Therapeutic effect of autologous compact bone-derived mesenchymal stem cell transplantation on prion disease

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Abstract

Prion diseases are fatal neurodegenerative disorders of humans and animals and no effective treatments are currently available. Allogenic transplantation of immortalized human mesenchymal stem cells (MSCs) can prolong the survival of mice infected with prions. However, autologous transplantation is an appropriate model for evaluating the effects of MSCs on prion diseases. Therefore, we isolated and purified MSCs from the femur and tibia of mice as compact bone-derived MSCs (CB-MSCs). Flow cytometric analysis showed that CB-MSCs were negative for myeloid stem cell-derived cell markers CD11b and CD45, but positive for molecules such as Sca-1, CD105 and CD90.2, which are reported to be expressed on MSCs. The ability of CB-MSCs to migrate to brain extracts from prion-infected mice was confirmed by an in vitro migration assay. Intra-hippocampus transplantation of CB-MSCs at 120 days post-inoculation marginally but significantly prolonged the survival of mice infected with the Chandler prion strain. The transplantation of CB-MSCs did not influence the accumulation of disease-specific prion protein. However, the CB-MSC transplantation enhanced microglial activation, which appeared to be polarized to the M2-type activation state. These results suggest that autologous MSC transplantation is a possible treatment for prion diseases, while the modification of microglial activation may be a therapeutic target for neurodegenerative diseases.

INTRODUCTION

Prion diseases are neurodegenerative disorders, notable examples of which include bovine spongiform encephalopathy, scrapie in sheep and goats, chronic wasting disease in deer and Creutzfeldt–Jakob disease in humans. The diseases are characterized by vacuolation of neurons and neuropil, astrocytosis, microglial activation and the deposition of disease-specific prion protein (PrPSc) in the central nervous system. Prion diseases have a long incubation period; however, after clinical onset, the diseases are subacutely progressive and inevitably fatal. No therapeutics are available at present. The process of the conversion of the cellular form of prion protein (PrPC) into PrPSc is believed to be associated with the propagation of prions, the causative agent of the disease, and neurodegeneration [1].

Stem cells provide a new approach for the treatment of degenerative diseases because of their potential applications in regenerative medicine. Mesenchymal stem cells (MSCs) are pluripotent cells that can be isolated from adult tissues of mesodermal origin, such as bone marrow, adipose tissue, compact bones and umbilical cord blood [2]. MSCs can be differentiated into various cell types, including not only mesodermal lineages (osteocytes, chondrocytes, adipocytes and skeletal muscle cells), but also ectodermal lineages (neuronal cells, glial cells and Schwann cells) and endodermal lineages (hepatocytes and insulin-producing pancreatic cells) [3, 4]. MSCs have some advantages for use in cell therapy, such as minimal ethical problems and extensive sources. The safety of MSCs is also empirically recognized, because few adverse effects have been reported in clinical trials of MSCs for neurological diseases such as stroke, spinal cord injury and amyotrophic lateral sclerosis [5, 6].

MSCs have been reported to have beneficial effects as cell therapies in animal models of stroke [7, 8], spinal cord injury [9],...
brain tumour [10], and neurodegenerative diseases such as Alzheimer’s disease (AD) [11, 12], Parkinson’s disease [13–15] and amyotrophic lateral sclerosis [16, 17]. In clinical trials, MSC transplantations for neurological disorders also tended to achieve functional recovery or partial improvement in patients with stroke [18], spinal cord injury [19] and multiple sclerosis [20]. In neurodegenerative and neurological disorders, MSCs transplanted via the intracerebral or intravenous route migrate to brain lesions and ameliorate functional deficits or exhibit neuroprotective potential. This may be attributable to immunomodulatory and anti-inflammatory effects from the production of cytokines and chemokines, or through the modulation of microglial activation [11, 21]; neuroprotection by producing neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and hepatocyte growth factor [22, 23]; neuronal differentiation or the stimulation of differentiation of endogenous neural stem cells [24–26]; or neurovascularization from producing vascular endothelial growth factor (VEGF) [27] or decreased oxidative stress [28]. These findings demonstrate that MSCs are promising tools for cell therapy in neurodegenerative disorders. However, the detailed mechanisms that allow MSCs to relieve detrimental conditions and restore functional deficits remain to be elucidated.

