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A closer look at prion strains
Characterization and important implications

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Abbreviations: PrP^C, cellular prion protein; PrP^{Sc}, scrapie prion protein; TSEs, transmissible spongiform encephalopathies; TME, transmissible mink encephalopathy; CJD, Creutzfeldt-Jakob disease; sCJD, sporadic CJD; vCJD, variant CJD; FFI, fatal familial insomnia; BSE, bovine spongiform encephalopathy; CWD, chronic wasting disease; PK, proteinase K; SAF, scrapie-associated fibrils; CNS, central nervous system; WB, western blot; PE, phosphatidylethanolamine; sPMCA, serial protein misfolding cyclic amplification; CPA, cell panel assay

Prions are infectious proteins that are responsible for transmissible spongiform encephalopathies (TSEs) and consist primarily of scrapie prion protein (PrP^{Sc}), a pathogenic isoform of the host-encoded cellular prion protein (PrP^C). The absence of nucleic acids as essential components of the infectious prions is the most striking feature associated to these diseases. Additionally, different prion strains have been isolated from animal diseases despite the lack of DNA or RNA molecules. Mounting evidence suggests that prion-strain-specific features segregate with different PrP^{Sc} conformational and aggregation states.

Strains are of practical relevance in prion diseases as they can drastically differ in many aspects, such as incubation period, PrP^{Sc} biochemical profile (e.g., electrophoretic mobility and glycoform ratio) and distribution of brain lesions. Importantly, such different features are maintained after inoculation of a prion strain into genetically identical hosts and are relatively stable across serial passages.

This review focuses on the characterization of prion strains and on the wide range of important implications that the study of prion strains involves.

Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases, such as Creutzfeldt-Jakob disease (CJD) in human, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids and scrapie in sheep, are a group of fatal neurodegenerative disorders. The major neuropathological hallmarks of TSEs are extensive spongiosis, neuronal cell loss in the central nervous system, gliosis,[1] and deposition of amyloid plaques.[2]

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Central to prion disease pathogenesis is the conversion of the host-encoded cellular prion protein (PrP^C) into a partially protease-resistant disease-associated isoform of PrP (PrP^{Sc}), which aggregates in the brain and is associated with infectivity.[3,4] According to the protein-only hypothesis, PrP^{Sc} is the essential causative agent of prion disease and transmission.[3-9] Once generated, PrP^{Sc} acts as a conformational template to promote the conversion of PrP^C into nascent PrP^{Sc},[9] possibly with the aid of one or more additional cellular cofactor(s).[10-13] Despite the fact that PrP^C and PrP^{Sc} have the same amino acid sequence, they differ from each other in several aspects: unlike PrP^C, PrP^{Sc} is insoluble in non-ionic detergents, partially resistant to proteinase K (PK) digestion and presents an increased content of β-sheet structure.[14-17]

Prion diseases are characterized by an extreme variability in their clinical presentation, neuropathological pattern and molecular subtype, implying the existence of different prion strains despite the absence of a nucleic acid as a part of the infectious prions.[18,19] Within the context of the protein-only hypothesis, prion strain specificity is believed to be encoded at the level of protein conformation, particularly of PrP^{Sc} tertiary structure.[20-23] This conformational diversity may arise from different factors, including the amino acid sequence of substrate PrP^C, the cell and tissue environment where the conversion takes place[11,13,24] and the process leading to the selection of the successful strain from the initiating PrP^{Sc} population.[25,26]

This review focuses on the principal experimental evidences that have led to the identification of prion strains, on their main biochemical features and on the phenomenon of prion strain mutation.

Evidence of the Existence of Prion Strains

The existence of different prion strains was first observed in 1961 by Pattison and Millson in goats.[27] In this report, goats infected with the same batch of scrapie agent developed different clinical

phenotypes, which were termed by the authors "scratching" and "drowsy," according to the pathological symptoms. After this first evidence, many studies have been conducted to understand the molecular basis underlying prion strain diversity. Initially the existence of prion strains seemed to be in conflict with the protein-only hypothesis,[28] as it was assumed that the different phenotypes found in animals were due to differences in the genetic information conveyed by the TSE-causing agent. Currently, however, several pieces of evidence support the hypothesis that the differences in clinical phenotypes between prion strains are encoded by different conformations of the various PrP^{Sc} molecules, which in turn result in differences in their biochemical properties.[20-23] In this paragraph some historical pieces of evidence that have led to the acceptance of this hypothesis will be analyzed.

