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Review article

Cell models of prion infection

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Abstract – Due to recent renewal of interest and concerns in prion diseases, a number of cell systems permissive to prion multiplication have been generated in the last years. These include established cell lines, neuronal stem cells and primary neuronal cultures. While most of these models are permissive to experimental, mouse-adapted strains of prions, the propagation of natural field isolates from sheep scrapie and chronic wasting disease has been recently achieved. These models have improved our knowledge on the molecular and cellular events controlling the conversion of the PrP^C protein into abnormal isoforms and on the cell-to-cell spreading of prions. Infected cultured cells will also facilitate investigations on the molecular basis of strain identity and on the mechanisms that lead to neurodegeneration. The ongoing development of new cell models with improved characteristics will certainly be useful for a number of unanswered critical issues in the prion field.

prion diseases / cell models / prion dissemination / apoptosis / PrP protein

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1. INTRODUCTION

The first successful attempt to establish scrapie-infected cultures was obtained as early as 1970 when low levels of prion multiplication was reported in a mouse brain-derived cell line called SMB [31]. Since then, and despite different repeated attempts to maintain scrapie-infected cultures [114], stable and consistent propagation of prions was achieved in a very limited number of cell cultures. As a result, very few permissive cell lines were available in the early 2000s and all of them were permissive only to rodent-adapted prion strains [132]. The first exception occurred in 2001 when it was shown that expression of ovine PrP^C in rabbit epithelial RK13 cells rendered them permissive to the multiplication of a sheep scrapie agent [146]. This was the first example of successful transmission of a natural transmissible spongiform encephalopathy (TSE) agent (i.e. not previously adapted to rodents) to cultured cells, and this also provided evidence that PrP^{res} can be readily detected in infected cells from the non-neuronal lineage. Since then, prion multiplication was demonstrated in several other non-neuronal cell systems, including fibroblasts [116, 147], muscle cells [39], microglial cells [61], and another natural isolate (from chronic wasting disease) was successfully transmitted to a mule deer cell line [116].

2. CULTURED CELLS PERMISSIVE TO PRION MULTIPLICATION

Cell models can be classified depending on the prion species propagated (Tab. I).

2.1. Cell models permissive to experimental, rodent-adapted scrapie strains

Mouse neuroblastoma cell lines have been the most extensively characterised model for propagation of mouse-adapted strains of prions. They include N2a [19,115], C-1300 [115] and NIE-115 [86] lines, all of them deriving from the same cell line [114]. Subclones of the N2a cell line with higher permissiveness were subsequently identified [16, 41, 95] in which subcloning of the infected cultures was no longer necessary to stably maintain high levels of infection. GT1 cells are hypothalamic cells also widely used to multiply mouse prions [5, 95, 124]. Some GT1 subclones may undergo apoptosis following infection [124], a finding not consistently reported. PC12 rat pheochromocytoma cells can be differentiated into neuron-like cells which can then be infected with mouse prions [118]. SMB cells were originally isolated from Chandler strain-infected mouse brain culture [31]. However, Chandler-infected SMB cells can be cured with pentosan sulphate and infected de novo with other mouse-adapted prion strains [13, 65]. SN56 is a mouse cholinergic septal neuronal cell line which is permissive to the multiplication of several mouse-adapted prion strains [7]. Neuronal stem cells isolated from conventional or transgenic mice were recently shown to propagate mouse-adapted prions [53,90]. Recently, hippocampal-derived HpL3-4 cells obtained from a PrP^C knock-out mouse and transfected with mouse PrP^C were shown to be permissive to the 22L strain of the mouse prion [80]. Finally, cells from the peripheral nervous system, such as MSC80 Schwann-like cells, replicate low levels of the RML strain [47].

Recent studies confirmed the original finding [146] that non-neuronal cell lines can efficiently propagate prions. Common fibroblast cell lines [147] and a microglial cell line (MG20) established from transgenic mice overexpressing PrP are susceptible to various murine prion strains [61]. Notably, the mouse-adapted bovine spongiform encephalopathy (BSE) agent was successfully propagated in MG20 cells. Infection of a skeletal myoblast cell line (C2C12) has been described recently [39], and could be used to investigate prion infection of muscles observed in sheep and cervids. While all the aforementioned cell models are permissive to mouse-adapted prion strains, a cell line (HaB) stably infected with hamster prions has been described in the past [136].

Generally, more than one strain can be propagated in a single cell line (Tab. I). N2a and GT1 cells are susceptible to Chandler, Fukuoka-1, RML, 22L, and 139-A

Table I. Cultured cells permissive to rodent-adapted or natural TSE agents.

