A neuronal cell line that does not express either prion or doppel proteins

Boe-Hyun Kim, Jae-II Kim, Eun-Kyoung Choi, Richard I. Carp and Yong-Sun Kim

Prions have been extensively studied since they represent a new class of infectious agents, the pathogenic prion protein (PrPSc). However, a central question on the physiological function of the normal prion protein (PrPc) remains unresolved. A cell model which was previously established from Rikn mice (PrPSc−/−) remains problematic because of its ectopic expression of the doppel (Dpl) which may have a neurotoxic effect. Here we established neuronal cell lines from Zürich I (PrPc−/−) which do not express Dpl protein and ICR mice (PrPc+/+) by transfecting with plasmid encoding for the large T antigen of SV40. The transformed cells have shown neuronal characteristics and, thus, these cell lines may provide a useful model to explore the function of neuronal PrPc. NeuroReport 16:425–429 © 2005 Lippincott Williams & Wilkins.

Key words: Prion protein-deficient neuronal cell line; Doppel protein; Transformed cells; Zürich I mice

INTRODUCTION

Transmissibility is the property that separates transmissible spongiform encephalopathies (TSE or prion diseases) from other neurodegenerative diseases such as Alzheimer’s and Parkinson’s diseases; this property seems to reside, at least in part, in an aberrant form, PrPSc, of a constitutively expressed protein, PrPc [1]. It has been proposed that interaction of the conformer proteins, PrPc and PrPSc, generate more PrPSc via an undefined post-translational process [2]. PrPc is known to express constitutively in both neuronal and non-neuronal cells, but is at the highest level in neurons in the central nervous system (CNS). Despite the wide distribution of expression in various tissues and across numerous species, there is no consensus regarding the cellular functions of this protein [3–5].

Previously, PrP-deficient (PrPc−/−) cell lines were established from Rikn mice to provide a cellular model for the study of physiological function of PrPSc [6], however, these cells express Dpl protein which has 30% homology to the C-terminus of PrPc, and Dpl protein is reported to have neurotoxic effects [7–9]. The aim of the present work was to establish, as an experimental tool, a continuous cell line of neuronal cells from Zürich I (PrPc−/−) which do not express Dpl protein and ICR mice (PrPc+/+) by transfecting with plasmid encoding for the large T antigen of SV40. The transformed cells have shown neuronal characteristics and, thus, these cell lines may provide a useful model to explore the function of neuronal PrPc.

MATERIALS AND METHODS

Establishment of hippocampal neuronal cell lines from Zürich I (PrPc−/−) and control ICR mice: Primary hippocampal neuronal cells were cultured from 14-day-old embryos from Zürich I and ICR mice (Daehan Biolink, Korea). Cells were cultured on poly-L-lysine (P-L-L)-coated dishes with culture media (DMEM with 20% FBS, 100 unit/ml penicillin and 100 unit/ml streptomycin, Gibco BRL) incubated at 37°C in 5% CO2 and transfected with SV40 large T antigen containing vector (pSV3neo) using lipofectamine as described in the manufacturer (Invitrogen). After 1 week, the media was replaced by selective media (DMEM with 0.25 mg/ml G418, Boehringer Mannheim, Mannheim, Germany), which was then changed every 3 days. When colonies were visible and confluent, they were transferred to 24 well P-L-L-coated culture dishes and later transferred to 6 well P-L-L-coated culture dishes. The origin of the mouse lines and characteristics of cell lines used in the present study are shown in Table 1.

Estimation of the cell growth cycle and morphological analysis: Each cell lines were seeded at a density of 1 × 10⁵ cells and incubated at 37°C in 5% CO2; the cells were stained and counted each day for 6 days [10]. Cell growth and morphological analysis was assessed using inverted microscopy (Zeiss, Oberkochen, Germany).