In 1973, in a pioneering study aimed at demonstrating the existence of different prion strains, Fraser[29] showed that inbred C57BL and VM mice inoculated with brain homogenates from different Scrapie prion strains consistently developed a disease with distinct incubation times and histopathological lesions, and, importantly, that these differences could be stably propagated in subsequent passages. When prion strains were first described, the pathogenetic characteristics, such as incubation period, clinical signs and lesion profile, were the only means to discriminate one prion strain from another.[27,30] A first indication of the molecular basis of prion strain diversity came from a study in which scrapie-associated fibrils (SAF) were isolated and purified from animals infected with three different scrapie agents, ME7 and 139A in mice and 263K in hamster.[30] Mouse ME7 and 139A SAF differed from hamster 263K in terms of morphology, erythrocyte sedimentation rate and sensitivity to PK digestion. Importantly, SAF co-purified with infectivity in both animal systems; thus, it was hypothesized that the different molecular properties characterizing the different SAF may correlate with the biological and pathological differences, which are found among these agents.

Subsequently, the isolation and characterization of two biologically distinct strains of hamster-adapted transmissible mink encephalopathy (TME), the hyper (HY) and the drowsy (DY), significantly improved the understanding of the molecular bases of prion strains.[31] Indeed, purification and analysis of PrP^{Sc} molecules from hamsters infected with the HY and the DY strains revealed differences in terms of PrP^{Sc} sedimentation in N-lauroylsarcosine, sensitivity to PK digestion and electrophoretic mobility. Moreover, antigenic mapping of PrP^{Sc} with antibodies raised against different synthetic peptides showed a strain-specific difference in immunoreactivity in the N-terminus of the two PrP^{Sc} molecules. Taken together, these observations indicated that PrP^{Sc} from the two agent strains, although originating from the same host, differ in composition, conformation, or possibly both.

Further evidence providing a correlation between specific pathologic phenotypes and different biochemical characteristics of PrP^{Sc} molecules comes from a study by Medori et al.[32] In this work, they demonstrated that fatal familial insomnia (FFI)[33] of humans is a genetic prion disease linked to a specific PRNP gene mutation that causes a substitution of aspartic acid with asparagine at PrP position 178 (D178N). When the PrP^{Sc} associated with FFI was compared with the one associated with sporadic CJD (sCJD), the two proteins were found to be characterized by different electrophoretic mobility, allowing to speculate that these differences could be associated with different conformations of the two PrP^{Sc} analyzed.

In a parallel study, the D178N mutation was also associated to a familial CJD (fCJD) phenotype.[34] Therefore, since FFI and familial CJD (fCJD) were found to be associated to the same PRNP mutation (D178N), the possibility of a second genetic component capable of driving the development of the two different phenotypes was investigated. Indeed, it was found that the presence of a methionine in position 129 segregates with the D178N mutation, giving rise to FFI, while the presence of a valine in such position associated with the D178N mutation causes fCJD; this proves that the 129M/V polymorphism associated with a single mutation can determine different phenotypes. Furthermore, western blot (WB) analyses showed that the PK-resistant PrP^{Sc} has a different electrophoretic mobility in FFI and fCJD. In particular, the unglycosylated form of the PK-resistant PrP^{Sc} had an electrophoretic mobility of 19 kDa in FFI, whereas in fCJD it was approximately 21 kDa; thus, the phenotype differences among fCJD and FFI are associated to prion proteins with different biochemical features.[35]

Although initially the existence of prion strains in the context of the protein-only hypothesis was met with skepticism, the experimental evidence reviewed here, together with other studies,[27,29-31] clearly indicate that the different clinical features of prion diseases are associated to biochemical differences of the pathogenic protein.

Biochemical Aspects of Prion Strains

Prion strains can be classified according to different parameters. Incubation periods, profiles of histological damage and clinical signs are the main in vivo hallmarks that are used to differentiate prion strains.[36-38] In addition to the in vivo differences, each prion strain is associated to a specific cluster of biochemical features characterizing PrP^{Sc}. Among them, the most commonly used are electrophoretic mobility after PK digestion,[31,39,40] glycosylation pattern,[39-41] extent of PK resistance,[31] sedimentation[31] and resistance to denaturation by chaotropic agents.[31,42] Recently, differences among strains in the binding affinity for copper have been also described.[43]

These different strain-dependent features can be the consequence of different conformations of the PrP^{Sc} molecules. Indeed, several studies support the possibility that the different size of the fragments obtained after digestion with PK reflects the exposure of unique PK cleavage sites arising from different PrP^{Sc}.[44-46]

In particular, robust evidence comes from the analysis of two prion strains of hamster-adapted TME, the HY and the DY, which are biochemically distinguishable by a difference of approximately 2 kDa in the electrophoretic mobility of their PK-digested PrP^{Sc} fragments.[31] The Fourier transform infrared spectroscopy evaluation revealed distinct conformations in the two isolates as a result of differences in their β-sheet content, possibly explaining the biochemical difference in molecular weight

between the two strains.[46] Earlier investigations indicated that the HY and DY strains are characterized also by other important biochemical differences.[31] In particular it was shown that the sedimentation properties significantly differ between PrP isolated from uninfected, HY-infected and DY-infected brains. Indeed, after ultracentrifugation, PrP from uninfected brains remained in the supernatant, while PrP from DY- and HY-infected brains were found in both the supernatant and the pellet, although DY PrP was present at much lower levels in the pellet compared with HY PrP. Such findings suggest that different prion strains aggregate to a different extent, possibly due to differences in their tertiary and quaternary structure.[16,42]