Cell designation	Tissue of origin or cell type	Species	Strain of prion	Comments
Rodent-adapted strains				
C-1300	Neuroblastoma	Mouse	Chandler [19]	
NIE-115	Neuroblastoma	Mouse	Chandler [86]	
N2a	Neuroblastoma	Mouse	Chandler [115], Fukuoka-1 [19], RML [16,41]	
N2a#58	Neuroblastoma	Mouse	Chandler, 139A, 22L [95]	Overexpression of PrP^C
GT1	Hypothalamic cells	Mouse	SY-CJD, FU-CJD [5], Chandler [95,96], RML [124], 22L [89,95,96], Fukuoka-1 [4]	
PC12	Pheochromocytoma	Rat	139A [118]	
SMB	Brain cells	Mouse	139A, 22F [13], 79A [65], Chandler [31]	
SN56	Septal neuronal cells	Mouse	Chandler, ME7, 22L [7]	
NSC	Neuronal stem cells	Mouse	RML [53] 22L [90]	From transgenic or conventional mice
MSC80	Schwann like cells	Mouse	Chandler [47]	
HpL3-4	Hippocampal cells	Mouse	22L [80]	From $PrP^{0/0}$ mouse transduced for mouse PrP^C
L929, NIH/3T3	Fibroblasts	Mouse	22L, ME7, RML [147]	
MG20	Microglial	Mouse	Chandler, ME7, mouse BSE [61]	
C2C12	Myoblasts	Mouse	22L [39]	
moRK13	Epithelial RK13	Rabbit	Fukuoka-1, 22L, Chandler [33], M100 [143]	RK13 cells expressing mouse PrP^C
voRK13	Epithelial RK13	Rabbit	Vole-adapted BSE [33]	RK13 cells expressing vole PrP^C
HaB	Brain cells	Hamster	Hamster prions [136]	
Natural TSE isolates				
ovRK13 (Rov)	Epithelial RK13	Rabbit	Natural sheep scrapie [146]	RK13 cells expressing ovine PrP^C
MovS	Schwann-like from Dorsal Root Ganglia	tgov Mice	Natural sheep scrapie [3]	From ovine transgenic mice
CGN^{ov}	Neuronal primary cultures (cerebellum)	tgov Mice	Natural sheep scrapie [34]	From ovine transgenic mice
MDB	Fibroblast-like	Deer	Chronic wasting disease [116]	

murine prion strains. L929 mouse fibroblasts propagate 22L, ME7, and RML while MG20 microglial cultures can be infected by ME7 and Chandler strains and by the mouse-adapted BSE agent. RK13 cells expressing mouse PrP^C are susceptible to several murine prion strains as well [33]. However, there is also evidence that prion multiplication show cell tropism ex vivo. This is best illustrated for the ME7 strain which does not replicate in N2a or GT1 cell lines [16, 67] but does replicate in SN56 [7], L929 [147], and MG20 [61] cell lines.

2.2. Cell models propagating naturally-occurring prion isolates

The first natural TSE agent (i.e. non rodent-adapted) to be successfully propagated in cultured cells was from sheep scrapie. The epithelial rabbit RK13 cell line, expressing no detectable endogenous rabbit PrP^C, was genetically engineered to stably express an ovine PrP^C variant ($^{136}V^{154}R^{171}Q$) associated with a very high susceptibility to scrapie [59]. The resulting cultures, known as Rov cells, can be readily infected with and replicate to high titres some sheep scrapie isolates [146]. Neuroglial cell lines with Schwann-like features [3] and primary cultures of neurons and astrocytes [34], both of which were derived from transgenic mice expressing the VRQ allele of ovine PrP^C, were later shown to be permissive to sheep scrapie as well. Finally, prion propagation and PrP^{res} accumulation were also demonstrated in an astrocyte-like, sheep-derived cell line [145] inoculated with the same set of ovine prion agents ([75] and our unpublished results). These cell models have distinct and complementary features that proved useful to study various aspects of sheep scrapie infection. More recently, a chronic wasting disease isolate was successfully transmitted to a mule deer-derived fibroblast cell line [116]. Although multiplication of the human prion was reported on one occasion [73], there is no available cell model permissive to human Creutzfeldt-Jakob disease (CJD) or cattle BSE agents.

3. CELL BIOLOGY OF ABNORMAL PRP

Prion-infected cultures have been powerful and useful experimental tools to study the cell biology of abnormal PrP and to investigate the molecular and cellular events controlling the conversion of PrP^C into PrP^{Sc}.

Cell surface PrP^{Sc} cannot be released into the milieu by phosphatidylinositol-specific phospholipase C (PIPLC) treatment of intact infected cells [24, 77], in contrast to what is readily observed for PrP^C in uninfected cells. Thus, and although PrP^{Sc} has a glycosylphosphatidylinositol (GPI) anchor [134], the manner in which PrP^{Sc} associates with membranes differs from that of PrP^C and has yet to be determined. While PrP^C is rapidly synthesised and degraded [14, 23, 97, 100], the abnormal form is much more stable. Earlier pulse-chase experiments did not yield evidence that infected cells could eliminate PrP^{Sc} [14, 25]. Experiments blocking prion multiplication with anti PrP antibodies later showed that infected N2a cells do get rid of PrP^{Sc} [41, 44, 102, 104]. Similar findings were obtained with sheep scrapie-infected Rov cells in which the disappearance of pre-formed PrP^{Sc} can be monitored after arrest of inducible PrP^C expression [12]. These findings indicate that, although PrP^{Sc} is much more stable than PrP^C, the infected cells do have the capacity to degrade PrP^{Sc}. Little is known on the cellular proteases involved in processing and/or degradation of abnormal PrP. However, some protease inhibitors can modulate the processing [152] or the degradation [79] of PrP^{Sc} in infected cultures and the tyrosine kinase inhibitor STI571 promotes lysosomal degradation of PrP^{Sc} [42]. A delicate balance between synthesis and degradation of PrP^{Sc} could be a determining factor for successful multiplication in cultured cells [150]. Some strains may not accumulate in cultured cells if degraded too rapidly by the cellular machinery while others may more readily resist degradation and replicate to detectable levels.

