Subclinical prion infection in humans and animals

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Transmission of prion diseases between mammalian species is limited by a so-called ‘species’ or ‘transmission’ barrier. Recognition of prion transmission usually relies on the appearance of clinical symptoms in inoculated animals and the interval between inoculation and appearance of clinical disease is designated incubation period. At some point during this clinically silent period, neuropathological and biochemical changes as well as accumulation of prions in the brain can be detected and this stage can be called preclinical prion disease. Recently, several lines of evidence have suggested that subclinical forms of prion disease exist, in which high levels of infectivity and PrPSc are found in animals that do not develop clinically apparent disease during a normal life-span. Such asymptomatic prion ‘carrier’ states challenge our current understanding of pathogenesis as well as of the molecular basis of barriers to transmission. Subclinical as well as preclinical/clinical prion disease may be relevant when analysing the risk to public health of potential sources of prion exposure.

Prion diseases of humans and animals are transmissible disorders associated with distinct neuropathological lesions and the presence in the central nervous system (CNS) of an abnormal isoform, PrPSc, of the host-encoded prion protein, PrPC. PrPSc differs from PrPC in being partially protease-resistant, rich in β-sheet structure, and occurring in an aggregated form. Prion diseases are unique in that they may be at the same time inherited and transmissible. Much evidence exists to suggest that the principal or sole component of the prion is PrPSc, although the exact structure of the infectious particle has yet to be determined. Prions are unique infectious agents in that they do not appear to contain any nucleic acid and are relatively resistant to many physical and chemical treatments that readily inactivate conventional infectious agents. These observations led to the protein-only hypothesis of prion replication, which proposes that prions are propagated by autocatalytic conversion of PrPC to PrPSc, leading to accumulation of infectivity, PrPSc and to the development of clinical disease. One of the key observations supporting the role of PrPC in the pathogenesis of prion diseases is the demonstration that mice lacking PrPC are resistant to infection with prions.
Prion strains and transmission barriers

The existence of multiple isolates, or strains, of prions was difficult to accommodate within the protein-only hypothesis, that in its current form excludes the presence of a conventional informational molecule other than PrP. Different prion strains produce distinct phenotypes, as defined by the pattern of neuropathological lesions (the ‘lesion profile’) and length of the incubation period when inoculated into susceptible animals. Several strains of naturally occurring sheep scrapie have been isolated by biological cloning in mice. The finding that different prion strains can be propagated in in-bred mice expressing prion protein with the same primary sequence and that at least some strains can be re-isolated unchanged after passage through an intermediate host expressing PrP with a different primary sequence shows that strain variation is not necessarily dependent on the primary sequence of the host prion protein gene. This highlights the remarkable biology underlying these diseases, whose causative agent, a protein entity apparently devoid of a nucleic acid genome, somehow encodes heritable properties.

Several lines of evidence now suggest that prion strain information is encoded by PrPSc itself. For example, the hyper (HY) and drowsy (DY) strains of transmissible mink encephalopathy (TME), which can be passaged in hamsters and distinguished by their disease phenotype, differ in the physicochemical properties of PrPSc deposited in the brains of the affected animals. Limited proteolytic cleavage of PrPSc from infected hamsters, followed by Western blot analysis, reveals distinct strain-specific banding patterns. The different mobilities are due to cleavage at different amino-proximal sites, whose exposure to proteinase may reflect distinct conformations of PrPSc characteristic for each strain. Differences in the banding pattern of protease-treated PrPSc are also observed in cases of CJD in humans that present with distinct phenotypes; crucially, these biochemical differences can be transmitted to PrP in transgenic mice expressing human PrP.

Experiments performed since the early 1960s have demonstrated that transmission of prion disease from one species to another is considerably less efficient than within species, and the term ‘species barrier’ was coined by Pattison in 1965. The effect of a species barrier is to lengthen the mean incubation period, increase the range of incubation periods and to reduce the fraction (in some examples to zero) of inoculated animals succumbing to clinical disease. When, following transmission across a species barrier, brain homogenate from the sick recipient is passaged through the same species, the incubation period shortens and becomes much more consistent. This marked difference in transmission parameters between primary and second passage is diagnostic for the
existence of a species barrier and the extent of the fall provides a guide to the size of the barrier.