An important point is that if these biochemical characteristics are strain-dependent, then they would have to be maintained through serial passages in the same host. Indeed, it has been shown that when transgenic mice expressing a chimeric mouse/human PrP^{C} are inoculated with different human prion strains, the electrophoretic profile of PrP^{Sc} after digestion with PK and the glycoform ratio of every specific strain are maintained,[22] indicating that the chimeric PrP^{C} can adopt different conformations depending on the source of inoculated PrP^{Sc}. However, it is not clear how the glycoform ratio can be preserved. Additionally, using monoclonal antibodies that efficiently immunoprecipitated native PrP^{Sc}, it has been shown that the differentially glycosylated molecules of native PrP^{Sc} are closely associated and always immunoprecipitate together, while PrP^{C} glycoforms can be selectively immunoprecipitated.[41] Furthermore, the ratio of glycoforms comprising immunoprecipitated native PrP^{Sc} from diverse prion strains was similar to those observed on denaturing WB. These studies are consistent with the view that the proportion of each glycoform incorporated into PrP^{Sc} is controlled in a strain-specific manner and that each PrP^{Sc} particle contains a mixture of glycoforms.

Another interesting feature characterizing different prion strains is represented by their binding selectivity for different metal ions.[43] Support for this observation comes from in vitro conversion studies of two different human prion strains isolated from two clinically different cases of classical CJD; according to these studies, the two strains can be converted one into the other merely by changing their metal ion occupancy. Possibly, copper and zinc binding can influence PrP^{Sc} conformation in a manner that is dependent on the bound metal ion. Thus, metal binding represents a possible strain-specific mechanism of post-translational modification of the PrP, as well as a potential mechanism for the generation of multiple prion strains.

Considering that the electrophoretic mobility of PrP^{Sc} molecules after PK digestion has been the main biochemical parameter used to discriminate between strains, but that distinct prion strains can display similar patterns of protease resistance, the development of a very sensitive conformation-dependent immunoassay (CDI) was pivotal to prove and study the different conformations of PrP^{Sc} molecules. This assay evaluates PrP isoforms by simultaneously following antibody binding to the denatured and native forms of the protein.[45] The analysis of different prion strains by means of this assay yields unique binding profiles, indicating that each prion strain has a PrP^{Sc} molecule with a specific conformation and that the biological and pathogenetic features of each strain can possibly be attributed to the different conformations of the protein.

In summary, the experimental data available to date strongly support the hypothesis that differences in prion strains lie in the diversity of structures that PrP^{Sc} can acquire, although the definitive evidence for the structural nature of the differences between prion strains is still missing.

Relationship Between Prion Strains Stability, Replication and Incubation Time

The contribution given by the studies of yeast prions has been of fundamental importance to better understand the phenomenon of prion strains. In fact, this model makes it possible to study in a simplified way the mechanisms that regulate prion replication. One of the most important features of prion strains discovered with yeast prions is that the frangibility (propensity to break) of the PrP^{Sc} fibrils is a strain-dependent characteristic.[47] Yeast prion strains have shown a heritable frangibility that is substantially different from strain to strain, but almost constant in PrP^{Sc} fibrils of the same strain.[20,47,48] For instance, some of the most infective yeast prion strains form amyloid fibrils that are likely to break, leading to a more aggressive tendency to replicate in vivo. Indeed, the analysis of three distinct prion conformations of yeast Sup35 and their in vivo phenotypes revealed that the Sup35 amyloid that caused the most aggressive replicating phenotype was actually the one displaying the slowest growth. Still, it was demonstrated that such slow growth was more than compensated for by an increase frangibility, which promoted the formation of new seeds and prion replication.[47]

These important observations in yeast have made it possible to find a correlation between the frangibility of prion aggregates and the rate of replication and therefore the strength of the strain's phenotype.

Although prion propagation in mammalian tissue may be a more complex process, recent evidence indicates that a kinetic description, similar to that validated for yeast, could be important for the understanding of the propagation of mammalian prion strains. Indeed, a recent study in mouse revealed that the degree of stability of the different prion strains can play a key role in determining the rate of PrP^{Sc} formation and the ensuing differences in the incubation times observed among different strains.[49] In particular, the conformational stability of 30 different prion isolates was assessed in a chaotrope-based assay, revealing a linear relationship between the concentration of guanidine hydrochloride (Gdn.HCl) required to denature 50% of PrP^{Sc} molecules ($[Gdn.HCl]_{1/2}$) and the incubation times, which ranged from 60 to 523 d in mice. Although the chaotrope-based assay does not measure protein fragmentation directly, such results suggest that decreasing PrP^{Sc} stability increases the fragmentation of PrP^{Sc} molecules, therefore allowing the exposure of more PrP^{Sc} surface, which is able to bind to PrP^{C}, resulting in an increased rate of PrP^{Sc} formation and hence a shortening of incubation times. This kind of correlation was also reproduced when a mathematical model was employed to investigate how the structural stability

of different aggregated forms can influence the kinetics of prion replication.[50] The simulation indicated that prion strains with higher conformational stability exhibit lower rates of breakage and vice versa.