Cellular heparan sulphates are sulphated linear polysaccharides typically linked to proteins to form heparan sulphate proteoglycans (HSPG) located at the cell surface [141]. A number of studies are consistent with the

involvement of heparan sulphates in the biogenesis of prions. A variety of sulphated glycans, including pentosan polysulphate [13,28], dextran sulphate 500 [8, 12, 28], heparin [49], and various heparan mimicking molecules [1, 126] are potent inhibitors of PrP^{Sc} accumulation in several cell lines infected with murine prions and in Rov cells infected with a sheep scrapie agent [98], presumably by competitive inhibition of cellular heparan sulphates for the binding to PrP^{C} [49]. More direct evidence for a role of cellular heparan sulphates in the formation of abnormal PrP was obtained by showing that treatment of infected N2a cells with heparinase III resulted in a marked reduction of PrP^{res} [11]. The underlying mechanisms have not been determined. Since heparan sulphates can bind numerous molecules, including PrP^{C} [29, 49, 149], one possibility is that the heparan sulphate moieties of proteoglycans could help bring together partners critically involved in the conversion process, such as PrP^{C}, PrP^{Sc}, and other possible cofactors. Inhibition of the expression (either by antisense RNA and siRNA) or function (by antibodies) of the 37 kDa/67 kDa laminin receptor (LRP/LR) prevents PrP^{res} accumulation in infected N2a and GT1 cells [78]. Again, the mechanisms are unclear. As speculated above, LRP/LR, PrP^{C}, and PrP^{Sc} might form, along with heparan sulphates a functional complex required for conversion. Alternatively LRP/LR inhibition could prevent prion multiplication by perturbing the metabolism of PrP^{C}, a possibility supported by the fact that LRP/LR is involved in PrP^{C} trafficking [50] and that inhibition of LRP/LR decreases cellular PrP^{C} levels [78].

Detergent resistant microdomains (DRM) or rafts are specialised, cholesterol-rich membrane microdomains that resist solubilisation in cold non-ionic detergent such as Triton X-100 [131]. As in the brain, PrP^{C} from cultured cells is incorporated into rafts [94, 135, 144, 148], as many GPI-anchored proteins. In infected cells, normal and abnormal forms of PrP are found in rafts [94, 144], raising the possibility that these domains might be involved in the conversion process. Indeed it was shown that perturbing the association of PrP^{C} with rafts via cholesterol depletion reduces conversion [138]. In addition, genetically engineered PrP^{C} containing a trans-membrane domain was no longer incorporated into rafts and was poorly converted [63, 138]. Although the mechanisms by which rafts might control the conversion process are not known, several hypotheses have been proposed [20]. Rafts could bring together the different molecular partners required for efficient conversion. Rafts could also be used as vehicles to convey the required molecules to specific subcellular sites in which conversion occurs.

The subcellular distribution of abnormal PrP in infected cells is extremely difficult to assess, primarily due to the poor immunoreactivity of PrP^{Sc}. Immunodetection requires treatments with strong denaturants, such as guanidium, which have deleterious effects on cell morphology and also make colocalisation with organelle markers difficult to interpret. Nevertheless, analysis of several types of infected cells, including N2a [124, 136], GT1 [124], and HaB [136] made it clear that PrP^{Sc} resides intracellularly. While an accurate and exhaustive picture of PrP^{Sc}-containing intracellular compartment is lacking, PrP^{Sc} accumulation has been observed repeatedly in late endosomes and/or lysosomes [88, 106] where trimming of PrP^{Sc} may occur [26]. PrP^{Sc} has been seldom visualised in the Golgi apparatus [136] and in the nucleus [84]. Labelling with membrane-impermeant reagents has demonstrated that abnormal PrP is also present at the cell surface of infected N2a cells [77, 144]. The presence of abnormal PrP at the plasma membrane of infected cells was also observed in at least another paradigm, MovS cells infected with a strain of sheep scrapie (our own unpublished results). Exosomes are microvesicles from endosomal origin that are secreted by a variety of cells [45], including neurons [43]. It was recently demonstrated that exosomes released by MovS and Rov cells do contain abnormal PrP and prion infectivity when the cells were infected with a sheep scrapie agent [46]. A similar observation was subsequently reported for GT1 cells infected with a mouse-adapted prion strain [143]. Exosomes are formed in multivesicular bodies (MVB),

which could therefore represent an intracellular site for PrP^{Sc} accumulation. Since PrP^{C} permanently cycles between the plasma membrane and intracellular compartments [109], it is important to stress that at least some of the aforementioned sites may merely represent sites where PrP^{Sc} accumulates rather than sites where conversion actually occurs. However, as detailed in section 4, the presence of PrP^{Sc} in some specific subcellular compartments, such as the cell surface or MVB, may crucially affect the cell-to-cell spreading of prion agents.