Western blot analysis and immunocytochemistry: For Western blot analysis, 50 μg protein from each cell lysate was prepared and separated on 12% Tris-glycine gels and transferred to nitrocellulose membrane (Amersham). The membrane was blocked with 5% nonfat dry milk in TBST for 1 h at room temperature and then probed with one of the primary antibodies: anti-PrP 3F10 at dilution of 1:1000 [11], anti-NeuN (neuron-specific nuclear protein) at 1:1000.
(Chemicon, Temecula, California, USA), anti-GFAP (glial fibrillary acidic protein) at 1:5000 (DAKO), anti-CD11b (Integrin αM) at 1:1000 (Serotec, Oxford, UK), anti-CNPase (2’,3’-cyclic nucleotide 3’-phosphodiesterase) at 1:1000 (Sigma, St. Louis, Missouri, USA). Incubation with primary antibody was overnight at 4°C, and appropriate secondary antibodies conjugated with horseradish peroxidase (Zymed, San Francisco, California, USA) were then added. Bound antibodies were visualized by chemiluminescence (Pierce, Rockford, Illinois, USA). Anti-actin at 1:300 (Santa Cruz Biotechnology, INC, Santa Cruz, California, USA) was used as a cellular marker. For immunocytochemistry, cells were plated on 1% gelatin coated cover slips and cultured for 24 h. Cells were fixed, permeabilized with 0.2% Triton X-100 (Sigma) at room temperature for 10 min, treated with 2.5% normal donkey serum (Jackson, West Grove, Pennsylvania, USA) and 1% BSA (Sigma) in PBS at RT for 1 h, and then rinsed with PBS. Cells were incubated with primary antibodies against MAP2 (microtubule-associated protein 2) at 1:50 (Upstate, Charlottesville, Virginia, USA) as a neuronal marker; GFAP at 1:100 (DAKO, Glostrup, Denmark) as an astrocyte marker; CD11b at 1:50 (Serotec) as a microglia marker and CNPase at 1:50 (Sigma) as an oligodendrocyte marker overnight at 4°C. Appropriate secondary antibodies conjugated with fluorochromes (Zymed) were then applied. After washing with PBS, cells were incubated with Hoechst (10μM, Sigma) at 37°C for 1 min and observed using confocal microscopy (Zeiss). Hoechst staining was used as a cellular marker.

Table 1. Mouse origin and characteristics of cell lines used in this study.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Mouse origin</th>
<th>Cell line expression</th>
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<tbody>
<tr>
<td>HW 8-2</td>
<td>C57BL</td>
<td>+</td>
</tr>
<tr>
<td>Hpl 3-4</td>
<td>Rikn (from C57BL)</td>
<td>−</td>
</tr>
<tr>
<td>ZW 13-1, 13-2, 13-3</td>
<td>ICR</td>
<td>+</td>
</tr>
<tr>
<td>Zpl 2-1, 2-4, 3-4</td>
<td>Zürich I (from ICR)</td>
<td>−</td>
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Fig. 1. Hippocampal neuronal Zpl and ZW cell lines expressing SV40 T antigen. (a) Confirmation of SV40 T antigen expression (105 bp) in Zpl and ZW cell lines by PCR analysis. M: 100 bp ladder marker; Zür: a negative control; Hpl 3-4: a positive control. (b) Comparison of the growth rate in Zpl and ZW cell lines. (c) Representative doubling time of each cell lines. Proliferation time was estimated from the growth rate of each cell lines presented in (b). The numbers above each column represent the doubling time of each cell line.
Reverse transcriptase polymerase chain reaction (RT-PCR): To confirm the PrP and Dpl expression, total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, California, USA), and cDNA was synthesized. To test for integration of SV40 large T antigen, genomic DNA was extracted from cultured cells using DNA extraction kit (Qiagen, Hilden, Germany). PCR was performed with the following primers (Bioneer, Daejeon, Korea): PrP, sense: 5'-ATGGCGAACCTTGGCTACTGG-3'; antisense: 5'-CCCTCATCCACGCATAGAAATG-3'; and Dpl, sense: 5'-GGGGATCCATGAAGAACCGGCTGGG-3'; antisense: 5'-CCGCTCGAGTTACTTCACAATGAACCA-3'; GAPDH, sense: 5'-TGGTATCGTGGAAGGACTCATGAC-3'; antisense: 5'-ATGCCAGTGAGCTTCCCGTTCAGC-3'; SV40 large T antigen, sense: 5'-TGAGGCTACTGCTGACTCTCAACA-3'; antisense: 5'-GCATGACTCAAAAACTTAGCAATTCTG-3'.

RESULTS
PrP−/− hippocampal neuronal cell lines established from Zür I and control mice: Three distinct neuronal cell lines were established from the hippocampus of Zür I (PrP−/−) mice (Zpl 2-1, 2-4, 3-4) and from control ICR (PrP+/+) mice (ZW 13-1, 13-2, 13-3). The presence of the gene for SV40 large T antigen was examined with PCR analysis using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the housekeeping control gene. As shown in Fig. 1a, PrP+/+ cell lines had more copies of SV40 large T antigen than PrP−/− cell lines. Despite this, the proliferation rates of PrP−/− cells were higher and the doubling times were shorter than those values for PrP+/+ cell lines (Fig. 1b,c). There were marked differences in the growth rates by the sixth day: the four PrP−/− cell lines (Zpl 2-1, 2-4, 3-4 and Hpl 3-4) were faster than the growth rates for the PrP+/+ lines (ZW 13-1, 13-2, 13-3 and HW 8-2). The doubling times are shown graphically in Fig. 1c. These data showed that the PrP+/+ cell lines had longer doubling times than the PrP−/− cell lines.

