Transmissible spongiform encephalopathies and tissue cell culture

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Abstract

The discovery of prion proteins and the diseases which are associated with them still present scientists and clinicians with a number of problems. There are clearly risks with the use of living cells and materials of animal origin to produce therapeutic compounds with respect to the transmission of prion protein. However the medical benefit many of these compounds has to be weighed against this. It is clear a number of groups are continuing to unravel the highly complex relationships of prion biology and pathology and it is only when this is clearly established that the community can decide on these issues. Until this time the scientific community must rely on the best research available and provide guidance from this.

Introduction

The biology of Transmissible Spongiform Encephalopathies (TSE's) is still surrounded by controversy as even the mechanism of the disease pathology and the transmission of the agent still remains to be fully explained. The reason for our lack of understanding of this disease is due to the complexity of the condition and the difficulty with the models we currently have. The incubation times of the disease are prolonged and are often variable (Tateishi et al., 1996). This chapter will not seek to discuss the many different hypotheses concerning the concept of prions or prion disease, however the chapter will endeavour to clarify some of the known risks of transmission of TSE in particular prion proteins with particular reference to the production of biotherapeutic proteins.

There are only a few biological features of TSE disease which are generally agreed. The disease is principally characterised as a neurodegenerative condition that is invariably fatal in mammals. The condition can be genetic, sporadic or transmissible in nature. The various diseases (Listed in Table 1) in different species have a common marker where a normal cellular protein [PrP^C] undergoes a conformational change to its structure. The main biochemical feature of the

change is the remarkable resistance of the changed protein [PrP^{Sc}] to degradation by a number of physical and chemical agents (Gadjusek, 1988). The structural characteristic of this protein transformation is changed to a beta sheet structure at the expense of the alpha helical formation of the normal protein. The beta sheet structure is thought to be more stable than the normal isoform and results in the accumulation of the protein. How the protein undergoes the conformational change is still unclear; some suggestions have been made that there is an interaction with a second biological moiety, however, this still remains to be fully described (Kaneko et al., 1996). The level of the PrP^C in the cells of the brain and nervous tissue is much higher than in all other tissues and this may explain the predominance of the neurological features of the disease (Kretzschmar et al., 1986).

The agents causing TSE are collectively known as prion proteins. The definition of prion is 'a **proteinaceous infectious** particle that lacks nucleic acid'; composes largely if not entirely of PrP^{Sc} (PrP^{Sc} is an abnormal pathogenic isoform of the protein that causes disease; the only identifiable molecule in purified preparations of prions). The PrP^{Sc} molecule is thought to act as a catalyst in an enzymatic-like form that can convert the PrP^C molecules to PrP^{Sc}. Ac-

Table 1. Described transmissible spongiform encephalopathy diseases in mammals

Disease	Species affected	Comments
Scrapie	Sheep and goats	Reported disease as early as 1732. Worldwide distribution (except Australasia).
Transmissible mink encephalopathy	Mink	Sporadic disease of ranched mink, outbreaks are principally in the United States but cases world-wide are recorded.
Chronic wasting disease	Deer	Prion disease limited to the United States limited to three known species of deer.
Bovine spongiform encephalopathy	Cattle	Disease predominately present in Europe with the United Kingdom having the overwhelming majority of cases.
Feline spongiform encephalopathy	Domestic and wild cats	First described in 1989, thought to be linked to the consumption of BSE contaminated foodstuffs.
Kuru	Human	Disease is limited to the Fore people of New Guinea and is linked to the consumption of human remains.
Sporadic Creutzfeldt-Jakob disease	Human	Incidence of this disease appears to be consistent worldwide to 1 in 10 ⁶ individuals.
Variant Creutzfeldt-Jakob disease	Human	First described in 1995, thought to be linked to the consumption of BSE contaminated foodstuffs.
Familial Creutzfeldt-Jakob disease	Human	World-wide distribution. Features vary depending on the polymorphisms associated with the condition.