Several parameters are known to influence the transmissibility of prions both across and within species. These include polymorphisms in the prion protein gene that give rise to differences in PrPC primary structure between donor and host, prion strain type, the route of inoculation (e.g. peripheral versus intracerebral) and the dose. The effect of a very substantial species barrier is that few, if any, animals succumb to disease on primary passage, and if they do so, then only after incubation periods approaching the natural life-span of the animal.

Abrogation of species barriers has been achieved using transgenic mice. Inoculation of transgenic mice that overexpress hamster PrPC (and continue to express normal levels of mouse PrP C) with either mouse or hamster prions results in clinical disease and the accumulation of PrPSc derived from mouse or hamster PrPC, respectively12. These findings led to the suggestion that the PrP primary structure is the major determinant of species barriers. Further experiments with transgenic mice expressing a variety of mammalian PrP genes have been useful in assessing barriers to prion disease. Transgenic mice devoid of mouse PrP but expressing human PrP lack a barrier to infection with human prions from sporadic CJD (sCJD), but not variant CJD (vCJD), cases7,13. Such mice have been used to assess risk factors associated with human exposure to BSE and to support the view that vCJD and BSE are caused by exposure to the same prion strain10,13.

Although a strong barrier to the development of clinical disease has frequently been observed when prions are transferred from one mammalian species to another, there are also instances where this is much less the case. An important example is the transmission, both natural and experimental, of cattle BSE prions to a wide variety of other species, where in some cases only a relatively low barrier to clinical disease is observed. This was observed in the transmission of cattle BSE into wild-type mice, which succumb to clinical disease with a low, but distinct, barrier to the development of clinical disease10,14. On the other hand, a barrier to transmission, as determined by appearance of clinical disease, may also be due to differences in the prion strain rather than to differences in the PrPC of donor and recipient. For example, transgenic mice devoid of mouse PrP but expressing human PrP show no species barrier to human sCJD prions but a pronounced barrier to human vCJD prions, even though the human donors (of the PRNP 129MM genotype) expressed prion proteins of identical primary structure10. Hence, it has been proposed that barriers to prion transmissibility be referred to as ‘transmission barriers’, to reflect the complex relationship between species, PrP primary structure and prion strain type, and the contribution these parameters make to the disease process15.
Subclinical prion infection in animal models

It has long been argued that the incubation period of a prion disease can exceed the natural life-span of an animal. After inoculation of mice with mouse-adapted scrapie prions, prion titres typically rise first in spleen and later in brain, and histopathological changes develop in the CNS during a long incubation period devoid of clinical symptoms. Under certain circumstances, the incubation period approaches the natural life-span of the animal and, if it were to exceed it, the brain and other tissues could harbour significant levels of infectious prions even though clinical signs of infection never appear. In reality, one can of course not determine whether or not clinical symptoms would have appeared had the animals lived longer, underlining the difficulty in distinguishing between preclinical and subclinical disease. Thus, clinically, asymptomatic animals may have significant infectious titres in brain and other tissues. However, there may also be subclinical, as distinct from such preclinical, forms of prion infection, where animals become asymptomatic carriers of infectivity and would not develop clinical disease. Such carrier states are well recognised in other infectious diseases. For example, chronic hepatitis C may in some cases persist for a life-time, as evidenced by constant replication of the virus and histopathological changes in the liver, without causing clinical symptoms and thus represents a subclinical disease state, while AIDS presents with a long preclinical phase that almost invariably results in severe clinical disease. In prion diseases, where incubation periods are extremely prolonged, distinction between subclinical and preclinical states is difficult. It certainly can be argued that animals dying after a typical life-span without clinical signs of prion disease but harbouring high levels of infectivity represent the late preclinical stage of ‘transmissions’ where the ‘incubation period’ exceeds the normal life-span. The distinction between the terms subclinical and preclinical is essentially a semantic one in this context. We defined the term subclinical prion infection operationally to refer to animals in which prion replication is occurring, but which have not developed clinical signs of prion disease during a normal life-span.

