Establishment of a Cell Line Persistently Infected with Chronic Wasting Disease Prions

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ABSTRACT: Elk prion protein (PrPSc) has been confirmed to be capable of rendering rabbit epithelial RK13 cells permissive to temporal infection by chronic wasting disease (CWD) prions. The present study satisfactorily generated persistently CWD prion-affected RK13 cells (RKC1-11) using elk PrPSc expressing cells (elkRK13) that were generated via the lentiviral expression system with high efficiency. The elkRK13 cells have been shown to be permissive to accumulation of abnormal isoforms of prion protein (PrPSc) resulting from CWD prions up to 97 serial passages thus far. This novel prion-affected cell line will help facilitate investigation of the molecular basis of CWD prion pathogenesis and confirmation of CWD prion infectivity in vitro.

KEY WORDS: CWD, infection, lentivirus, prion, RK13 cells.


Chronic wasting disease (CWD) is a neurodegenerative disorder in cervids and a member of the transmissible spongiform encephalopathies (TSEs), also known as prion diseases. TSEs are characterized by abnormal prion proteins (PrPSc) acting as infectious agents, which are generated by posttranslational modification of normal prion proteins (PrPC) that accumulate in the brain and lead to diseases [10, 13]. No TSEs have been found in any animal species in the Republic of Korea except for CWD, which is known to have originated from imported elk from Canada [12]. National CWD surveillance has been conducted since 2001, and subsequent cases were found in farmed elk in 2004 and 2005 [6]. More recently, CWD cases were detected in farmed elk, sika deer, red deer, and crossbred sika and red deer in 2010. This is the first report of natural CWD cases in red deer and crossbred sika and red deer in 2010. Epidemiologically speaking, it is possible that these indigenous CWD cases are associated with the imported CWD-affected elk.

Several lines of persistently prion-affected cells have represented relevant and useful experimental models [14]. Mouse neuroblastoma cell lines (N2a) are the most commonly used; however, their use is limited by the fact that they are susceptible only to mouse-adapted prion strains. In contrast, rabbit kidney epithelial cells (RK13), which overexpress exogenous sheep PrPC, have been confirmed to be susceptible to natural sheep scrapie [3]. On the other hand, CWD prion persistently infected cell lines have been established from transformed primary mule deer brain cells [11]. Attempts to establish persistent CWD prion infection in a cell line have previously been unsuccessful, resulting in transient PrPSc accumulation in elk PrPSc-expressing RK13 cells [1].

In this study, we attempted to establish a CWD prion persistently infected cell line to help characterize CWD prions, which are currently causing sporadic disease in South Korea. We tested the lentiviral protein expression system [5] as an approach method because it has broad tropism to a range of species and cell types, a stably expressed cell line is easily selected using safe laboratory practices without lentivirus production [4], and there are a variety of cell types available that allow for PrPSc expression. The Prnp gene, with Met at codon 132, was amplified from elk genomic DNA by polymerase chain reaction and cloned into the pLEX-MCS vector (Open Biosystems, Huntsville, AL, U.S.A.) to generate a lentiviral transfer vector. Recombinant lentivirus was produced as described previously [5]. Briefly, a lentiviral transfer vector, a VSV-G expression vector (Addgene, Cambridge, MA, U.S.A.) and a gag-pol expression vector (Addgene) were cotransfected into 293T cells, and the supernatant was used as recombinant lentivirus stock [5]. HeLa cells were used to check the viral titers.

To develop CWD prion-susceptible cell lines, we inoculated recombinant lentivirus into a rabbit kidney epithelial cell line (RK13) that does not express endogenous PrPSc (Fig. 1). To select the stable PrPSc expressing cells, lentivirus-infected cells were incubated with puromycin for 6 passages. PrPSc expression was confirmed using an indirect fluorescence assay (IFA). The cells were fixed with 4% paraformaldehyde for 30 min and then permeabilized with 0.1% Triton X-100. The PrP was detected using mAb 6H4 (Prionics, Zurich, Switzerland) and an anti-mouse IgG-FITC conjugate. All of the surviving cells, those that were puromycin resistant, expressed PrPSc on their cell mem-
branes (Fig. 1). The cells were lysed with buffer containing 150 mM NaCl, 1% Zwittergent 3–14, 50 mM Tris-HCl (pH 7.5), and 2 mM EDTA. The supernatants were mixed using magnetic beads (Dynabeads M-280 Sheep Anti-Mouse IgG; Invitrogen, Carlsbad, CA, U.S.A.) coated with mAb 6H4 (Prionics) for 1 hr, and then the beads were washed with PBS for 3 times. The captured PrP\textsubscript{C} was detected by Western blotting (WB) by using polyclonal antibody S1, which was obtained by immunization of rabbits against the synthetic peptide corresponding to the bovine PrP106-122 (CTH-GQWNKPSKPKTNMK). Molecular weights of 28–37 kDa for the PrP signal were observed. This signal disappeared with 10 \(\mu g/ml\) of Proteinase K (PK) digestion, which led us to classify it as PrP\textsubscript{C} (Fig. 2A). The cells stably expressed PrP\textsubscript{C} without puromycin treatment. We therefore concluded that the elk PrP\textsubscript{C}-expressing RK13 cell (elkRK13) was established with high efficiency through use of the lentiviral expression system.