Previously, we reported that intracerebral or intravenous transplantation of immortalized human bone marrow-derived MSCs (BM-MSCs) prolonged the survival of mice infected with prions. The transplanted MSCs migrated to brain lesions and produced neurotrophic factors such as BDNF, VEGF, and neurotrophin (NT) 3 and 4/5 [29]. These results suggest that human BM-MSCs have neuroprotective effects in prion disease. However, our experiments involved allogenic transplantation and thus instead of the human MSCs themselves, human PrPSc expressed in human BM-MSCs might have impeded prion propagation in mouse brains by interfering with the intracellular interaction between mouse PrPSc and PrPSc [30–32]. Alternatively, immortalized human BM-MSCs that can proliferate more effectively than primary MSCs are expected to exist for a longer period in the brain lesions, and this might have resulted in an overestimation of the effect.

Therefore, in this study, to evaluate the effect of autologous MSC transplantation on prion disease, we isolated MSCs from compact bone from the femur and tibia of mice, and analysed the therapeutic potential of compact bone-derived MSCs (CB-MSCs) for prion disease.

RESULT

Expression of cell surface markers on CB-MSCs

After removing CD11b- and CD 45-positive cells by magnetic activated cell sorting (MACS), CB-MSCs were serially passaged to expand cell numbers. At the fourth passage, the expression of surface markers was analysed by flow cytometry (Fig. 1). The cells were confirmed as negative for the myeloid-derived markers CD11b and CD45, but molecules that are reportedly expressed on the surface of MSCs, i.e. CD29, CD44, CD73, CD90.2, CD105, Sca-1 [33, 34] and CD106 [35, 36], were expressed on the surface of the CB-MSCs. However, the histograms for CD73, CD90.2, CD105 and CD106 showed that the CB-MSCs included cells that were both positive and negative for these molecules, indicating that CB-MSCs comprise heterologous cell populations.

Migration of CB-MSCs to brain extracts from prion-infected mice

MSCs are known to migrate to brain lesions in animal models of neurodegenerative disorders, such as Parkinson’s disease [13], ischemia [7], glioma [10] and brain tumour [37]. Previously, we reported that when immortalized human BM-MSCs are transplanted intracerebrally or intravenously, they migrate to brain lesions in prion disease [29]. Human BM-MSCs migrated to brain extracts from prion-infected mice according to an in vitro migration assay, so the capability of BM-MSCs to migrate to lesions in prion disease in vivo can be assessed using an in vitro migration assay [38]. Thus, we assessed the ability of CB-MSCs to migrate to brain extracts from Chandler strain-infected mice using the in vitro migration assay (Fig. 2). Fig. 2(a) shows that the CB-MSCs migrated to the bottom side of the insert wells. Quantitative analysis of the migrated CB-MSCs showed that 30 and 2.1 times as many CB-MSCs migrated to the 1 and 0.1 % brain extracts from prion-infected mice, respectively, compared with those from the corresponding uninfected mice (Fig. 2b).

Effect of CB-MSCs on survival of prion-infected mice

The results of the migration assay suggested that CB-MSCs are capable of migration into lesions of prion disease. Thus, we examined the therapeutic potential of CB-MSCs for prion disease. Under the experimental conditions used in the current study, early clinical signs, such as ataxia of hind limbs and changes in pelage and posture, appear at around 120 days post-inoculation (p.i.). A decrease in body weight also becomes apparent at around 110–120 days p.i. Thus, CB-MSCs were transplanted into the left hippocampus of Chandler strain-infected mice at 120 days p.i. in order to evaluate the therapeutic potential of CB-MCSs in the clinical phase. The intra-hippocampal transplantation of CB-MSCs significantly prolonged the survival of mice infected with the Chandler strain (163.8±6.2 days, n=9) compared with that of the PBS-injected group (155.0±2.4 days, n=7) (Fig. 3a, P<0.01).