Although the instability and breakage rate of PrP^{Sc} seem to have a key role in determining the incubation period of a prion strain, there are probably other mechanisms that further complicate the understanding of this strain-specific feature. In 2011 Ayers et al.[51] sought to understand the relationship between prion strain characteristics and the predictability of the incubation period. The amplification efficiency and the stability of eight hamster-adapted prion strains were examined and correlated with the resulting incubation period of disease and processing of PrP^{Sc} in neurons and glia. In contrast to what was observed in murine prion strains, short incubation period strains were characterized by a higher conformational stability of the PrP^{Sc} and a more efficient replication. As was also speculated by the authors, this could be due to the presence of a minor subpopulation of PrP^{Sc} molecules that is conformationally less stable, and that may be masked by an excess of conformationally more stable PrP^{Sc}. However, other factors influencing the incubation time such as differences in prion structure or cellular processing, cannot be excluded. Indeed, strain- and cell-specific variations in the proteolytic processing of PrP^{Sc} was observed for the strains under study, suggesting a relationship between the extent of truncation of PrP^{Sc} within the soma of neurons and the corresponding incubation periods. The short-incubation period strains were characterized by the accumulation of longer intact portion of C-terminal protein in the soma of neurons, astrocytes and microglia, while long-incubation prion strains PrP^{Sc} did not accumulate to detectable levels in the soma of neurons but were detected in glia similar to short incubation period strains, suggesting a strain-specific clearance of PrP^{Sc} in neurons, more efficient for the latter. These pieces of evidence strongly suggest that the strain-encoded relationship between PrP^{Sc} replication, stability and processing in neurons is predictive of the incubation period of disease.

Prion stability, in addition to the incubation time, has been also correlated with the ability of the different prion strains to invade the central nervous system (CNS).[52] Using mouse-adapted prion strains it was demonstrated that highly neuroinvasive prion strains are conformationally unstable in denaturing conditions and efficiently generate PrP^{Sc} molecules within a short incubation period. Additionally, the neuroinvasive strains formed diffuse, non-fibrillar PrP aggregates in the CNS and mice rapidly progressed to terminal disease, while weakly neuroinvasive strains form dense, congophilic, fibrillar plaques and mice progressed to terminal disease more slowly. These findings suggest that the non-fibrillar PrP aggregates are more toxic and this observation is consistent with recent studies indicating that smaller aggregates of the mammalian prion protein, which should be more readily generated by strains that form fragile particles, are markedly more infectious than larger aggregates.[53] Moreover, this study can have important clinical implications: indeed, a natural infection with more stable, weakly or non-neuroinvasive strains with long incubation times may yield asymptomatic, long-term carriers of infectious, posing a risk for transmission to other humans or animal.

Taken together, the available experimental evidence suggests that the variability in the brittleness of the aggregates could represent a key mechanism by which prion conformations dictate the strength of strain phenotype. These observations can have important therapeutic implications; indeed, efforts to minimize the impact of protein aggregation could be designed not only to slow aggregate formation and growth, but also to decrease the rate of fragmentation by increasing fiber stability.

Strain Mutation

Typically, referring to viruses or bacteria, a strain mutation corresponds to a modification of the genetic information. It has already been explained in this review that the genetic content of the PrP^{C} and its pathological isoforms, PrP^{Sc}, is the same. In fact, in the case of prions, "strain mutation" refers to a conformational change of the PrP^{Sc} during its propagation in a host.[54]

This phenomenon has been known for a long time[55,56] and can be demonstrated through different approaches of strain typization, such as sedimentation,[31] light scattering,[57] transmission electron microscopy,[58] and atomic force microscopy[58] for assembly; through structural change studies, by circular dichroism,[42] limited proteolysis,[59] and dye binding,[60-62] and through stability studies, by SDS solubility.[37] Strain mutations occur when a propagated strain does not maintain the same biochemical and pathogenetic characteristic of the inoculum, thereby resulting in the propagation of a new strain.

