Cell density-dependent increase in the level of protease-resistant prion protein in prion-infected Neuro2a mouse neuroblastoma cells

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Cells persistently infected with prions continuously produce protease-resistant prion protein (PrP-res). Here, we show that the PrP-res level in prion-infected Neuro2a (N2a) neuroblastoma cells decreased to 50 % of their initial level over the first 48 h and then recovered by 96 h after seeding. The level of cellular prion protein (PrPSc) also appeared to fluctuate, but did not influence the fluctuation of the PrP-res level. 

Prion-infected N2a cells, co-cultured with a higher number of prion-unsusceptible cells, had twice as much PrP-res as those cultured without unsusceptible cells, suggesting that cell density influences the fluctuation of PrP-res formation. Direct cell-to-cell contact between cells, rather than soluble factors, was involved in the cell density-dependent increase in the PrP-res level. The cholesterol content, which is known to influence PrP-res formation, also changed depending on cell density. Our data suggest that alterations in cellular microenvironments controlled by cell density influence PrP-res formation.

The causative agent of prion diseases, prion, is believed to be composed mainly of an abnormal isoform of prion protein (PrPSc). During the progression of the disease, a host-encoded cellular prion protein (PrPC) undergoes a conformational change to form PrPSc, which accumulates in the central nervous system. It is known that PrPSc is composed of protease-sensitive and -resistant forms (Tzaban et al., 2002; Safar et al., 2005; Pastrana et al., 2006). The PrPSc form that is resistant to limited proteolysis is referred to as PrP-res, and PrP-res is used as a diagnostic marker of prion diseases.

Studies using prion-infected cells have provided many insights into prion biology, including the mechanism of PrP biosynthesis and the cell biological mechanism of prion propagation (Caughey & Raymond, 1991; Borchelt et al., 1992; Vey et al., 1996; Naslavsky et al., 1997; Marijanovic et al., 2009), and transmission of prions (Fevrier et al., 2004; Gousset et al., 2009; Mattei et al., 2009). Although cells persistently infected with prions produce PrP-res and possess prion infectivity, the PrP-res level is known to change depending on the culture conditions, such as composition of the medium and differentiation state of cells (Rubenstein et al., 1990; Bate et al., 2004). The PrP-res level also changes during routine cell passage, possibly caused by subtle differences in the culture conditions.

PrPSc in prion-infected cells is believed to exist in a dynamic equilibrium between synthesis and degradation.

Theoretically, after each cell division, the amount of PrPSc per cell must halve, after which it increases again to an appropriate level until the next cell division (Weissmann, 2004). Indeed, a decrease in PrP-res levels in cultured cells 48–72 h after passage followed by recovery of the PrP-res level thereafter has been reported (Ghaemmaghami et al., 2007). During this process, certain host factors can modulate the equilibrium level of PrPSc by changing the rate of PrP-res formation, although the factors remain to be elucidated.

To understand the mechanism of fluctuation in the PrP-res levels in prion-infected cells, we analysed the kinetics of PrP-res levels in N2a subclones, N2a-5 and N2a-3 persistently infected with the Chandler strain (ScN2a-5-Ch and ScN2a-3-Ch; Uryu et al., 2007). In addition, N2a-5 and N2a-3 cells persistently infected with the 22L strain (ScN2a-5-22L and ScN2a-3-22L) were used. Methods of immunoblotting and dot blotting for detecting PrP are described in Supplementary Material (available in JGV Online).

When ScN2a-5-Ch cells were seeded at a split ratio of 1:10, the culture was 40–50 % confluent 48 h after seeding, and 80–90 % confluent at 72 h after seeding (Fig. 1a). For analysing PrP-res levels, cells were first seeded into several 60 mm dishes at a 1:10 split ratio. At 72 h after seeding, the cells grown in one dish were harvested and used as the 0 h sample. Cells grown in other dishes were seeded again into new 60 mm dishes at a 1:10 or 1:40 split ratio, and...
were harvested every 24 h for quantification of PrP-res by immunoblotting. In N2a-5 and N2a-3 cells infected with the Chandler strain, the level of PrP-res decreased to about 50% of that of the 0 h PrP-res sample over the first 48 h, following which PrP-res levels recovered to the initial level by 72–96 h after seeding. If the cells were maintained in the same dishes thereafter, PrP-res levels further increased and then reached a plateau (Fig. 1b; Supplementary Fig. S1a, available in JGV Online). When ScN2a-5-Ch cells were seeded at a 1:40 split ratio, under which the cultures became confluent at 120–144 h after seeding, PrP-res levels began to increase from 96 h after seeding, and had recovered to the initial level by 120–144 h (Fig. 1c). This result indicates that fluctuation in the level of PrP-res does not simply depend on the time period after the seeding of cells. Although a pattern of the kinetic change of the PrP-res level in N2a cells infected with the 22L strain did not appear to be the same as that of the PrP-res level in the