Bona fide PrP^{Sc} has been operationally defined for years as resistant to proteinase K (PK) treatment and as insoluble in non-denaturing detergents. Recent studies have revealed a more complex picture. A PK-sensitive PrP^{Sc} fraction was first described by Safar et al. [122] and later isolated and characterised from infected brain tissues [101, 142]. However, the biological significance of PK-sensitive forms of PrP^{Sc} regarding prion infectivity and/or neurotoxicity needs to be clarified. The identification of such forms in infected N2a cells [142] indicates that cell-based models may be useful to investigate the cell biology of PK-sensitive PrP^{Sc}.

4. DE NOVO INFECTION AND CELL-TO-CELL DISSEMINATION OF PRIONS

Surprisingly, little is known about the initial interactions of exogenous prions during de novo infection of a recipient cell. Likewise, the subcellular sites where conversion of PrP^{C} into PrP^{Sc} occurs remain to be identified. Moreover, whether conversion can proceed at the cell surface or whether internalisation of abnormal PrP is required for cell infection is an open question.

The easier and most classical way to transmit prions is to incubate prion preparations (e.g. infectious brain homogenate) with cultured target cells. However, several independent studies have recently shown that prion transmission is much more efficient when live infected biological material is used as the source of infectivity [39,65,99]. Further investigation into the underlying active biological processes will be necessary to optimise prion transmission to cultured cells.

Several studies have recently investigated the interaction of PrP^{Sc} with cultured recipient cells. Permissive (N2a, GT1, SN56, Rov) and non-permissive (CHO, CaCo2) cells were incubated in the presence of PrP^{Sc} from various strains or isolates, including RML and C506M3 murine prions, cattle BSE, and sheep or hamster prions. The abnormal PrP added to the cells was prepared in different ways. Some investigators used rods, the purest prion preparation known to date. Other studies used infected brain tissues homogenised in physiological buffers, arguing that PK-digested, highly aggregated forms have poor biological relevance. Despite varying experimental conditions which may hinder comparisons among them, all of these studies have shown that PrP^{Sc} is internalised by a process that does not require the presence of PrP^{C} at the cell surface [55, 81, 98]. The identification of putative cellular receptors for PrP^{Sc} led to a different outcome. Morel et al. reported that CaCo-2TC7 cells internalise PrP^{Sc} from BSE-infected cattle brain homogenate but not that from mouse-adapted C506M3 strain and suggested that LRP/LR was involved [92]. The uptake of purified PrP^{res} from the C506M3 strain in BHK21 cells was later shown to be in part LRP/LR-dependent [51]. Other studies provided evidence that cellular heparan sulphates are receptors for PrP^{Sc}. The enzymatic removal of cell surface heparan sulphates [58], the inhibition of their sulphation by chlorate [58] or competition with heparan mimetics [58,126] all prevented the binding and the uptake of detergent-extracted, aggregated forms of abnormal PrP in N2a, GT1, and CHO cells. A similar observation was reported for PrP^{Sc} from brain homogenate in CHO cells [55]. Finally, Paquet et al. demonstrated an efficient entry of abnormal PrP in permissive Rov cells by mechanisms that apparently did not rely on cellular heparan sulphates [98]. Collectively, these studies suggest that abnormal PrP can enter the cells by different routes. One possibility is that internalisation routes may depend on the cell type. Alternatively, the utilisation of heparan sulphates versus other macromolecules as cellular receptors might depend on the biochemical state of abnormal

PrP. Using fluorescently-labelled aggregated PrP^{res}, the internalised PrP^{res} was tracked intracellularly by cell imaging. Aggregates taken up by SN56 cells or neurons in primary cultures are dispersed in a large set of intracellular vesicles, including late endosomes and lysosomes, and transported along neurites [81]. However, the aforementioned studies did not address where in the cells is conversion initiated. Early pulse-chase experiments with infected N2a and HaB cells have revealed that conversion of PrP^{C} to the protease-resistant state is a late post-translational event that takes place after PrP^{C} has been delivered to the plasma membrane [15, 25]. More recently, it was shown that transmission of infection requires the presence of cellular heparan sulphates and the presence of PrP^{C} during exposure to the inoculum [58, 98]. While this suggests that molecular complexes between PrP^{Sc}, PrP^{C}, and cellular heparan sulphates are required at the plasma membrane, it remains unclear whether conversion can proceed further at the cell surface or if the putative complexes are driven to intracellular compartments for initiation of conversion. Further studies will be necessary to fully characterise the different internalisation routes of PrP^{Sc} and to clarify their biological significance regarding the initiation of cell infection. Identification of the prion multiplication sites is obviously important to define efficient strategies for therapeutic intervention.