There is an important aspect that has to be considered when studying prion strains. Indeed, it is still an open question whether strains undergo a real mutation process or whether the resulting mutants were already present in the inoculum but at a very low titer. For instance, in scrapie and CJD isolates, the coexistence of multiple PrP^{Sc} types is found.[63,64] Indeed, in a single isolate, considerable differences can be detected, in terms of glycosylation patterns and electrophoretic mobility of the PK-resistant PrP^{Sc} fragments.[39-41] This great heterogeneity is also highlighted by thermal inactivation studies in which PrP^{Sc} subpopulations with different stability can be appreciated.[65] As mentioned above, these observations suggest two possible explanations for the emergence of prion strain mutants: the first one considers that strains exist as a single clone and if a different strain is propagated it can be assumed that a strain shift has occurred (**Fig. 1A**); the second possible explanation instead considers that strains exist as a pool of different molecular species with a dominant type of PrP^{Sc} that is preferentially propagated in a given host, but in a different host a minor PrP^{Sc} type can be favored, causing a shift in the strain (**Fig. 1B**). The second theory seems to better explain the high level of heterogeneity that is registered from experimental data, although the possibility that prion strains can infect the host as a single clone cannot be excluded.

It can be hypothesized that among the different conformations of PrP^{Sc} coexisting in the same isolate, only a fraction is able to replicate in a certain species, in a manner that is dependent on the sequence and conformation of the PrP^{C}, on the natural clearance

capacity of the infected cells[66-69] and on the presence of cofactors.[11,13,24] In such a model a prion strain behaves as a quasi-species and represents a pool of molecules that are kept under control by the host.[70] Hence, in a given host, a strain will be constituted of a principal molecular component and a minor one. Accordingly, transmission between species is possible primarily when there is compatibility between the conformation of PrP^{Sc} of the infectious agent and of the PrP^{C} of the new host, but it also depends on cell and tissue environment.[71-73] This phenomenon is known as species barrier. Although the primary structure of the PrP^{C} is very conserved between species, some amino acidic residues are different, resulting in PrP^{C} with distinct molecular conformations. As a consequence, when a prion strain of one species infects an animal of a different species there are two possible scenarios. The first is that the infectious PrP^{Sc} has a conformation that is not compatible with the PrP^{C} conformation of the host, resulting in non-conversion; in this case, the species barrier is defined as absolute. The second possibility is that the PrP^{Sc} conformation is compatible with the PrP^{C} conformation of the host, allowing conversion and, therefore, infection. In this case the propagated strain can be identical to the infecting unit[74] or can change into a different prion strain characterized by a different conformation.[20,42] Thus, this type of transmission can facilitate the replication of the minor molecular component, if it is favored in the new host (apparent mutation), or the generation of a new PrP^{Sc} different from the one of the inoculum (direct mutation)[36,75] (**Fig. 1B**).

The mutation of a strain can arise both during the transmission of the inoculum to a host of a different species, and within a single species.[76] In the first case the sequence of the PrP^{C} of the host is different from the one of the