Subclinical prion infection has been described in rodent models that exhibit a transmission barrier between hamster prions (263K or Sc237 strain) and wild-type mice. As shown by two independent studies, wild-type mice inoculated intracerebrally with high doses of 263K hamster prions do not develop clinical disease. However, 1–2 years after inoculation, the brains contain high levels of prions that, when inoculated into further mice and hamsters, cause clinical prion disease (summarised in Table 1). The PrPSc found in the brains of the inoculated mice was derived from mouse and not hamster PrPSc, suggesting...
replication of mouse PrPSc and not due to the presence of the residual inoculum\textsuperscript{17,18}. The prion content of the brains was significantly higher than the amount originally inoculated, showing that there had been prion replication, and approached levels found in animals at terminal stages of clinical prion disease. The fact that these mice were able to

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*263K and Sc237 hamster prions are reported to be the same strain (Scott \textit{et al}.\textsuperscript{12}).
tolerate such high levels of infectious prions without showing clinical symptoms is remarkable and underlines how little we currently know about the neurotoxic pathway(s) involved in prion diseases. The findings also stress that prion infection may, unless revealed by bio-assay or by the presence of PrPSc, go undetected in apparently healthy animals.

The occurrence of subclinical prion infections is not restricted to instances involving species barriers. In studies investigating the role of the lymphoreticular system in the pathogenesis of prion disease, B-cell-deficient mice appeared resistant to peripheral prion infection as judged by their failure to develop clinical disease. They were, however, susceptible to prion infection when inoculated intracerebrally, exhibiting incubation periods similar to those seen in wild-type control animals. Although peripherally challenged immunodeficient mice showed no clinical signs of scrapie, marked accumulation of PrPSc in their brains was observed. The subclinically infected animals harboured significant prion levels in their brain that in some cases exceeded those in terminally sick, wild-type, controls. Subclinical prion infection has also been described in Tga20 mice (transgenic mice overexpressing mouse PrP) injected with low doses of RML or ME-7 prions. The response of these animals oscillated between a healthy appearance and mild scrapie symptoms. However, animals that developed an ataxic syndrome always progressed to terminal stages of disease. Surprisingly, the subclinically infected animals that were sacrificed at over 200 days after inoculation contained similar levels of infectivity as terminally sick animals. This again shows that prion titres may reach maximum levels without eliciting clinical disease. Therefore, in judging susceptibility to infection of an animal exposed to prions, it is not sufficient to monitor for clinical signs, but it is necessary to assay for PrPSc and/or prion infectivity.

**Subclinical prion infection and risks to public health**

The occurrence of clinically silent prion infection, be it sub- or preclinical, has several implications for public health, most notably regarding the iatrogenic transmission from apparently healthy individuals.

Iatrogenic CJD in humans can have incubation periods longer than 30 years. Such cases have resulted from the use of pituitary-derived hormone treatments, dura mater grafts, corneal grafts and contaminated neurosurgical instruments. Although such cases are relatively rare, they underline the need for identifying potential sources of infection and the establishment of effective control procedures. It is possible, although not formally demonstrated, that some cases of iatrogenic CJD may have been caused by infection from silent carriers.

Of critical importance to humans is the emergence of vCJD, which has been epidemiologically and experimentally linked to dietary exposure to
cattle BSE prions\textsuperscript{10,14}. In sCJD, PrP\textsuperscript{Sc} is undetectable using current methods in tissues peripheral to the CNS\textsuperscript{23,24}, and inoculation into primates of these tissues only occasionally results in clinical disease\textsuperscript{25}. However, the tissue distribution of PrP\textsuperscript{Sc} is starkly different in cases of vCJD, where lymphoreticular tissues such as spleen, lymph nodes and tonsils as well as specific regions of the eye show PrP\textsuperscript{Sc} positivity\textsuperscript{23,24}. This feature of vCJD has been exploited diagnostically, with tonsil biopsy testing for the presence of PrP\textsuperscript{Sc} proving to be a sensitive and specific \textit{ante-mortem} test for vCJD\textsuperscript{23,26}. With the large number of individuals exposed to BSE prions and the uncertainty of the number of clinical cases of vCJD that will occur in the future, clinically silent vCJD prion infection must remain a consideration when applying risk assessments to the iatrogenic transmission of this disease. Importantly, the demonstration of infectivity and PrP\textsuperscript{Sc} in tonsil tissue provides a strong case for the use of disposable instruments in tonsillectomy procedures, one of the most common surgical procedures.

