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Correlation of cellular factors and differential scrapie prion permissiveness in ovine microglia

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ABSTRACT

Prion diseases are fatal neurodegenerative disorders by which the native cellular prion protein (PrP^C) is misfolded into an accumulating, disease-associated isoform (PrP^D). To improve the understanding of prion pathogenesis and develop effective treatments, it is essential to elucidate factors contributing to cellular permissiveness. We previously isolated five clones from an immortalized subline of ovine microglia, two of which had demonstrated differential permissiveness to a natural isolate of sheep scrapie and distinct transcriptomic profiles. To more robustly identify factors contributing to this activity, relative permissiveness, cell proliferation, selected gene transcript level, and matrix metalloproteinase 2 (MMP2) activity were compared amongst all five clones. Differences in cell proliferation were not detected between clones; however, significant correlations were identified between relative permissiveness and genes associated with cell growth (i.e., *RARRES1* and *PTN*), protein degradation (i.e., *CTSB* and *SQSTM1*), and heparin binding (i.e., *SEPP1*). MMP2 activity varied amongst clones, but did not correlate with permissiveness. These associations support the contribution of cell division and protein degradation on the permissiveness of cultured ovine microglia to PrP^D.

1. Introduction

Prion diseases are fatal neurodegenerative disorders that affect humans (e.g., Creutzfeldt-Jakob disease) and several animal species (e.g., scrapie in sheep) (Aguzzi and Calella, 2009). A key event in pathogenesis is the conversion of the host-encoded, cellular form of the prion protein (PrP^C; C superscript for "cellular") into misfolded isoforms (PrP^D; D superscript for "disease-associated") (Prusiner, 1998). PrP^C is a cell-surface protein encoded by the prion gene (*PRNP*) and is highly expressed in multiple cell types, notably those of the central nervous system (CNS) (e.g., neurons and microglia) (Aguzzi and Calella, 2009; Prusiner, 1998). Accumulation of PrP^D within the CNS leads to slowly progressive neurodegeneration and death (Aguzzi and Calella, 2009; Prusiner, 1998). The development of therapeutics is in part hampered by incomplete knowledge about cellular pathogenesis, including mechanisms underlying cellular permissiveness to prions.

While it is well established that cellular expression of PrP^C is required for infection (Bueler et al., 1993; Vilette et al., 2001), expression level alone is insufficient to explain variation in permissibility. Certain changes to the amino acid sequence of PrP^C dramatically affect relative susceptibility to infection and disease incubation time (Belt et al., 1995; Goldmann et al., 1994). However, other factors must also play a role given the differences in susceptibility and disease observed between mice bearing the same *PRNP* genotype (Lloyd et al., 2001) and the differential permissiveness observed between clones of cultured cell lines similarly expressing sequence-matched PrP^C (Klohn et al., 2003; Munoz-Gutierrez et al., 2015; Neale et al., 2010; Polymenidou et al., 2008). Such observations clearly suggest the existence of non-PrP^C factors that affect cellular permissiveness to prions.

Factors that impact PrP conversion, localization, and degradation may affect cellular permissiveness to prion infection (Ghaemmaghami et al., 2007; Grassmann et al., 2013). Cell division (Ghaemmaghami et al., 2007) and the expression of extracellular matrix (ECM) components (Marbiah et al., 2014) are specifically known to influence prion propagation in a murine neuroblastoma cell line. For example, prion permissiveness of this model system was related to a gene network that

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in part regulates homeostasis of the ECM (Marbiah et al., 2014). Functions of the identified genes were dependent on cellular differentiation and included activation of matrix metalloproteinase 2 (MMP2) (e.g., through *Fn1* expression), sulfation of glycosaminoglycans (e.g., through *Papss2* expression), and regulation of cell shape and motility (e.g., through *NCKAP1L* expression (Weiner et al., 2007)). These studies did not, however, determine if there were key hyposulfated proteoglycans nor relate variation of ECM components with the mitotic rate. Furthermore, the above studies were limited to murine neuroblastoma cells.

We have similarly investigated an immortalized ovine microglia subline for factors that affect permissiveness to a *PRNP*-homologous source of sheep brain-derived scrapie prions. In an initial study comparing two clones of this subline (Munoz-Gutierrez et al., 2016), we demonstrated that differential permissiveness was not due to differences in PrP^{C} expression and, by analysis of the transcriptome, revealed an association with differential regulation of numerous genes, including those with roles specific to heparin binding (e.g., *SEPP1*), cell proliferation (e.g., *RARRES1*), protein degradation (e.g., *SQSTM1*), and the ECM (e.g., *MMP14*).

In the study described herein, these and other factors were more closely and robustly evaluated by comparing the capacity of prion propagation in five clones of the immortalized ovine microglia subline described above. Permissibility was measured using the Standard Scrapie Cell Assay (SSCA) and tested for correlation with cell proliferation rate, MMP2 activity, and transcript levels from a selected subset of genes previously correlated with permissiveness to prions (Munoz-Gutierrez et al., 2016).

2. Materials and methods

2.1. Cell lines and inoculation

The ovine cell line used in this study was originally isolated, immortalized, cloned, and characterized in previous studies (Munoz-Gutierrez et al., 2016; Munoz-Gutierrez et al., 2015), which were approved by the Institutional Animal Care and Use Committees of Washington State University (ASAF04575). For these experiments, five clones (434, 438, 439, 440, and 441) previously generated from human telomerase (hTERT) immortalized ovine microglia subline H were utilized (Munoz-Gutierrez et al., 2015). Naïve clones were maintained in culture at 37 °C in OMEM (Opti-MEM [Gibco] medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 IU of penicillin, and 10 mg streptomycin ml^{-1}) and passaged every 5 days. For passaging, cells were lifted with 1 × trypsin-EDTA in 1 × PBS (Gibco).