cumulation of the abnormal form in neural tissue is thought to be the principal cause of the clinical pathology exemplified by the disease. The gene encoding the PrP protein consists of a single open reading frame in all of the human genomes thus far examined. Hamster, mouse and sheep in contrast have varying numbers of introns present within the gene. All of the mammalian PrP genes thus far examined show homology (Schatzl et al., 1995), which may explain the ability of the agent to be transmissible between species. The chicken PrP gene, however, does appear to be significantly different (Gabriel et al., 1992). The expression of the PrP gene is known to be closely regulated, but its biological function is not clear. Post-translationally the protein is changed to remove an amino terminal signal peptide of 22 amino acids and 23 peptides are removed from the carboxyl terminus during the addition of the glycoposphatidyl inositol anchor during transport through the Golgi apparatus. The protein finally emerges on the cell surface after transportation *via* secretory vesicles. The normal trafficking of PrP results in the relatively rapid proteolytic cleavage and degradation of the molecules. In the formation of the abnormal form of the protein the same post translational changes occur but the abnormal form is not rapidly turned over in the same way as the native form. The accumulation of this protein results in the formation of vesicles. These changes seem to be lim-

ited to the nervous system (vacuolation, accumulation of amyloid fibrils).

Biosafety issues of prions

The resistance of the abnormal isoform of the protein to inactivation and the ability of the TSE to cross the species barrier causes the greatest concern with respect to biosafety. The risk of transmission of TSE by bio-therapeutic products continues to cause concern due to the continued use of materials with the potential to contain TSE's. The principal issue is estimating the risk of the transmission of TSE's and how this risk is reduced to prove the safety of such products.

Iatrogenic transmission of CJD was first postulated in 1974 by a case of CJD in a recipient of a corneal transplant, the corneal donor having died from CJD (Duffy et al., 1974). There have been two further cases in the literature describing similar tragic events. In the neurosurgical field there have been a number of incidents where there has been strong circumstantial evidence presented of person to person transmission of CJD. The follow-up on patients, where the repeated use of surgical instruments after invasive procedures had been previously carried out on patients who had subsequently been shown to die of CJD, demonstrated a number where unexplained CJD was observed. There are two further groups of patients

where transmission of CJD has been recorded. Children with short stature due to a lack of human growth hormone were treated with hormones recovered from the pituitary glands of cadavers. The practice was stopped in the 1980's due to the increasing incidence of CJD in the treated patients. The incidence of disease has been reported as between 1 in 500 in the United States to 1 in 50 in France (Brown, 1995). In total, it has been estimated, 103 individuals have contracted the condition from a treated population of in excess of 30 000. The last remaining group, where the transmission of CJD has been recorded, is those patients, who, during their neurosurgical procedures, have received *dura mater* grafts. *Dura mater* has been sourced from cadavers and was used to assist in the healing process post surgery. The cases reported were principally identified in Japan, where the use of *dura mater* grafts appears to be more commonplace, but the material was used worldwide (Will et al., 1999). The biomanufacturing community in collaboration with the regulatory authorities has taken a number of steps to eliminate the risks of transmission of CJD by these methods. For example, in the case of donated materials from cadavers the clinical and familial history of the donor is rigorously screened prior to permission being given for donation and use in the treatment of patients. Human growth hormone is not now harvested from cadavers but is made as a recombinant protein, thereby removing the risks of TSE transmission.

Despite steps being taken to reduce the risk of transmission of CJD from donated neurological material it is the risk from the transmission of CJD from human blood and blood products and the risk to public health which continues to give concern to clinicians and regulatory authorities world-wide. The cases of transmission of TSE reported above all involve materials that are known to contain high levels of the abnormal isoform of the protein. The risk from blood and blood products is much more difficult to determine. It is extremely difficult to detect PrP^{Sc} in the blood of even end stage human patients suffering from TSE disease. Laboratory experiments have demonstrated transmitted TSE from infected patients, in rodent models at least (Manuelidis et al., 1985) but not in primates (Brown et al., 1994). This contrasting laboratory evidence indicates therefore, that there is a theoretical possibility of transmission of the prion protein *via* blood. However, perhaps stronger evidence, that there is little risk from blood products, is the low incidence of CJD in haemophiliacs. In the past this patient group, because of the constant requirements