Loss of body weight with disease progression is one of the prominent features in Chandler strain-infected mice [39, 40]. To evaluate the effects of CB-MSCs more objectively, we weighed the mice every week from 120 days p.i. At 141 days p.i., the decrease in body weight appeared to slow down in the CB-MSC transplanted group compared with the PBS-injected group (Fig. 3b, P=0.05), and the differences in body weight were more obvious at 148 and 155 days p.i. (Fig. 3b, P<0.01).
We also transplanted CB-MSCs to mice infected with the Obihiro strain, as another scrapie strain (Fig. S1, available with the online Supplementary Material). The effect of CB-MSCs on prolongation of the survival of mice infected with the Obihiro strain was not statistically significant (154.4 ±7.5 days, n=7) compared with that of the PBS-injected group (151.5±1.9 days, n=4). We reported that intraventricular administration of anti-PrP mAbs mitigated the disease progression of mice infected with the Chandler strain even when the administration started at 120 days p.i. However, no protective effect was observed in mice infected with the Obihiro strain under the same conditions [39]. This fact suggests that the Obihiro strain is more resistant to the treatment against prion diseases than the Chandler strain. Nonetheless, three out of seven Obihiro strain-infected mice with transplanted CB-MSCs apparently survived longer than those undergoing the sham operation. This suggests that the therapeutic potential of CB-MSCs is not limited to the Chandler strain.

**Effects of CB-MSCs on PrPSc accumulation and neuropathology**

To analyse whether CB-MSCs influence the accumulation of PrPSc, we transplanted CB-MSCs into the hippocampus of Chandler-infected mice at 120 days p.i. and the kinetics of the total PrP and proteinase K-resistant PrPSc levels were analysed by immunoblotting (Fig. 4). When we initiated the intravenous administration of anti-PrP mAb to the Chandler strain-infected mice at 120 days p.i., the decrease in body weight became significantly slower compared to that in the control mice at around 140 days p.i. [40]. A similar tendency was observed for the decrease in body weight in the Chandler strain-infected mice transplanted with CB-MSCs (Fig. 3b). Thus we set 145 days p.i. as an examination point in the following experiments. At 145 days p.i., the mean PrPSc level in the PBS-injected group increased nearly twofold compared with the level at 120 days p.i.; however, no difference was observed in the PrPSc levels in the CB-MSC-transplanted and PBS-injected groups. The PrPSc levels increased further at the terminal stage of the disease, but no difference was observed in the PrPSc levels between the CB-MSC-transplanted and PBS-injected groups (Fig. 4a, c). The mean total PrP levels of the CB-MSC-transplanted group increased 1.3- and 1.8-fold at 145 days p.i. and at the terminal stage, respectively, compared with the levels at 120 days p.i.; however, there were no differences in the total PrP levels observed for the CB-MSC-transplanted group and PBS-injected groups (Fig. 4b, d). These results indicate that CB-MSCs did not influence the accumulation of PrPSc in brains.

Consistent with the results of PrPSc accumulation obtained by immunoblotting, no obvious differences in the accumulation of PrPSc in the hippocampus of CB-MSC-transplanted and PBS-injected groups were observed by immunohistochemistry (IHC) for PrPSc at 145 days p.i. and at the terminal stage (Fig. 5a, PrPSc). No difference was observed in the astrogliosis in the hippocampus of CB-MSC-transplanted and PBS-injected groups according to IHC for glial fibrillary acidic protein (GFAP) (Fig. 5a, GFAP). By contrast, there were differences in microglial activation, which was assessed by the ionized calcium binding adaptor molecule 1 (Iba-1) (Fig. 5a, Iba-1). The quantitative analysis detected more Iba-1-positive cells in the CB-MSC-transplanted group than in the PBS-injected group in the hippocampus at 145 days p.i. (Fig. 5b, P<0.05). However, there were no differences in the number of microglia at the terminal stage of the disease (Fig. 5b). No difference was observed in vacuolation in the hippocampus of the CB-MSC-transplanted and PBS-injected groups (Fig. 5a, HE and Fig. 5c).
In the HE staining section, large cells with abundant pale cytoplasm were observed in the MSC-transplanted side of the hippocampi of Chandler strain-infected mice (Fig. S2a; 145 days p.i., 25 days after MSC-transplantation). Similar cells were observed in the ipsilateral side of the hippocampus of mock-infected mice transplanted with CB-MSCs. However, such cells were not observed in the Chandler-infected or mock-infected mice with sham operation. Thus, the large cells with pale cytoplasm are likely to be transplanted CM-MSCs. At the terminal stage of the disease, such cells were not observed, but instead aggregates of necrotic cells that were not observed in sham-operated mice were observed in the hippocampi of the Chandler strain-infected mice transplanted with CM-MSCs (Fig. S2b). These results suggest that the transplanted CB-MSCs survived for at least 25 days after transplantation.