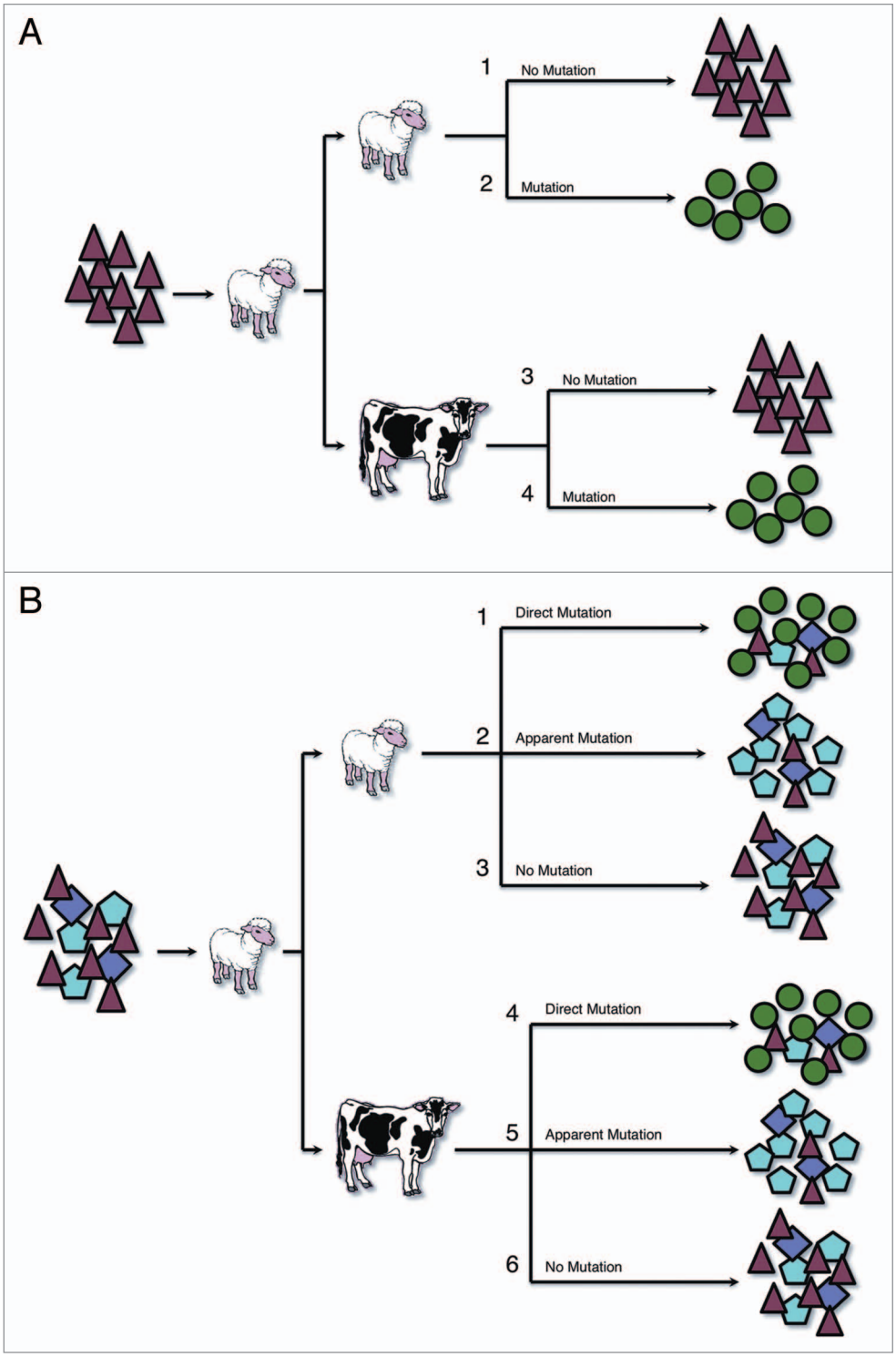

Figure 1. Hypothesis of prion selection during propagation. (**A**) Prions infect as a single clone. The strain can remain the same of the inoculum (**1 and 3**) or it can undergo an adaptation process due to the presence of different cofactors or a different cellular environment (transmission within the same species) (**2**), or due to the species barrier (transmission between different species) (**4**). (**B**) Prions infect as a mixture of different conformations of PrP^{Sc}. When the transmission is within the same species a new PrP^{Sc} type can be generated (**1**), a minor type can emerge becoming the major one (**2**), or the pool of PrP^{Sc} can remain the same of the inoculum (**3**). An analogous process can occur when the transmission is between different species and the pool of different conformations of PrP^{Sc} must adapt to the new host, resulting in the propagation of a different pool of PrP^{Sc} from which a new PrP^{Sc} type can be generated (**4**), or in the selection of a minor strain that becomes the major one (**5**). Possibly, the propagated pool of PrP^{Sc} can be identical to the infecting unit (**6**).

inoculum and the result of the conversion process can be a different strain[39] because of the species barrier, as described above. In the second case the PRNP gene, coding for the PrP^{C}, is identical between donor and host, suggesting that there are possibly also other mechanisms involved in strain selection,[77] such as cellular environment, polymorphisms and cofactors.[78] Many studies have been performed to reveal the nature of the cofactors that may be involved. It has been demonstrated that RNA molecules are important cofactors for the propagation of hamster prions in vitro,[11,13,79] but they are not necessary for mouse and vole prions,[80] whose replication in vitro is supported by phosphatidylethanolamine (PE).[80,81] Protein chaperones, such as Hsp104 and GroEL, which employ different mechanisms to affect the conformation and physical state of other proteins,[82] are able to promote in vitro the conversion process of hamster PrP, while chemical chaperones, such as sucrose, trehalose and dimethyl sulfoxide, inhibit the conversion process.[24] Additionally, a very recent study showed that a change in cofactors might be sufficient to cause a change in prion strain properties. Indeed, strain properties of recombinant PrP^{Sc} generated from recombinant PrP^{C} by sPMCA (serial protein misfolding cyclic amplification)[83] in the presence of cofactors such as 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) and RNA molecules, could be altered in subsequent in vitro passages by changing the cofactor element to phosphatidylethanolamine (PE).[13] Furthermore, in vitro propagation with only PE induced the conversion of three different strains into a single strain with unique properties, indicating that a single cofactor can modulate the propagation of different strains and lead to the selection of a single, phenotypically distinct new strain. Such observations strongly indicate that cofactors are important constituent of infectious prions.