Concerns in this regard were highlighted by the demonstration that transgenic mice expressing human PrP encoding methionine at codon 129 (the \textit{PRNP} genotype of all cases of vCJD recorded to date) demonstrated a very high level of subclinical infection on challenge with BSE or vCJD prions, raising the possibility that both primary BSE prion infection of humans and iatrogenic exposure to vCJD prions could lead to more subclinical infections than clinically apparent cases\textsuperscript{9}.

The issue of clinically silent prion infection in cattle and other species exposed to BSE prions is also of obvious importance to public health. PrP\textsuperscript{Sc} can, unsurprisingly, be demonstrated in cattle with no obvious symptoms of BSE, using a Western blot assay\textsuperscript{27}. Another study, which examined specific regions of bovine brain with a tissue-slice-based immunoassay, also gave PrP\textsuperscript{Sc}-positive results in a screen of apparently healthy cattle\textsuperscript{28}. The results from experimental studies of subclinical prion disease, demonstrating that animals can harbour high levels of infectious prions in their brains without showing any clinical signs of disease, emphasises the need to test for prion infection as opposed to merely clinically-apparent prion disease in slaughtered cattle. In humans, because of the tissue distribution of PrP\textsuperscript{Sc} outside the CNS in vCJD, prevalence screening for the presence of PrP\textsuperscript{Sc} in tonsil tissue taken from routine tonsillectomies may provide information as to the number of individuals who may be incubating this disease\textsuperscript{15}.

**Biological implications of subclinical forms of prion disease**

Interesting biological implications are raised by the recognition of subclinical forms of prion infection. In view of the fact that high levels of prions and PrP\textsuperscript{Sc} accumulate in the brains of clinically healthy
animals, the question of whether PrPSc itself is neurotoxic or not needs to be addressed. Several lines of evidence suggest that PrPSc itself may not be highly neurotoxic. Büeler et al. reported that by 20 weeks after inoculation with RML scrapie prions, mice carrying only one functional PrP allele accumulated levels of infectivity and PrPSc as high as wild-type animals, but remained healthy until almost a year after inoculation, while wild-type animals died by about 26 weeks. In another experiment, PrP over-expressing brain tissue was grafted into PrP knockout mice which were then inoculated with prions. While the grafted, PrP-overexpressing tissue developed hallmark signs of prion disease, such as spongiform change and accumulation of PrPSc, the surrounding tissue (in which PrP was not expressed) suffered no deleterious effects. Besides confirming the absolute requirement for PrP expression to propagate these diseases, these experiments failed to demonstrate any neurotoxicity to surrounding areas of the graft. Also, in the human prion disease fatal familial insomnial there are severe clinical symptoms although the levels of PrPSc are low or undetectable; some of these cases can be transmitted to susceptible animals, confirming the presence of infectious prions. Transmission of BSE to wild-type mice resulting in characteristic prion disease in the absence of detectable PrPSc has also been reported.

What these observations suggest is that PrPSc, as defined by its physicochemical properties of insolubility and partial resistance to protease treatment, while being an accurate marker of prion infection, may not be the neurotoxic molecule responsible for the pathogenesis of these diseases. It is possible that the neurotoxic species may be a different form of PrP, possibly an intermediate formed in the conversion of PrPC to PrPSc, which remains to be defined at the molecular level. Perhaps PrPSc is a relatively inert end-product, with the rate of neurodegeneration governed by the rate at which the neurotoxic intermediate is formed.

**Conclusions**

The demonstration of PrPSc and infectivity in mice thought to be resistant to prions from a different species as well as clinically silent prion infection after same-species passage questions our current understanding of prion ‘species barriers’ and mechanisms of pathogenicity. Assessment of ‘species barriers’ have relied on the failure of inoculated animals to develop clinical symptoms. From the studies discussed above, it now appears prudent to include molecular and neuropathological assessment of clinically healthy inoculated animals to exclude the possibility of subclinical prion infection. The
possibility of iatrogenic transmission of vCJD is of obvious concern in public health issues and, given the results obtained from the experimental studies discussed above, warrants appropriate infection control guidelines to be considered when using surgical instruments and ‘at-risk’ tissues from apparently healthy humans for medical procedures. Further research into developing early, non-invasive diagnostic tests and defining molecular pathways involved in the long, clinically silent period of these diseases will be of great importance. Resulting insights could lead to early therapeutic strategies for these diseases and perhaps help solve the most enigmatic problem in the prion field at present – defining the neurotoxic and infectious entity at the molecular level.

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