Several observations suggest that prions can disseminate in vivo through different modes. Consistent with step-by-step infection of closely apposed cells, prions can multiply and spread along particular neuroanatomical pathways to reach the brain ([9] and references therein). Extracellular forms of PrP^{Sc} can also be detected in infected tissues [62], the diffusion of which could transmit infection to more distant cells [17]. Cell surface abnormal PrP could potentially infect contacting cells as suggested by infection of recipient cells by paraformaldehyde-fixed infected SMB cells [65]. On the contrary, the presence of infectivity in the cell culture media of numerous prion-infected cell lines, including N2a, GT1, HpL3-4, SN56, MovS, and Rov [7, 34, 46, 80, 124] raised the possibility that infection can be transmitted, at least among cultured cells, through the release of cell-free forms of prions. The physical nature of released prion infectivity has remained uncertain for years. Février et al. first reported that abnormal PrP and infectivity actively released from sheep scrapie-infected MovS and Rov cells was associated with small vesicles (50–100 nm) called exosomes [46], raising the possibility that infected exosomes could serve as a vehicle for prion dissemination [108]. Similar findings were later obtained for GT1 cells infected with a mouse-adapted prion strain, indicating that potential spread of infection through cell-free mechanisms is probably not restricted to a particular type of cell or strain [143]. A further support for cell-free transmission of prions was gained by Leblanc et al. who showed that retrovirus infection enhances prion infectivity release [76]. To investigate the biological significance of cell-associated and cell-free infectivity regarding the spread of prions, co-cultures of infected donor cells and uninfected recipient cells were analysed. The spatial distribution of the newly infected cells revealed preferential infection of nearby cells, although infection of distant cells also occurred [99]. While the underlying mechanisms remain to be characterised, it seems that prion spreading among cultured cells can occur through different modes. Further studies will be necessary to test the interesting possibility that mechanisms of cell-to-cell prion spreading may vary depending on the cell type [39, 99] and/or the prion strain.

5. BIOLOGICAL PROPERTIES OF CELL-PASSAGED PRION STRAINS

The mechanisms that lead to the reproducible production of stable, yet distinct, strains in a given host are not understood. Distribution of PrP^{res} deposition in the infected brain, as assessed by PETblot or histoblot can vary in a strain-dependent manner [127, 137], raising the possibility that distinct strains multiply in distinct brain cell types [36]. Since the central nervous system is extremely complex, the basis of prion identity and diversity should be advantageously investigated in single cell

lines faithfully propagating several strains of prions.

Several studies have examined the biological characteristics of strains propagated in cultured cells. Incubation times, brain vacuolisation, and clinical signs of several murine strains (22F, Chandler, Fukuoka, and SY) remain unchanged following multiplication in N2a, GT1 or SMB cell lines [4, 5, 13]. Biological characterisation of sheep, mouse, and vole strains of prions propagated in RK13 cells expressing the ovine, murine or vole PrP^C, respectively, suggests that these strains are not modified through multiplication in non-neuronal cells as well [33]. In the absence of nucleic acids, strain biological properties are proposed to be encoded by the abnormal PrP [150]. The PrP^{res} banding patterns of strains generated in cultured cells are often, but not always [34], different from those generated in the brain [3, 4, 26, 90, 95, 146] but return to the original brain pattern upon animal inoculation [3, 4]. This confirmed that the cellular context in which multiplication occurs has a determinant effect on the glycoforms and on the size of the abnormal PrP species and that modifications of the PrP^{res} molecular profile are not necessarily associated with changes in their biological properties [133]. This was taken by some investigators as an indication that abnormal PrP does not encode the strain characteristics [5]. Interestingly and importantly, although brain- and cell-derived PrP^{res} are different, strain-specific banding pattern differences observed for brain PrP^{res} were also detected after serial propagation in cultured cells [4, 13, 33, 61]. All these findings demonstrate that different prion strains can propagate in a single cell type, indicating that multiplication in different brain cells is not necessary for prion diversity. The available data suggest that the biological properties of the strains are not modified after propagation in cultured cells. Importantly, strain-specific features of abnormal PrP are maintained upon multiplication in cultured cells, indicating that these models represent interesting cell biological systems to study how distinct abnormal PrP from various prion strains are generated in a single cell type. There are suggestions that the subcellular distribution of PrP^{Sc} may vary depending on the cell type in which a given strain is replicated [106]. Conversely, it would be interesting to study PrP^{Sc} intracellular trafficking in a single cell line infected with several distinct strains.