Fig. 1. Kinetics of the change in PrP levels in N2a subclone. (a) Phase-contrast microscopic images of ScN2a-5-Ch cells at the indicated culture times. (b) Changes in PrP-res levels in ScN2a-5-Ch cells. For the detection of β-actin, 10 μg post-nuclear cell lysates was loaded. For the detection of PrP-res, the cell lysates were treated with proteinase K (PK) and 100 μg cell lysate equivalents was loaded. The PrP-res level relative to that of PrP-res at 0 h was calculated, and graphs on the right represent the mean ± SD of the relative PrP-res levels (n=3). (c) The kinetics of PrP-res level in ScN2a-5-Ch cells passaged at a 1:40 split ratio (n=1, filled diamonds). Changes in the PrP-res level obtained after the passage at a 1:10 split ratio (open diamonds) were overlaid. (d) Changes in PrP C levels in N2a-5 cells. Cell lysates (10 μg) without PK treatment were loaded. Graphs show the PrP C level relative to the PrP C level at 0 h (mean ± SD, n=3).
Chandler-infected cells, the fluctuation in PrP-res level was also observed (Supplementary Fig. S1b, available in JGV Online).

The kinetics of PrP<sub>C</sub> levels in N2a-5 and N2a-3 cells, especially by 96 h after seeding, were similar to those of PrP-res levels (Fig. 1d; Supplementary Fig. S1c, available in JGV Online). The PrP<sub>C</sub> levels decreased to about 50% of that of the control (the amount of PrP<sub>C</sub> at 0 h) over the first 48 h, but had recovered by 96 h after seeding, suggesting that fluctuation in the PrP-res level might be caused by a change in the level of PrP<sub>C</sub>. However, the kinetics of PrP<sub>C</sub> and PrP-res levels in PrP-overexpressing cells showed that even though the cells expressed a level of PrP<sub>C</sub> that would be sufficient for PrP-res formation, the PrP-res level still decreased after seeding of the cells (Supplementary Fig. S2, available in JGV Online). Therefore, the observed fluctuation in the PrP-res level appears to be independent of that in the PrP<sub>C</sub> level.

The results described above suggested that cell density is one of the factors for the increase in PrP-res level. To determine if cell density is indeed related to the increase in the PrP-res level, ScN2a-3-Ch cells were co-cultured with various numbers of N2a-1 cells to produce conditions under which the same number of ScN2a-3-Ch cells was present in cultures of different cell densities. N2a-1 cells express the same level of PrP<sub>C</sub> as N2a-3 cells but are not susceptible to prions (Uryu et al., 2007). Therefore, changes in the PrP-res level in these co-cultures should represent changes in PrP-res level in ScN2a-3-Ch cells.

First, we analysed the effect of the presence of N2a-1 cells at different densities on the growth of ScN2a-3-Ch cells. ScN2a-3-Ch cells, labelled with the PKH26 fluorescent dye, were co-cultured for 72 h with various numbers of N2a-1 cells (Fig. 2a). Counting of cells labelled with red revealed no significant difference in the number of ScN2a-3-Ch cells among co-cultures. This suggested that different numbers of counterpart cells did not influence the growth of ScN2a-3-Ch cells at least over 72 h. Therefore, the experimental condition was considered to be suitable for comparison of the PrP-res level in ScN2a-3-Ch cells cultured at different cell densities.

To analyse the effect of cell density on the PrP-res level, a uniform number of ScN2a-3-Ch cells (1:20 split ratio) was cultured for 3 days with various numbers of N2a-1 cells. ScN2a-3-Ch cells cultured with higher numbers of N2a-1 cells (Fig. 2b, 1:10 to 1:80 split ratio) had PrP-res at a level that was almost twice as high as the same number of ScN2a-3-Ch cells cultured in the absence of N2a-1 cells. These results suggest that a higher cell density facilitates PrP-res formation. Consistent with this observation, cells in areas with a higher cell density showed more intense fluorescence signals of PrP<sup>sc</sup> than cells with a lower cell density (Supplementary Fig. S3, available in JGV Online).