Expression of the PrP and Dpl genes in Zpl and ZW cells: To examine the gene expression of each cell, we used RT-PCR analysis. The expression of PrP gene was not detected in wild-type ZW and HW cells but not in Zpl cells (Fig. 2a). In contrast GAPDH was expressed to the same extent in all samples. Subsequently, to analyze the expression of PrP we performed Western blot analysis (Fig. 2b). As expected, the 3 Zpl lines did not contain PrPC, whereas the ZW lines contained ample amounts of PrPC. The same amount of total protein was analyzed as shown by the similar levels of actin expression in the various cell lines.

Characterization of established cell lines: As reported [6], the Hpl 3-4 and HW 8-2 cell lines showed significant differences in their morphological appearance. The HW 8-2 cells have much longer neurite extension, and the cells are narrower than Hpl 3-4 cells (Fig. 3). The morphological appearance of Zpl and ZW cell lines were similar, although the neurites of the ZW cells are slightly longer than those of the Zpl cells. We used different cell-type marker antibodies that described in Materials and Methods to characterize the transformed cell lines. The results were negative for the oligodendrocyte and microglial markers in both Western
blot and immunofluorescence experiments (data not shown). C6 and HW 8-2 cell lines were used as positive controls for the astrocyte marker and the neuronal marker, respectively. Zpl and ZW cells were neuron-positive in Western blot analysis using antibodies against NeuN, a 66 kDa protein band; which did not react with C6 cell lysates (Fig. 4a). Using anti-GFAP antibody, a 50 kDa protein band was detected only in C6 cell lysates (Fig. 4a). Thus, these newly established lines were shown to be composed of neuronal cells. In agreement with the Western blot findings, the Zpl (a and d) and ZW (b and e) cells positive for MAP-2 staining but not GFAP whereas C6 cells (c and f) were positive for GFAP but not MAP-2 using immunofluorescence analysis (Fig. 4b).

**DISCUSSION**

We established hippocampal neuronal cell lines from Zür I PrP<sup>−/−</sup> and ICR PrP<sup>+/+</sup> mice; which do not express the Dpl gene. The fact that expression of Dpl in the absence of PrP expression in vivo can cause neuropathological and clinical changes will certainly affect the usefulness of a cell line that is PrP<sup>−/−</sup> and Dpl<sup>−/−</sup> in studying the role of PrP in cellular physiology.

The proliferation rate of PrP<sup>−/−</sup> cell lines (Zpl and Hpl) is faster than those of PrP<sup>+/+</sup> cell lines (ZW and HW); the difference was ~2.8-fold for the Zpl vs ZW cell line that does not express either PrP and Dpl and ~1.5-fold for the Hpl 3-4 vs HW 8-2 cell line that does express Dpl. Although the results of Western blot, RT-PCR, and PCR analysis showed no differences in the expression levels of PrP and SV40 large T antigen among the PrP<sup>+/+</sup> cell lines, the exact doubling time of each cell line differed slightly. Furthermore, even though PrP<sup>−/−</sup> cell lines expressed a similar level of mRNA of SV40 large T antigen each of their doubling times differed from one another; the exact reason for the doubling time difference is unknown, but it could be a function of the region of insertion of the SV40 large T antigen gene or the number of copies of the gene.

With the development of the PrP<sup>−/−</sup> cell line, future effort could involve transfection of both Dpl-negative
cell lines (derived from Zpl) and Dpl-positive cell lines (derived from Hpl 3-4) with or without PrP C expression. These cell lines will be useful experimental systems to analyze the physiological function(s) of both PrP C and Dpl proteins, and to investigate their relationship in prion diseases.

CONCLUSION
Attempts to analyze the physiological function of PrP C in a comparatively simple neuronal tissue culture system have been compounded by the fact that the previously available PrP C cells overexpress the Dpl which may affect cellular function of PrP C [7,8,12–14]. The newly established cell lines, which do not express either PrP or Dpl retained the properties of neuronal cells, thus may provide a useful model to explore the functions of neuronal PrP C.

REFERENCES

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