6. PRP^C POLYMORPHISMS, AMINO ACID SUBSTITUTION, AND PRION PROPAGATION

Some PrP^C amino acid substitutions have a crucial effect on prion disease. So far, only humans homozygous for methionine at position 129 of PrP^C expressed clinical disease following infection with the BSE agent [32]. In sheep also, there is a strong influence of PrP^C polymorphism on individual susceptibility to sheep scrapie. Animals bearing the $^{VRQ}PrP^C$ variant are extremely susceptible while sheep homozygous for the $^{ARR}PrP^C$ are resistant to classical scrapie strains [59]. However, it is important to stress that PrP^C-dependent susceptibility is not absolute and can vary depending on the strain of the agent. The newly described atypical sheep scrapie cases (Nor98, [10]) can infect sheep homozygous for $^{ARR}PrP^C$ [18] and epidemiological data indicate that the more susceptible PrP^C allele for atypical strains is not VRQ [123] but $^{F141LARQ}PrP^C$ [93]. This emphasizes that PrP^C-dependant genetic control of prion disease is a complex phenomenon that depends on both PrP^C and the strain of the agent. Sabuncu et al. generated Rov cells in which $^{VRQ}PrP^C$ or $^{ARR}PrP^C$ can be alternatively expressed. They noticed that the two PrP^C variants showed distinct cell biological properties [121] and that the cells expressing the $^{ARR}PrP^C$ were at least 10 000-fold less permissive to sheep prion multiplication than VRQ Rov cells [120]. Recently, Maas et al. described stable expression of mouse PrP^C in a hippocampal-derived cell line established from a PrP^C knock-out mouse and showed that substitutions of amino acid on the outer surface of helix 2 and 3 dramatically impaired cell infection by a strain (22L) of mouse prion [80]. It is not yet known whether HpL3-4 cells genetically engineered to express PrP^C from other species will propagate other prion species as well. However, these studies demonstrate that reverse genetic approaches

on cell models can be fruitful to assess the impact of PrP^C amino acid substitution on prion multiplication.

Conversion of substituted PrP^C has also been investigated by transiently transfecting epitopically-tagged PrP^C in infected N2a cells [110, 128]. While heterologous PrP^C differing from endogenous PrP^C by as little as one amino acid may no longer be converted [111], such studies also showed that the homology between PrP^C and PrP^{Sc} is not always required for efficient conversion and indicate that the effects of amino acid substitution can strongly vary depending on the strain propagated by the infected N2a cells [6]. Importantly, these studies also showed that introduction of a heterologous PrP^C can dramatically inhibit the conversion of the endogenous PrP^C. This led to the notion of a dominant negative effect, a process by which expression of a heterologous PrP^C can block in trans the conversion of the wild type PrP^C in infected cultures [35,56,64,110] or in mice [103]. Although the mechanisms by which the interaction between heterologous PrP^C and PrP^{Sc} can impair prion infection are not fully understood [57,117], experiments conducted with infected N2a cells have shown that lentiviral transduction of heterologous PrP^C can inhibit PrP^{Sc} formation [35]. Such cell-based models might help to develop gene therapy approaches to prion disease.

7. MECHANISMS OF NEURODEGENERATION

Prion multiplication in cultured cells can result in specific alterations in cellular metabolism, some of which can affect cell survival. For instance, infection with several murine prion strains impairs the cell response of GT1 and N2a cells to oxidative stress [89], presumably through a decrease in superoxide dismutase activity. RML infection was also shown to sensitise N2a and GT1 cells to pro-apoptotic stimuli such as endoplasmic reticulum stress or to mild proteasome inhibition [54, 71]. However, it is important to point out that prion-infected cell lines accumulating infectious titres similar to those in brain tissue do not show any obvious cytopathic effect, with the possible exception of RML-infected GT1 cells that inconsistently undergo apoptosis. Thus, to date, prion-infected cell lines have been of limited value for exploring the mechanisms leading to neuronal cell death. A significant breakthrough was recently obtained with infection of primary cultures of neurons and astrocytes by a sheep scrapie agent. Importantly, while both cell types were infected, neurons gradually underwent apoptosis [34] involving JNK-c-Jun signalling [21]. This strongly suggests that such primary cultures are pertinent models to explore the mechanisms responsible for prion-induced neurodegeneration. In addition, they should be of great value to investigate the relative contribution of astrocytes and microglial cells to infected neuron injury [85].

8. INFECTED CULTURES FOR THE IDENTIFICATION OF ANTI-PRION MOLECULES

More than 25 years ago, amphotericin B was shown to delay pathogenesis in scrapie-infected rodents and CJD-infected monkeys [2]. Polyanion pentosan polysulphate (PPS) was also identified as a retardant of disease onset in mice and hamsters [40, 72]. The fact that these molecules were subsequently shown to inhibit prion multiplication in infected cultures [28, 83] demonstrated that molecules with therapeutic potential can be identified ex vivo and prompted the hope that ex vivo screening could be of potential value. Since the early 1990s a large number of molecules have been found to inhibit PrP^{Sc} accumulation in prion-infected cultures, mainly in N2a cells (see for example [68]). However, it turned out that most of them showed none or very limited effects when subsequently tested in infected animals (for a review, see [139]). In fact, the results obtained with infected cells cannot be extrapolated to the in vivo situation. These disappointing, but not totally unexpected, findings underscore the need to test in vivo compounds identified ex vivo. Finally, it is worth noting that some compounds inhibiting prion PrP^{Sc} accumulation in infected cells are active in other models of amyloid diseases [125] or against yeast prions [140], suggesting

common biochemical pathways and possible common therapeutic targets. Yet, as detailed below, some molecules originally identified for their inhibitory effect in cell culture do also delay disease onset in animals. The fact that in most cases the effects are quite limited does not necessarily mean that infected cell models are not adequate to screen for anti-prion drugs but rather indicates that prion propagation in organisms is a complex biological process difficult to stop or to delay.