The influence of different cofactors in determining prion strains propagation properties can possibly justify the phenomenon of the cellular prion tropism that characterizes different prion strains; this was elegantly shown by a work of Weissmann's group through the cell panel assay (CPA).[84] Indeed, they showed how distinct prion strains are able to replicate differently depending on the cell line that is examined. All the cell lines employed in this study (CAD5, R33, LD9 e PK1) supported the replication of the 22L strain (scrapie prion adapted to mice), but only the CAD5 cell line supported the replication of the 301C strain (BSE prion adapted to mice). Moreover, each tested strain was able to replicate in the CAD5 cell line, but in the R33 cell line only the 22L strain could replicate. Thus, different cell types in one host can offer different environments, possibly resulting in a different selection pressure on the strains.[84-86] It has been long debated whether the infection of a lymphotropic strain, which colonizes the lymphoid tissue with a long latency before the neuroinvasion, is the result of a selection process whereby a strain capable of invading the CNS with a high efficacy must be selected. This hypothesis could be investigated in vCJD patients in which the presence of PrP^{Sc} molecules have been already demonstrated in various lymphoid tissues in the early stages of the disease, before the onset of the neurological disorders.[87,88]

Support for this hypothesis comes from a recent study[54] which demonstrated that the propagation of the same prion strain (22L) in cultured cells and in mice results in two different prion strains, termed by the authors cell-derived and brain-derived prions. In order to determine the differences between the two derived prion strains, CPA was employed.[84] The analysis showed that cell-derived prions were unable to infect R33 cells or PK1 cells in the presence of swainsonine (an inhibitor of Golgi α-mannosidase II that impairs formation of complex N-linked glycans), while brain-derived prions were able to infect both cell types in the presence of swainsonine. Interestingly, a time-point analysis showed that nine days after infection of PK1 cells with brain-derived prions, and after a first split, the secreted prions maintain the same characteristics of the infectious unit; however, after subsequent splits, corresponding to 40 cell replication cycles, the properties of these prions were those of the cell-derived prions. This result indicates that the brain-derived prion population may be heterogeneous and that it undergoes a "Darwinian evolution" process when transferred into cells, in which the most advantaged prion is selectively amplified.

From a physical point of view, the great heterogeneity within prion strains is likely due to conformational fluctuations of the PrP^{Sc}; given the reversibility and the frequency of this phenomenon, it can be hypothesized that the activation energy required for the conformation transition among PrP^{Sc} molecules of the same strain is low, thus allowing for the formation of a considerable number of different conformations.[70] If the environment changes, then the free energy profile can also change, and a subtype, which at first was less represented, can be favored, causing a shift of the dominant molecular species.[89]

To conclude, viruses and prion populations have a common aspect in that they both exist in a heterogeneous state due to mutations: viruses by changing their genetic content, and prions by modifying their conformation. As a consequence, they are both under the host's selective pressure, as viruses need to evade the control of the immune system, while prions must avoid cellular clearance mechanisms.

Prions and TSEs: Strain Divergence or Convergence?

In this paragraph, the behavior of prion strains in two human TSEs, sCJD and vCJD, will be analyzed. In particular the aim of this paragraph is to analyze the evolution of prion strains to understand whether, in these two TSEs, strains tend to converge or to diverge in their evolution.

Before analyzing this important characteristic, it is fundamental to explain the terminology that will be used. We are going to refer to the molecular classification by Parchi et al.,[90] which is based on the polymorphism in position 129 (MM, MV or VV) and the type of PrP^{Sc} (type 1, in which the unglycosylated form of the protease-resistant PrP^{Sc} has an electrophoretic mobility of 21 kDa; type 2, in which the unglycosylated form of the protease-resistant PrP^{Sc} has an electrophoretic mobility of 19 kDa). This classification arises from the hypothesis that if the polymorphism 129 can modulate the phenotype of the familial prion diseases (fCJD and FFI, as explained earlier in this review), then probably it can modulate also that of sporadic prion diseases,

justifying their heterogeneity. According to this hypothesis, the cases affected by sCJD were divided into six groups according to the genotype of the polymorphism in position 129 and the type of PrP^{Sc}. Then, the phenotypes of every group were analyzed to evaluate the homogeneity within every group. The results have permitted a molecular sub-classification of the sCJD.[90,91] However, this classification seems not to be sufficient to explain the complexity of the sporadic form of CJD. In fact, in some molecular subtypes, additional variants have been reported, such as MM or VV patients with amyloid plaques, which are absent in the majority of patients with these genotypes.[44] Moreover, among patients belonging to the same subgroup, important phenotypic differences can be found, such as, for instance, the extent of neuronal loss or PrP^{Sc} deposition differences.[92]

Even at the biochemical level the complexity is higher: indeed, aside from the migratory differences of the PrP^{Sc} of types 1 and 2, there are other properties that could be important during the propagation of the strain, like the presence of other fragments derived from differential cleavage at the C- and N-terminus of the protein, which probably coincide with the presence of other forms of PrP^{Sc} with different resistance to PK digestion.[44] All these molecular classifications are based upon the principle that in all CNS districts the type of PrP^{Sc} is the same, but there are pieces of evidence pointing to the fact that different types of PrP^{Sc} can be found in different brain areas.[64,93] The first evidence of the presence of more than one form of PrP^{Sc} in the brain of a sCJD patient was reported by Puoti in 1999.[94] These different types of PrP^{Sc} can be found to coexist in the same brain region or they can infect distinct districts. Such co-infection influences the vacuolization and the amyloid aggregates formation.[95] Even the ratio between the different glycoforms is determined in a region-specific manner according to the type of PrP^{Sc} (1 or 2) and the genotype of codon 129.