for blood products to sustain survival, have been susceptible to blood borne pathogens. Infection from both *Human Immunodeficiency virus* and *Hepatitis viruses* has an extremely high incidence in these patients. In contrast, CJD has not been identified in this group, indicating a very low risk of transmission *via* blood products (Baron et al., 1999). Most countries require the blood donating organisations to ask the donors for a familial risk of CJD that would exclude donation. In addition, the United Kingdom, in common with some other countries leucodeplete the blood used in treatment. The removal of the non-red blood cell component of the blood is thought to reduce the risk of transmission of the CJD agent. There can never be a zero risk in the use of blood and blood products from the transmission of CJD; however, the use of these products continue to be a necessary part of medical treatment where removal would almost certainly put lives at risk. Therefore, a risk benefit analysis requires to be done, balancing the patients need for immediate treatment with the very low risk of transmission of this agent.

There are two further potential biosafety issues concerning prion proteins with respect to the risk with the production of biotherapeutic materials rather than blood products. These are:

1. The introduction of TSE with the supplements derived from animals used in their production.
2. The risk of the living cells used in the production of the materials producing PrP^{Sc} protein.

The risk of TSE in animal derived supplements

Risks from foetal bovine serum and other bovine components:

The public has become acutely aware of the risks from TSEs of animal derived materials by the emergence of bovine spongiform encephalopathy ('mad cow disease') in the United Kingdom and mainland Europe. This epidemic has had profound medical, economic and public health ramifications world-wide.

The discovery of a novel TSE in cows in the United Kingdom during the 1980's has dramatically changed the biological risks associated with the use of calf serum as a supplement when producing therapeutic proteins. The previously unrecognised disease was first defined in 1986 with spongiform pathologic changes in the brain, but clinical signs had been reported in cows with the condition the previous year. Histologically the brains exhibited the spongiform lesions

and astrogliosis characteristic pathologic changes in the brain (Wells et al., 1987). The initial cases heralded an epidemic that peaked in January 1993 when the number of cases exceeded 3500 per month. The epidemic was modelled and it was suggested the common exposure to a dietary protein supplement produced from the rendering of ruminant offal which was estimated to occur during 1980–1981 (Wilesmith et al., 1991). The link between the protein supplement and the condition was discovered over the following 10 years. It appears two main factors coalesced to initiate the epidemic.

1. The method of production of the bovine protein supplement had changed during the late 1970's and early 1980's.
2. There was an increase in the use of protein supplements to young animals.

The protein supplement was a mixture of sheep and bovine sourced material and two hypotheses were equally considered for the source of the abnormal prion proteins. The United Kingdom is recognised to have a significant number of sheep infected with scrapie. The first hypothesis was that sheep scrapie prion proteins may have been transmitted to the cows in the feed. Alternatively the abnormal proteins may have been sourced from a spontaneous conversion of normal prion proteins in cows. Regardless of the initiating source of PrP^{Sc} the agent appears to be transited *via* the foodstuffs and was able to induce the disease in infected animals. During 1988 the U.K. initiated a feed ban of the implicated supplements whereas continental Europe initiated the bans in the following few years. The disease, finally termed bovine spongiform encephalopathy (BSE) has been reported in a number of European countries, although no country ever reported the high incidence that was observed in the United Kingdom. The spread of disease is clearly linked to the transportation of animals and the feeding of dietary food supplements. There is less strong evidence that the agent can pass between animals either vertically or horizontally. If the agent can pass between animals the incidence must be extremely low since the incidence in infected herds seldom reaches greater than 5%. The data on vertical transmission is even less substantial. The epidemic was controlled in the United Kingdom by a number of means. The ban on the feeding of ruminant remains to cows probably represented the singular most important step taken to prevent further spread of the disease. There was, in addition, a whole-

sale cull of animals with a high potential of being infected by the agent.