8.1. Heparan sulphate mimetics

Heparan sulphate mimetics, synthesised from dextran polymers and modified with varying amounts of sulphate, carboxymethyl and benzylamide groups, were initially identified as molecules with interesting properties in wound healing [38]. HM2602 was found to inhibit PrP^{res} accumulation in prion infected N2a [126] and GT1 [1] cells. HM2602 delayed the onset of the disease (14% increase in survival time) in experimentally-infected hamsters [1]. HM2602 treatment also reduced PrP^{Sc} levels in prion-infected mice, although the effect on the survival time was not reported. However, two heparan mimetics that inhibited prion multiplication in infected cells (HM5004 and CR-36), displayed no efficacy in vivo [1, 74].

8.2. Phosphorothioate oligonucleotide

Degenerate single-stranded phosphorothioated analogs of natural nucleic acids were screened in Rov cells infected with sheep scrapie and in N2a infected with mouse prions [70]. The ability of these randomers to inhibit the accumulation of abnormal PrP depended mostly on their length. Treatment of hamster transgenic mice inoculated peripherally led to a dramatic increase (from 200% to 300%) in survival time [70].

8.3. Congo Red

Congo Red physically interacts with amyloid fibre protein in prion diseases and other proteinopathies. Since the initial report that Congo Red can inhibit accumulation of PrP^{res} in infected neuroblastoma cells [27], there have been numerous studies on the effects of Congo Red or derivatives with improved properties in various prion-infected cultures [22, 28, 37, 83, 107, 119, 129]. Congo Red treatment induced a small delay of the disease onset (around 10%) in infected hamsters provided the treatment had started around the time of infection [60, 107].

8.4. Tetrapyrrole compounds

Tetrapyrrole compounds affect protein conformation and share some structural similarities with Congo Red. A variety of these molecules were tested in infected N2a cultures and some showed inhibitory activity on abnormal PrP accumulation [30]. Another porphyrin compound was subsequently found to be active in the MDB fibroblast-like cell line infected with a CWD isolate [116]. The most efficient molecules were tested in several animal models, including tg7 transgenic mice infected with hamster prions [69, 112] and mice infected with the RML strain of the murine prion [113]. The survival time of treated animals was markedly increased (from 50% to 400% increase in survival time). Of note, intracerebral delivery of Fe-TSP was reported to be as effective as a similar treatment with PPS [69].

8.5. Passive immunisation with anti- PrP^{C} antibodies

Since the interaction of PrP^{C} with antibodies might preclude the further conversion of PrP^{C} into PrP^{Sc}, a large number of monoclonal antibodies (mAb) were screened on various prion-infected cells to identify antibodies of potential therapeutic value. Monoclonal antibody 6H4 (recognising residues 144-152) and Fab fragments of D18 and D13 mAbs (recognising residues 132-156 and 95-103, respectively) potently inhibit prion multiplication in N2a cells [41, 102]. SAF34 and SAF61 mAbs, which recognise the octarepeat region and residues 114-152 respectively, impaired prion propagation in infected neuroblastoma cells with an increase of the turn-over of PrP^{C} as a proposed mechanism for prion inhibition [104]. Mouse and rabbit polyclonal

antibodies against a dimeric form of PrP^C also interfere with PrP^{res} accumulation in infected N2a cells [52]. One study published by Kim et al. reported that mAbs that effectively reduce abnormal PrP in infected N2a cells bind to and stabilise PrP^C at the cell surface [66]. Miyamoto et al. investigated the effect of mAb in N2a cells infected with three different strains of prion. They identified the 3S9 mAb (recognising residues 141-161) as a potent inhibitor of the 3 strains while the clearing efficiency of 2H9 mAb was strain-dependent [91]. Epithelial Rov cells stably infected with a strain of natural sheep scrapie were also exposed to a panel of mAbs raised against alpha or beta forms of recombinant mouse PrP^C. ICSM35, ICSM37, ICSM42, and, to a lesser extent, ICSM18 and ICSM19, dramatically inhibit PrP^{res} accumulation in these infected cells [12]. In this study, the most powerful mAbs were those raised against beta PrP suggesting that antibody interaction with PrP^{Sc} can be an efficient mean to inhibit prion multiplication. Feraudet et al. screened a panel of 145 mAbs on N2a and Rov cells stably infected with a murine and an ovine prion strain, respectively. They found no evidence for an epitope-dependent inhibition but did show an excellent correlation between prevention of abnormal PrP propagation and binding to cell-surface PrP^C [44].