The clones were inoculated with a sheep-derived scrapie isolate (Utah isolate) as previously described (Munoz-Gutierrez et al., 2015). Briefly, cells were plated at a density of approximately 1×10^5 cells per well into a twelve-well tissue culture plate. After overnight incubation, media was exchanged for 500 µl of 2.5% (w/v) scrapie (Utah) brain homogenate in OMEM. Mock-inoculated (i.e., negative control) cells received 500 µl of OMEM. Cells were incubated for six hours after which 500 µl of OMEM were added to each well. Cells were incubated for 5 additional days prior to the replacement of inoculum with fresh OMEM. Following 9 additional days of incubation, cells were expanded to 25-cm^2 culture flasks and split 1/5 every 2 weeks with fresh OMEM added weekly.

2.2. Quantification of PrP^{res} accumulation by SSCA

To measure the permissiveness of clones to scrapie prion infection, the capacity of PrP^{res} propagation (res superscript for proteinase K [PK]-resistant PrP^{D}) was evaluated at post-inoculation passages 3 (P-3), 5 (P-5), and 8 (P-8) using the Standard Scrapie Cell Assay (SSCA) (Mahal et al., 2008) as adapted to sheep microglia (Dinkel et al., 2016). The SSCA was selected as the measure of prion permissibility since it measures the percent of a cell population that has detectable levels of PrP^{res}. Thus, a higher percentage of positive cells within a sample indicates that the cells are more permissive to prion propagation. This is in contrast to assays such as immunoblots, which measure PrP^{res} in a pooled sample, and thus may have the same number of positive cells, but with an increased amount of PrP^{res} in those positive cells. Cells were collected and plated at an estimated density of 20,000 cells/well into a 96-well ELISpot plate (MultiScreen-IP 0.45-µM filter plate, Millipore) (Mahal et al., 2008). For each clone, eight Utah-inoculated (i.e., scrapie-inoculated cells) and eight mock-inoculated (i.e., OMEM-treated cells) wells were plated. Cells were treated with 0.6 µg PK ml^{-1} (2.5 units mg^{-1}, Roche Applied Science, Indianapolis, IN, USA) in lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris-HCl [pH 8.0], 5 mM EDTA, and 150 mM NaCl) for 90 min shaking at 37 °C. Cells were subsequently labeled with the anti-PrP mAb 6H4 (0.5 µg ml^{-1}; Prionics, Zurich, Switzerland) and an alkaline-phosphatase-conjugated anti-mouse IgG_1 antibody (1:2000; Southern Biotechnology Associates, Birmingham, AL, USA) (Dinkel et al., 2016). A cutoff value representing the 95% upper confidence limit of the average spot number detected in all mock-inoculated wells (i.e., background spot number) was calculated (Frey et al., 1998) to objectively categorize clones as PrP^{res}-positive or −negative. Permissiveness was expressed as the average spot number determined from at least 3 independent replicate assays and statistically compared as described in subsection 2.7.

2.3. PrP^{res} evaluation by immunoblot

PrP^{res} accumulation was also evaluated by immunoblot, to confirm the molecular weight and glycoform pattern. At P-6, cells were collected by lifting and centrifugation at 1000 × *g* (JA50.5 rotor) for 7 min at room temperature (RT). Cells were washed with 1 × Dulbecco's-PBS (D-PBS) and pellets were frozen at −80 °C until testing. Cell pellets were lysed and lysates were electrophoresed, electroblotted, and immunoblotted as previously described (Stanton et al., 2008). Briefly, lysates were measured for total protein by the bicinchoninic acid protein assay (BCA kit; Thermo Scientific) and diluted to equivalent protein concentrations prior to proteinase K (50 µg $µl^{-1}$, 2.5 units mg^{-1}, Roche Applied Science) digestion and phosphotungstic acid (PTA) precipitation; both steps included a 1-h incubation at 37 °C. PrP^{res} bands were labeled using 3.5 µg ml^{-1} of the mAb F99/97.6.1 (O'Rourke et al., 2000) and a goat anti-mouse IgG_1 antibody conjugated to HRP (1:5000; Southern Biotechnology Associates). Membranes were incubated with chemiluminescent substrate (Luminata™ Forte Western HRP Substrate; Millipore) for 3 min and signals were detected by capture onto autoradiography film (GeneMate, Kaysville, UT, USA).

2.4. Measurement of cell proliferation

The BrdU Cell Proliferation ELISA Kit (Abcam, Cambridge, MA, USA) was used to measure the 24-h incorporation of BrdU into dividing cells in two experiments. First, cellular proliferation of naïve clones was measured by plating clones in duplicate into a 96-well plate at a density of approximately 11,000 cells per well and incubating with bromodeoxyuridine (BrdU) for 24 h. The cells were then fixed and BrdU content was assessed by spectrophotometry (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA) per manufacturer's instructions. Cell proliferation was expressed as the 24-h incorporation of BrdU ($OD_{450–550}$). To correct for variation between replicate assays, absorbance values ($OD_{450–550}$) for each clone were normalized to the scrapie negative clone 441 (interassay standard). In similar fashion, cell proliferation was measured for clones at P-8 following Utah- or mock-inoculation. For both naïve and inoculated clones, cell proliferation was expressed as the average normalized absorbance values from at least 3 independent replicate assays.