Since no difference was observed in the PrP-res levels in ScN2a-3-Ch cells cultured with or without the conditioned medium of N2a-1 cells grown at various densities (Fig. 2c), direct cell-to-cell contact rather than soluble factors is involved in the increase in PrP-res levels. In addition, coculture of ScN2a-3-Ch cells with methanol-fixed N2a-1 cells did not increase PrP-res level (Fig. 2d), indicating that direct cell-to-cell contact between live cells is necessary for the increase in PrP-res levels. We further analysed the effect of PrP<sub>C</sub> expression in the co-cultured counterpart cells on PrP-res formation, using N2a-24 cells that express trace levels of PrP<sub>C</sub> (Uryu et al., 2007). The cell density-dependent increase in PrP-res levels was again observed when ScN2a-3-Ch cells were cultured with high numbers of N2a-24 cells (Fig. 2e, 1:5 and 1:10 split ratio). We confirmed that the level of PrP<sub>C</sub> in N2a-3 cells did not change under co-culture (Supplementary Fig. S4, available in JGV Online).

It has been reported that cellular cholesterol contents are increased in several cells when the cells reach confluence (Cansell et al., 1997; Corvera et al., 2000; Takahashi et al., 2007). Since cholesterol depletion has been shown to affect PrP-res formation in prion-infected cell cultures (Taraboulos et al., 1995; Bate et al., 2004; Prior et al., 2007), we first stained N2a-5 cells with filipin III, a sterol-binding polyene antibiotic. Stronger fluorescent intensities were observed in cells at a higher cell density than in cells at a lower cell density (Fig. 3a, compare cells at 24 and 96 h after seeding at 1:10 split ratio, or cells 72 h after seeding at 1:5 or 1:40 split ratio). Consistent with this observation, the cellular cholesterol content of subconfluent N2a-5 cells (Fig. 3b, 24 h after seeding) was about 25% less than that in confluent N2a-5 cells (96 h after seeding). In addition, the cholesterol content in N2a-5 cells seeded at a higher split ratio (Fig. 3c, 1:40) was lower than that in cells seeded at a lower split ratio (1:5). These results demonstrated that the cellular cholesterol content of N2a-5 cells increased when the cells reached confluence.