Two separate studies have reported beneficial effects of passive immunisation with anti-PrP^C mAb on the survival time of infected animals. Sigurdsson et al. showed that weekly treatment with 8B4 and 8H4 mAb (50 μg per injection) of 139A-infected mice caused a 10% increase in survival time [130]. The effects of ICSM18 and ICSM35 mAb were tested in mice infected with the RML strain of the prion. While treatment was ineffective for animals infected by the intracerebral route, it strongly delayed the disease in peripherally-infected mice [151]. Importantly, increased survival time was also observed for treatments (2 mg per injection, twice weekly) beginning 30 days after infection.

Depletion of neuronal PrP^C through Cre-mediated recombination can prevent progression to clinical disease [82]. Cell-based models are therefore being used to define RNAi approaches with therapeutic potential [105].

9. CELL MODELS AS SENSITIVE BIOASSAY FOR PRION INFECTIVITY

Detection and quantification of prion infectivity in various biological samples is central to numerous fundamental and applied studies. So far, sensitive detection of infectivity relies on intracerebral inoculation into susceptible animals. However, these assays are long, very expensive and require numerous animals. The development of cell-based assays as an alternative way to titre prion infectivity has been hampered for years because of their low sensitivity. Following the observation that only a small percentage of cells are actually infected in a typical N2a culture population [115], N2a subclones with much higher permissiveness were isolated [16, 41]. These N2a sublines, along with improved detection of PrP^{res}, allowed the development of a quantitative, highly sensitive cell-based infectivity assay for the RML murine prion strain [67]. In the scrapie cell assay (SCA), N2a cells are exposed for a few days to the infectious inoculum, split three times at a 1:10 dilution, then spotted onto filters. Individual cells containing abnormal PrP are immunodetected and visualised after PK digestion and denaturation. Remarkably, SCA is almost as sensitive as the mouse bioassay while being much less expensive and ten times faster. It provided the proof of principle that cells could advantageously replace animal bioassay to titre prions. However, a significant limitation of the SCA is that N2a cells are not permissive to natural strains of prions. Cell-based titration of human-affecting prion strains will require the identification of cell culture systems permissive to BSE or CJD agents, a very demanding challenge for the coming years.

10. DEVELOPMENT OF NEW CELL SYSTEMS

As mentioned before, the molecular and cellular bases of permissiveness regarding prion multiplication in cultured cells are unknown. This severely impairs the development

of rationale strategies to (i) increase the number of pertinent cell models, and (ii) identify the urgently needed permissive models for human-affecting agents (i.e. BSE and CJD). In this difficult context, new experimental strategies are nevertheless being explored and promising results have already been obtained.

The proof of the principle that prions can multiply in transgenic mice-derived primary cultures of neurons [34] should stimulate the development of cultures from mice expressing PrP^C from various species. Such models may propagate prion strains that do not multiply to detectable levels in the currently available established cell lines and a critical issue is whether BSE or human CJD prions will multiply in these primary cultures. In addition, they should give valuable insight into the mechanisms underlying the strain-specific neurotoxic damage [48] observed in vivo.

Neuronal stem cells (NSC) isolated from the mammalian central nervous system represent another new cell culture system with potential benefits for prion propagation studies. NSC can be grown in tissue culture as multipotent cells but can also be induced to differentiate into neurons and/or astrocytes [87]. NSC and neurospheres (which contain NSC activity) from conventional and transgenic mice were successfully infected by 22L and RML murine prion strains [53,90]. In both cases, it was not clear which types of cells (e.g., progenitors or differentiated cells) were infected. As for cultured neurons, NCS from various PrP^C transgenic mice might increase the number of prion strains that could be transmitted to these new models. However, further work is necessary to establish if frozen stocks of NSC will be a valuable cell-based tool to titrate prion infectivity.

The development of a permissive cell model that could be genetically engineered to express various PrP^C could also be of great value to investigate different aspects of prion multiplication. RK13 cells, which express no detectable endogenous rabbit PrP^C, can be transfected with sheep PrP^C and become susceptible to sheep scrapie agent multiplication [146]. The fact that RK13 cells expressing rodent PrP^C are also permissive to several rodent-adapted prion strains [33] is a significant step towards obtaining a versatile cell model of prion infection. A further advance has recently been achieved by showing that transduced expression of mouse PrP^C conferred prion permissiveness to a hippocampal cell line (HpL3-4) derived from a PrP^C knock-out mouse [80]. It will be interesting to compare the panel of prion strains that will replicate in RK13 and HpL3-4 cells expressing a given PrP^C. However, these two cell models facilitate reverse-genetic approaches to assessing the influence of the PrP^C sequence on prion multiplication and strain identity.

11. CONCLUSION

The past ten years have greatly increased our basic understanding of prion biology. However, some fundamental questions are still unresolved. Ongoing work in different laboratories is currently leading to the development of more robust, versatile, and pertinent cell models of prion infections. It is anticipated that they will increase our knowledge on the fascinating properties of these infectious agents, including their precise molecular composition, the basis of cell permissiveness, and the identification of the molecular entities leading to neuronal cell damage.

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