When considering TSE risk in supplements to cell culture medium there are two main materials of bovine origin which are frequently used in the production of human biologics: serum and gelatin. Other materials such as pancreatic extract and muscle and tendon extracts are much less frequently used. Supplements made from other at risk species such as human or sheep are very rarely used. As the smallest recognised infectious prion particle is thought to be the dimer of the PrP^{Sc} (this however, has never been demonstrated to be true in animal infectivity assays), which is approximately 60 kDa in size, it is likely that any sterilising filters designed to retain viral or bacterial particles will not retain these proteins. The ability of prions to aggregate to varying degrees also hinders the prediction of the ability of filters or other mechanical removal steps to remove infectious material. Compounding the difficulty in using mechanical steps to remove TSEs is the observation that they are also resistant to many biological inactivation processes. In contrast to all other known infectious agents TSEs are known to resist detergents, nucleases, cationic and metal ion chelators, formalin, and hydroxylamine, as well as high temperatures. The combination of size and resistant features of TSEs presents severe difficulties in the production of supplements free of prion proteins.

During the manufacturing purification of these materials for inclusion in biologics there are frequently virus removal or inactivation steps that are designed to eliminate any potential contaminating viruses present in the in-process material. These steps frequently involve detergent treatment or extremes of pH or filtration using a small pore size filter. As can be quickly seen from the previous chapter these steps would have little value in the elimination of infectious prion protein should this be present.

As a result of the BSE outbreak the European regulatory authorities initiated steps to ensure there were no bovine risk materials used in the production of human biologics. Many other regulatory authorities followed suit and there is now a general agreement assuring the best risk management of BSE and other like agents. In general, there is a recommendation that if bovine (or human) sourced materials can be eliminated from the manufacturing process then this should be encouraged. The European Directive for the Quality of Medicine was set the task of producing a guideline for the use of bovine supplements for use in the man-

ufacture of human biologics. The guideline resulted in a document termed 'Certificate of Suitability', which provides a risk assessment on the bovine material under three general parameters.

1. The geographical origin of the material.
2. The part of the animal the material was sourced from.
3. The manufacturing process.

The United States and the European Agencies define countries from which ruminant material should not be sourced if these materials are to be used in any part of the production of a human biologic. Material should, therefore, be obtained only from countries that have not reported cases of BSE. It is also suggested the material should be harvested from young animals where the risk of BSE is lower. The World Health Organisation has classified tissue into four categories of risk of infectious PrP (Committee for Proprietary Medicinal Products: Ad Hoc Working Party on Biotechnology/Pharmacy and Working Party on Safety Medicines, 1992).

I High (Brain).

II Medium (Ileum, proximal colon, spleen).

III Low (Bone marrow, liver, pancreas).

IV Not detectable (Milk, muscle, skin, bone, serum).

Clearly, the higher risk materials are discouraged from use. Finally, the production process should use controlled sourcing of materials of low risk which is accepted as the most important criterion in achieving acceptable safety of the product due to the resistance of TSE to most inactivation procedures. The production process should use, where possible, methods which are shown to inactivate or remove TSE agents. By putting in place a risk assessment system such as this the European authorities have allowed the continued use of bovine materials in biotechnology with sufficient measures to prevent a risk to public safety. It should, however, be noted that the regulatory authorities around the world continually keep these matters under review and policy can change at any time in the light of new scientific data.

