.[51] However, evidence for a key role for B cells in prion propagation was produced using a panel of immunodeficient mice.[52] Whereas all of the other strains of mutant mice could be infected by intracerebral exposure to prions, mice with defects in B cell differentiation were extremely resistant to prion infection after intraperitoneal inoculation with scrapie prions. Because an absence of

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mature B cells is associated with defective FDC maturation, these investigators went on to demonstrate that mice deficient in tumour necrosis factor 1, which lack functional FDCs, were susceptible after peripheral inoculation, arguing that FDCs were not required for neuroinvasion.[52] Importantly, because B cells are circulating cells known (in common with most bone marrow derived cells) to express PrP^C, these data highlighted the need to consider the risks posed by blood transfusions from donors incubating prion disease.

Because both prion replication[31] and transport to the CNS[53] are dependent on PrP^C expression, it was anticipated that PrP^C expression on B cells would be required for their role in prion pathogenesis. However, this may not be the case because haematopoetic stem cells derived from PrP knockout mice are just as efficient in removing the block to neuroinvasion in B cell deficient mice as comparable cells from wild-type mice.[54]

Although it is possible that B cells could transport prions to the CNS via a non-PrP dependent process, or that they might secrete factors that bind to prions and enhance their neuropathogenicity, an alternative possibility is that FDCs (or a subset of cells not bearing the FCD-M1 or M2 immunophenotype) are in fact the cell type that promotes prion neuroinvasion. It is possible that prion propagation to high levels in FDCs simply increases the probability of prion uptake by the autonomic nerve terminals that extensively innervate lymphoid organs. Neuroinvasion via peripheral nerves has long been considered likely.[55] Thus, B cells need not be directly involved in either prion propagation or neuroinvasion, but rather are one of several necessary factors that promote FDC development. It must also be considered that more than one mechanism might be involved and that different prion strains may preferentially use these different routes, complicating any therapeutic strategies. Such studies aiming to dissect the cell type(s) involved in the peripheral pathogenesis of prion disease by various reconstitution experiments in $Prnp^{o/o}$ mice may also be complicated by the known ability of GPI anchored proteins to transfer from cell to cell ("GPI painting").[56]

The origins of neurotoxicity

It is worth noting that after nearly 20 years of research the precise nature of the infectious agent and the cause of cell death remain unclear. The current working hypothesis is that PrP^{Sc} is the sole infectious agent and, to date, the most highly enriched preparations contain one infectious unit/10^5 PrP monomers.[57] Various hypotheses have been proposed to explain the mechanism of spongiform change and neuronal cell loss. These have included direct neurotoxic effects from a region of the prion protein encompassing residues 106–126[58] to increased oxidative stress in neurones as a result of PrP^C depletion, which has been proposed to function as an antioxidant molecule.[59] It has also been suggested that PrP^C plays a role in regulating apoptosis, with the disturbance of normal cellular PrP values during infection leading to cell death.[60] Certainly, there have been numerous recent reports of apoptotic cells being identified in the neuronal tissue of prion disease brains,[61] although whether this is a direct consequence of infection or a secondary effect of localised necrosis is unclear.

Although PrP^C expression is required for susceptibility to the disease, a series of incisive experiments have been performed that demonstrate that PrP^{Sc} is not, by itself, cell toxic. Sections of neuroectodermal tissue from mice overexpressing PrP^C were grafted into the brains of $Prnp^{0/0}$ mice. After the administration of prions, there was extensive neuropathology and deposition of PrP^{Sc} in the graft. However, in the surrounding host tissue there was no detectable pathology even in the presence of PrP^{Sc}, which had migrated from the graft. PrP^{Sc} was seen to have invaded the host tissue and yet did not cause pathology in the absence of PrP^C: an observation that suggests that PrP^{Sc} is not directly cytotoxic.[62 63]

Conclusions

Important steps have been made in laying the foundations of an understanding of prion diseases at the structural level. These include the elucidation of the structure and folding dynamics of PrP^C and the characterisation of alternatively folded states, which may relate to the conversion to the PrP^{Sc} form. Proteolytic susceptibility and blot analysis of brain tissue have demonstrated that PrP^{Sc} can adopt different conformations and/or assembly states, so that we now have at least a rudimentary understanding of how different strains of the disease have arisen and breed true. These findings have been underlined by recruitment experiments in vitro that show that PrP^C can be recruited to PrP^{Sc} particles and be imprinted by parent PrP^{Sc} conformation. Interestingly, the ease of conversion to a given scrapie isoform reflects in vivo susceptibility to the given agent. The region of PrP necessary for prion propagation and which confers susceptibility to the disease has now been narrowed down to two individual segments, together comprising only 106 residues. However, despite a considerable increase in our understanding of prion conformation, no one has yet produced infective prions in vitro from purified recombinant material; such an achievement would not only prove the protein only hypothesis beyond doubt, it would also serve as the essential model by which the mechanism can be understood in molecular detail.

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