To obtain persistently CWD prion-affected cell lines, we infected elkRK13 with 0.2% (W/V) CWD brain homogenates. It was previously reported that phorbol 12-myristate 13-acetate (PMA) was applied to enhance PrP\textsubscript{C} and PrP\textsubscript{Sc} production in scrapie 22L-affected N2a cells [9]. After 6 hr of CWD prion infection, the cells were treated with 100 \(\mu g/ml\) of PMA for 4 days, and then PrP\textsubscript{Sc} accumulation was assessed using the standard scrapie cell assay (SSCA) [8]. ELISPOT plates (MSIPS4510; Millipore, Billerica, MA, U.S.A.) were activated using 70% ethanol for 2 min. Twenty-five thousand cells were seeded onto each plate, washed twice and then dried. The dried cells on the membranes were then treated with 2 \(\mu g/ml\) of Proteinase K (PK) digestion, which led us to classify it as PrP\textsubscript{C} (Fig. 2A). The cells stably expressed PrP\textsubscript{C} without puromycin treatment. We therefore concluded that the elk PrP\textsubscript{C}-expressing RK13 cell (elkRK13) was established with high efficiency through use of the lentiviral expression system.

![Fig. 1. Immunofluorescence staining of uninfected cells (RK13 and elkRK13) and persistently prion-infected RKC1-11 cells (passage 28). The PrP signal was detected by mAb 6H4.](image1)

![Fig. 2. Western blot analysis of the persistently CWD-infected cell line. A. RK13, elkRK13 and RKC1-11 (28 passages) cells were analyzed by Western blotting, with or without PK digestion. Molecular mass size markers are shown on the left. The arrows indicate un-, mono- and diglycosylated PrP\textsubscript{Sc} molecules from bottom to top, respectively. +=10 \(\mu g/ml\) PK, ++=20 \(\mu g/ml\) PK. PrP\textsubscript{C} from normal elk brain and PrP\textsubscript{Sc} from CWD elk brain, from which the original samples were collected to generate elkRK13 cells, were also used. B. Detection of the PrP\textsubscript{Sc} in the RKC1-11 cells at passages 87 and 97. The PrP signal was detected by mAb 6H4. GAPDH (glyceraldehydes-3-phosphate-dehydrogenase; Abcam, Cambridge, U.K.) antibody was used as a loading control.](image2)
The PrPSc was removed by RNase A for 15 min and centrifuged at 20,000 × g. In this study, we also experienced unsuccessful results during serial passages. In addition, we confirmed the PrPSc-positive cell lines (RKC1-1 to RKC1-12), and the PrPSc signals in these cells were detected up to 28 passages. For PrPSc analysis, the cells were lysed using 0.5% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, and 5 mM EDTA in 10 mM Tris-HCl (pH 7.5) and then incubated with 10 µl of PK. The digestion and, if necessary, 20 µg/ml of RNase A for 15 min and centrifuged at 20,000 × g for 45 min at 4°C. The pellets were then subjected to WB for analysis. The PrPSc-specific signal was observed after PK digestion. WB analysis showed the dominant diglycosylated PrPSc glycoform pattern in RKC1-11 cells was different from that of CWD elk brains (Fig. 2A).

To confirm persistent infection of CWD prions in RKC1-1 to RKC1-12 cells, the cells were serially passaged, and the accumulation of PrPSc was checked using a commercial ELISA-based CWD test kit (IDEXX HerdChek EIA). Only the RKC1-11 cells maintained PrPSc accumulation up to 95 serial passages (Table 1). The other 11 clones contained PrPSc but lost it during passage (data not shown). The OD values of the RKC1-11 cells were constantly over 1.0 during serial passages. Morphological changes in the RKC1-11 cells were not observed during serial passages. In addition, we confirmed the PrPSc accumulation in the RKC1-11 cells at passages 87 and 97 by WB (Fig. 2B).

This study showed that RKC1-13 cells that express exogenous elk PrPC are susceptible to CWD prion infection and are permissible to persistent accumulation of PrPSc. A previous study showed that temporal infection was observed in elk PrPC-expressing RKC13 cells; however, chronic infection was unsuccessful [1]. In this study, we also experienced disappearance of PrPSc during passage, with only 1 (RKC1-11) of the examined 12 clones maintaining PrPSc stably. Stable expression of exogenous PrPC in the cells may contribute to their susceptibility to CWD prions, as well as persistent infection by CWD prions. We used the lentiviral expression system, because it is known to be highly efficient for transfection of exogenous genes [2]. Our results demonstrated that 100% of the cells expressed PrPC after puromycin selection, which illustrates the importance of stable PrPC expression in the establishment of prion persistently affected cells. The difficulties in obtaining persistently CWD prion-infected cells may be elucidated as follows: RKC13 cells did not express PrPC, and thus we can exclude the possibility that endogenous PrPC inhibited the exogenous PrPC to PrPSc conversion in this study [15]. There is the possibility, however, that PrPSc formation by acute infection kills the host cells [16]. It is important to note that RKC13 cells were established as a cloned cell line that has been well characterized.

A detailed analysis of the RKC1-11 cells may contribute to further investigations.

We also cloned elkRKC13 cells and established cloned CWD-susceptible cells (RK-SEN-C10). The RKC1-11 cells at 34 serial passages were intracerebrally inoculated into transgenic mice that overexpress elk PrPC [7]. The transmission study is still in progress, and the results will be published along with identified characteristics of the PrPSc that accumulates in the RKC1-11 cells.

Currently, there are no reported cell lines permissive to bovine spongiform encephalitis (BSE) or human Creutzfeldt–Jakob disease (CJD) prions. By adopting our expression system, development of novel cell lines susceptible to BSE and variant CJD may be possible. Interestingly, a persistently classical BSE-infected cell line has been successfully developed using the lentiviral expression system (manuscript in preparation).

This study established a persistently CWD-infected cell line and CWD prion-susceptible cell lines. These cells may be advantageous used in a wide range of applications including confirmatory diagnosis, etiological identification, screening of anti-PrPSc substances and formation and inhibition of CWD prions in vitro.

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