The risk of prion protein in cells used in the manufacture of biotherapeutics

Few things are clear surrounding the risk of abnormal prion proteins being present in cells used for the

production of biotherapeutics and their potential contamination of the final therapeutic product. However, it is clear that all cells have the ability to produce the non-pathogenic form of the prion protein as part of the proteome (Borchelt et al., 1990). As there is clearly a genetic predisposition to some forms of prion disease, it may be a prudent step to investigate the genetic sequence of any production cell line to ensure there are no predisposing genetic factors which may make the production of abnormal prion proteins more likely. As cells such as neurones appear to have a higher level of production of this protein when compared to non-neural cells, consideration should be given to switching away from such higher risk cell lines. It is also clear that if the normal form of PrP is present this may be susceptible to transformation to the abnormal or pathogenic form, by whatever transmissible or sporadic mechanism. The estimation of the likelihood of these risks remains to be established. In humans there is an accepted incidence of sporadic CJD as 1 case in 10^6 individuals per year. The cause of these cases is not clear; it could be genetic or environmental or perhaps both. However, what remains indisputable is the fact that in a previously unaffected individual there are now abnormal prion proteins. The concern is that mammalian cells in culture may have the same risks of incidence of formation of the abnormal prion proteins. However, as these cells are in an *in vitro* environment where it is clear the differences may either reduce or increase the risks of transformation to the abnormal form, this still remains to be determined. Experiments carried out to transmit and propagate prion proteins *in vitro* are generally unsuccessful (Preusser et al., 1999). There are a limited number of cells which are capable of propagating prion proteins, including a number of mouse and sheep cell lines. The mouse neuroblastoma cell line N2a and the neuronal cell line GT-1 will propagate prion protein from sheep and humans *in vitro* (Butler et al., 1988; Schatzl et al., 1997). These cell lines have allowed the study of the biology of prion proteins, leading, principally, to the characterisation of the intra-cellular transport of the proteins. The biological function of the prion protein still remains to be fully determined. PrP knockout mice *in vivo* have not displayed significant physiological changes to non-knock-out animals. Studies, as described above, of over-expression of the PrP protein have indicated cells are more susceptible to oxidative stress, enzymatic activities like Cu/Zn superoxide dismutase or copper incorporation. However, the absence of any cytopathological effects during the propagation

of PrP^{Sc} *in vitro* is an extremely puzzling feature of the neurodegenerative disease. In the cell lines that have been shown to be able to be infected with prions, the only feature of infection appears to be apoptosis. Therefore, with the exception of cells that endogenously produce higher levels of PrP, there appears to be a low risk of these cells producing the abnormal protein.

The most controversial aspect to the BSE epidemic is the proposed transmission of the TSE to humans by the consumption of beef. This hypothesis was initially discounted as there had never been an association of scrapie with human disease and the ability of the agent to cause infection by ingestion was insignificant in murine studies. In 1996 a new variant form of Creutzfeldt-Jakob disease (vCJD) was described after a number of deaths. The vCJD was distinguished from the other forms by the early age of onset of the disease (generally under 40) and a clinical syndrome with distinct neurological symptoms. The earliest evidence of the link between BSE and vCJD was with the primate studies reported by Lasmez et al. (1996). In these studies a disease similar to vCJD can be induced by intracerebral inoculation of BSE into macaque monkeys. BSE, when inoculated into strains of mice, exhibited the same pathological features as mice inoculated with vCJD. A number of questions, however, still remain unanswered with regards the link between BSE and vCJD. The level of infectious prion protein in beef is likely to be extremely low and therefore what risk factors are associated with this transmission, and how do they relate to the risks in cell cultures used in biotechnology used to produce human therapeutics, needs to be determined. It is clear that some rodent cells can propagate abnormal prion protein but no clear studies have been reported on the cells used in the production of human biologics on their ability to produce the abnormal PrP. It is clear more investigations are necessary in this area.

Detection methods for prion proteins

Bioassays

The discovery that scrapie transmission can be accomplished and studied by *intra* cerebral inoculation of goats and the subsequent work on strains of the disease were the first true bioassays to be described (Cuille and Chelle, 1939; Pattison and Millson, 1961). However, it was not until the transmission to rodent models