To examine whether an approximately 25% decrease in cellular cholesterol content influences PrP-res formation, cholesterol depletion was carried out with lovastatin or methyl-β-cyclodextrin (MβCD) under non-cytotoxic conditions (Fig. 3d). Lovastatin treatment resulted in 26% decrease in the cholesterol content of ScN2a-5-Ch cells. Under this condition, the level of PrP-res decreased to 45% of that of the untreated control. Thus, it is possible that the approximately 25% lower content of cholesterol in subconfluent N2a-5 cells affects PrP-res formation. In turn, the cholesterol content of nearly confluent, or confluent N2a cells, may be sufficient to maintain the level of PrP-res. On the contrary, MβCD treatment reduced cellular cholesterol content to 35% of that of untreated cells, which drastically reduced the level of PrP-res. Although MβCD is known to remove cholesterol from the cell membrane (Christian et al., 1997; Simons et al., 1998), MβCD also directly binds to PrP<sub>C</sub> and inhibits the conversion of PrP<sub>C</sub> to PrP-res (Prior et al., 2007). Therefore, the efficient clearance of PrP-res appears to be due not only to cholesterol depletion, but also to a direct effect of MβCD on PrP<sub>C</sub>.
Fig. 2. Effect of cell density on PrP-res formation. (a) Co-cultured ScN2a-3-Ch cells with prion-unsusceptible N2a-1 cells. ScN2a-3-Ch cells labelled with PKH26 fluorescent dye (labelled with red) were co-cultured with various numbers of N2a-1 cells for 3 days. Phase-contrast images of the cells, overlaid with the corresponding fluorescent images, are shown. The ratios at the top indicate the split ratio of ScN2a-3-Ch and N2a-1 cells at seeding. The means ± SD of the number of labelled cells from four micrographs are shown below the images. The arrows indicate cells with threshold intensity that were counted as positive. For all (b–e), the PrP-res levels were measured by dot blotting and representative blots are shown. The split ratios of cells at seeding are indicated below each graph. (b) PrP-res levels in ScN2a-3-Ch cells co-cultured with N2a-1 cells for 3 days. The graph indicates the level of PrP-res relative to those in ScN2a-3-Ch cells without N2a-1 cells (n=4). Asterisks indicate significant difference (P<0.05) as analysed by Williams’ multiple comparison test. (c) Effect of conditioned medium (CM). N2a-1 cells were seeded into 60 mm dishes at a 1:5 to 1:80 split ratio and cultured for 3 days. The supernatants (CM) were then harvested and filtered through a 0.22 μm filter. An equal volume of CM was added to ScN2a-3-Ch cells that were seeded at a 1:10 split ratio 24 h before the addition of CM, and the cells were incubated for an additional 48 h. The graph represents the level of PrP-res relative to the amount of PrP-res in ScN2a-3-Ch cells cultured without CM (n=1). (d) PrP-res levels in ScN2a-3-Ch cells co-cultured with fixed N2a-1 cells. N2a-1 cells seeded into 24-well plates were cultured for 3 days and fixed with methanol. ScN2a-3-Ch cells were seeded at a 1:20 split ratio into wells containing fixed N2a-1 cells and cultured for 3 days. The graph shows the level of PrP-res relative to the amount of PrP-res in ScN2a-3-Ch cells without fixed N2a-1 (n=3). (e) PrP-res levels in ScN2a-3-Ch cells co-cultured with N2a-24 cells for 3 days (n=4). The co-culture was started at a ratio of 1:5 because growth rate of N2a-24 cells was slower than that of ScN2a-3 cells.
Fig. 3. The effect of cell density on cholesterol content. (a) Cellular localization of cholesterol. N2a-5 cells seeded at a 1:10 split ratio and cultured for 24 or 96 h, or cells seeded at a 1:5 or 1:40 split ratio and cultured for 72 h, were stained for cholesterol with filipin III. Top, fluorescence images; bottom, phase-contrast microscopic images. (b) Cholesterol content of subconfluent (24 h after seeding at a 1:10 split ratio) and confluent N2a-5 cells (96 and 120 h after seeding at a 1:10 split ratio). Cholesterol content is expressed as nmol per mg total protein (TP) (n=3). The asterisk indicates significant difference (P<0.05). (c) Cholesterol content of N2a-5 cells seeded at a 1:5 or 1:40 split ratio and cultured for 72 h. (d) Effect of cholesterol depletion on PrP-res formation. ScN2a-5-Ch cells were treated with 0.3 μM lovastatin or 2.0 μM M/iCD for 96 h. The graph on the left indicates cholesterol content (n=3). The image in the middle is a representative immunoblot of PrPSc and corresponding β-actin, and the graph on the right shows the PrP-res level (n=3).
Cell confluency has also been reported to affect intracellular distribution of cholesterol in Chinese hamster ovary cells (Takahashi et al., 2007). Treatment of prion-infected cells with a 24-dehydrocholesterol reductase inhibitor, U18666A, has been shown to inhibit PrP-res formation (Klingenste... et al., 2009). Although treatment with U18666A did not appear to alter cellular cholesterol content (data not shown), U18666A inhibited intracellular cholesterol transport, resulting in a redistribution of cholesterol from the plasma membrane to intracellular compartments (Liscum & Underwood, 1995; Sparrow et al., 1999; Klingenstein et al., 2006). As shown in Fig. 3(a), N2a cells in an area of high cell density showed more intense cholesterol staining on the plasma membrane than N2a cells in an area with lower cell density. Therefore, cell density-dependent cellular localization of cholesterol may also influence PrP-res formation.

Here, we showed that a higher cell density, which increases the opportunity of cell-to-cell contact, facilitated PrP-res formation in prion-infected N2a cells. We also showed the possibility that cell density-dependent cholesterol content and/or localization may influence fluctuation in the PrP-res level. However, it remains to be clarified how cholesterol influences PrP-res formation that is controlled by cell density. Prion propagation in continuously dividing cultured cells differs from prion propagation in non-dividing neuronal cells in the central nervous system. However, the acceleration of PrP-res formation by direct cell-to-cell contact is somewhat analogous to the deposition of PrPSc at neuro-muscular junction (Andréoletti et al., 2000; Hagiwara et al., 2007; Marijanovic et al., 2009). Although treatment with U18666A did not appear to alter cellular cholesterol content (data not shown), U18666A inhibited intracellular cholesterol transport, resulting in a redistribution of cholesterol from the plasma membrane to intracellular compartments (Liscum & Underwood, 1995; Sparrow et al., 1999; Klingenstein et al., 2006). As shown in Fig. 3(a), N2a cells in an area of high cell density showed more intense cholesterol staining on the plasma membrane than N2a cells in an area with lower cell density. Therefore, cell density-dependent cellular localization of cholesterol may also influence PrP-res formation.

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