The high degree of phenotypic heterogeneity characterizing sCJD[90] can lead to the conclusion that transmission studies will probably identify a broad panel of different prions with a great divergence between strains. However, quite surprisingly, many of the recent studies focusing on the characterization of sCJD subtypes have shown that there is a strong tendency to converge to a limited number of strains. This aspect can find an explanation considering the selection conditions, already described in this review, mediated by the environment in which the prion replicates and by the differences in the amino acid sequence of the PrP^{C}. In particular, studies with bank voles[96] and mice[97] lead to results that support the idea that there are two principal strains responsible of the sCJD, M1 and V2, and two potential strains, M2 and V1, which need further studies to be confirmed.

Different is the case of vCJD. vCJD has been observed in 12 different countries, but in every registered case the same clinical and pathological characteristics have been found.[39] In particular, the PrP^{Sc} responsible of the vCJD shows a peculiar WB profile, with the unglycosylated form of the protease-resistant PrP^{Sc} of 19 kDa (type 2) and a higher representation of the diglycosilated PrP^{Sc} (PrP^{Sc} 2B) compared with sCJD.[39] Nevertheless, using specific antibodies against type 1 PrP^{Sc}, a small amount of PrP^{Sc} type 1 with a high percentage of diglycosilated form can be detected in association with PrP^{Sc} 2B.[98] The 2B type is a useful marker for identifying the replication of BSE prions also in other species, including non-human primates.[99] In addition, unlike sporadic and genetic CJD, in vCJD the same biological marker (2B type) has been found in all the analyzed brain areas.[100] This strong biochemical and pathological homogeneity is in agreement with the hypothesis of the existence of a unique strain. However, unexpectedly, typization experiments of the strains in different transgenic models have given divergent results. In one of these studies, in a context of homotropic transmission, transgenic mice expressing high levels of human PrP^{C}-M129 were inoculated with vCJD isolates coming from France and from the UK.[101] All of the French isolates propagated as vCJD, with abundant amyloid plaques and presence of PrP^{Sc} 2B.[102] Instead, the isolates from the UK led to the propagation of either vCJD or sCJD.[103] In particular, the incubation time was shorter and the lesion profile was different compared with the one obtained with the propagation of the classical vCJD strain. Moreover, early replication of the typical agent of the vCJD in lymphoid tissues was detected, indicating that both strains were present in the inoculum.

This new strain with phenotypical features that were similar to sCJD was found to be of type 1 and the transmission in transgenic mice expressing the bovine PrP^{C} failed, unlike the vCJD classical strain (Type 2B).[26] The idea that the infection of vCJD contains a minor component of sCJD prions is supported by many pieces of evidence such as the presence of this prion strain at the first passage or the persistence of both types of PrP^{Sc} through serial passages in mice.[98] In conclusion, although vCJD is one of the most standardized phenotypes among the prion human diseases characterized by a typical form of PrP^{Sc}, the transmission studies of vCJD have shown the great potential of divergence of prions, contrary to the results obtained from the studies of sCJD. This data challenge our ability to recognize the pathologies that can derive from the divergence of the BSE strains when they infect humans, both at the pathological and at the biochemical level.

Conclusion

The discovery of prions has led to new interpretations of the pathogenetic mechanism of protein misfolding diseases. Indeed, the common thought was that a protein misfolding disease could only be caused by a mutation in the primary sequence of an endogenous protein, but the discovery of prions changed this view. In fact, it was demonstrated that a seed of misfolded protein can arise from an exogenous infectious protein, which is able to act as a template or as a catalyst for the formation of new aberrant protein.[5,6] Importantly, new evidence shows how processes similar to those described for prions could be implicated in the propagation of misfolded proteins of other neurodegenerative pathologies like Alzheimer disease, Parkinson disease, Huntington disease and amyotrophic lateral sclerosis.[104,105]

Certainly, one of the most puzzling aspects in the prion field is the existence of different strains of an infectious protein. Nevertheless, such diversity can be accommodated within the protein-only hypothesis, as several robust pieces of experimental

evidence indicate that strain-specificity is encoded at the level of the different conformations that the pathogenic protein can adopt. The identification of factors and mechanisms influencing the generation of new prion strains or the selection, from a conformationally heterogeneous PrP^{Sc} population, of the most suitable prion conformation in a specific environment, represents an important milestone toward the understanding of the mechanisms of prion strain diversity, which can have fundamental clinical and therapeutic implications. Although considerable advances have been made in the understanding of the phenomenon of prion strains, many pieces of information are still missing, foremost among them the definitive evidence for the structural nature of the differences between prion strains.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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