was successfully accomplished, that a clearer understanding of the pathogenesis of the disease was made. Today there are a number of mouse and hamster strains that have been used to study and detect the presence of prion proteins. There have also been a limited number of transgenic animals developed which express human or bovine PrP genes. These animal models currently represent the 'gold standard' for the detection of prions. There are, however, a number of drawbacks to the rodent studies; most of these experiments take at least one year to complete and frequently can exceed two years, the variation of the clinical outcomes of disease also requires large numbers of animals to be inoculated. This combination results in an inevitable high cost associated with the studies. The question of sensitivity of these assays has also arisen. When testing materials of human, bovine or ovine sources by using a rodent animal model there is clearly a species barrier which the invading prion protein must overcome before it can catalyse the conversion of prion protein to the abnormal isoform in the rodents. This may lead to the wrong interpretation of data generated from such assays and an underestimation of the infectivity levels of the test material. The reasons for this are unclear but may be due to the trafficking and processing of the abnormal prion protein, which is very poorly understood but would appear to be species specific. There may be as yet unrecognised compounds that are required for successful transmission of the disease. In the absence of these co-factors the inoculation dose would appear, from experimental studies, to be considerably increased to establish successful transmission. A second drawback to the animal studies is that TSE strains differ in infectivity and pathogenicity depending on the animal model chosen. This can vary mainly due to the genetic background of the animal, the transmission route and the volume of inoculum (smaller animals clearly have a limited volume which can be inoculated).

There are currently no reported reliable cell culture methods for the detection of prion proteins. Although a number of cell lines can be infected with prions there are no readily identifiable endpoints such as cytopathic changes for these assays. Again the question of sensitivity in cell culture studies remains poorly understood.

Molecular assays for the detection of abnormal prion proteins

Molecular assays for the detection of proteins have lagged behind those for the detection of nucleic acid for a number of years. Nucleic acid amplification techniques can now detect less than 10 copies of a specific nucleic acid species, something protein detection systems are unable to accomplish. The main detection methods applied to abnormal prion proteins are immunoassays. These rely on the specific interaction of an antibody probe with the target protein for detection. Interestingly, with natural transmission of prion disease there is no immune response, either cellular or humoral. As PrP^C is a normal component of the cell mechanisms of immune tolerance are present to prevent rejection of this protein by immunosurveillance. However, with the dramatic conformational changes to the protein some immuno-recognition may have been expected but this does not appear to be the case. The immune tolerance to the prion protein appears to have restricted the production of suitable antibodies for use in detection assays (Kasper et al., 1981). Today immune sera from a number of species have been generated and shown to react specifically with PrP in a number of different assays. In addition, monoclonal antibodies have been generated specific to a number of regions of the prion protein. Again, surprisingly, the generation of a conformationally specific PrP monoclonal antibody was not simple, although there are some excellent candidates (Kascak et al., 1987).

These reagents have been successfully used to monitor for transmissible prion proteins in the human food chain. A limited number of Enzyme Linked Immunoassay (ELISA) techniques are now marketed for the detection of BSE in bovine material.

In a relatively recent publication (Saborio et al., 2001) the authors describe a methodology of amplification of the PrP protein as a means of improving the detection rate of the protein. A 16-fold increase in the level of protein was reported by a cycling of the sample with incubation steps and sonication. This technique appears to have the potential to have limitless increase in the level of the prion protein from very low levels. There have been no subsequent publications from the group further validating the use of this assay.

Conclusions

Although the risks of microbial contamination of bio-therapeutic drugs are significant it is perhaps the risk from TSE agents that present the most significant and controversial issue today. Prion disease is unlike all other forms of known diseases, this disease can manifest as sporadic, genetic and transmitted forms. The common feature is the presence of infectious prion protein. The potential for these agents to contaminate medicinal products has been recognised by regulatory authorities around the world and a number of guidelines are already in place. Bovine and other ruminant products require stringent steps taken to ensure the absence of BSE. Measures are also in place to ensure that human prion disease proteins are not allowed to become part of any therapeutic compound. However, it should be noted that our knowledge of these agents is still in its infancy, we still know very little on how these agents are transmitted or even how they replicate. Clearly much of the basic biology of these agents has to be determined. Until then we should make use of the best available scientific advice to ensure products are as safe as possible.

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