**Effects of CB-MSCs on gene expression in the brains of prion-infected mice**

To analyse the activation state of microglia, quantitative RT-PCR (qRT-PCR) was carried out (Fig. 6). The expression of the *Aif1* gene, which encodes Iba-1, was up-regulated in prion-infected mice; however, greater up-regulation of *Aif1* gene was observed in prion-infected mice transplanted with CB-MSCs compared with those with sham operation. No difference was observed in *Aif1* gene expression between mock-infected mice transplanted with CB-MSCs and those with sham operation. The expression of *CD68*, which is used as a marker for activated microglia, was also up-regulated more significantly in prion-infected mice transplanted with CB-MSCs than those with sham operation. The expression of *IL-1β*, a representative pro-inflammatory cytokine, was up-regulated upon prion infection (compare mock PBS vs Chandler PBS) as reported. Transplantation of CB-MSCs itself up-regulated the expression of *IL-1β* in mock-infected mice: the expression level was 3.1 times higher in mock-infected mice with CB-MSC transplantation than those with sham operation. However, interestingly, compared to prion-infected mice with sham-operation, 5.3 times higher up-regulation was observed in prion-infected mice transplanted with CB-MSCs. The expression of another pro-inflammatory cytokine gene, *TNF-α*, exhibited a similar trend. The transplantation of CB-MSCs did not influence the gene expression of some marker genes for M2-type activation in macrophages, i.e. *Chil3*, *Retnla* and *Mrc1*, in mock-infected mice. However, the gene expression levels of *Chil3* and *Retnla* were up-regulated to a remarkable degree in prion-infected mice transplanted with CB-MSCs. A marginal but significant up-regulation of Mrc1 expression was also observed. These results suggest that the transplantation of CB-MSCs influenced the activation state of microglia in prion-infected mice. The gene expression level of *IL-10*, an anti-inflammatory cytokine that stimulates microglia to shift into the M2-type activation state, was up-regulated upon prion infection (compare mock PBS vs Chandler PBS) as reported. Transplantation of CB-MSCs in prion-infected mice transplanted with CB-MSCs. The expression level of *IL-10* also appeared to be up-regulated by CB-MSC transplantation in mock-infected mice, although the degree of up-regulation was smaller than that observed in prion-infected mice. No difference was observed in the gene expression levels of *NGF* and *BDNF*.

**DISCUSSION**

The primary aim of this study was to analyse the efficacy of autologous MSC transplantation for the treatment of prion diseases. Previously, we reported that the transplantation of immortalized human MSCs prolonged the survival of mice infected with prions [29]. That experiment involved the transplantation of heterologous MSCs, and thus human PrPSC from human MSCs was present. It is well known that heterologous PrPSC interferes with PrPSc formation of homologous PrPSC and PrPSc combinations [30, 31]. Thus, it
is possible that the extended survival might not have been caused by any direct or indirect neuroprotective effects of human MSCs, and it may instead have been attributable to the inhibition of PrPSc formation by human PrPc produced by human MSCs. In addition, the use of immortalized MSCs had an apparent protective effect due to their higher proliferation capacity [29]. However, in the present study, we showed that autologous and non-immortalized MSC transplantation prolonged the survival of mice infected with prions, even when they were transplanted in the early clinical phase of Chandler strain-infected mice (at 120 days p.i.). The kinetics of PrPSc accumulation indicated that there were no differences in the PrPSc levels between the MSC-transplanted and sham-operation groups (Fig. 4), suggesting that mouse CB-MSCs exerted their neuroprotective potential without inhibiting PrPSc formation.

The primary CB-MSCs used here comprised heterologous cell populations (Fig. 1), but were effective in mitigating the disease progression. Thus it is possible that certain sub-populations of CB-MSCs have a higher potential to exert neuroprotective effects. It was reported recently that cells that are positive for platelet-derived growth factor receptor α and Sca-1 are a more potent population of MSCs [41]. Cells that are positive for stage-specific embryonic antigen-3 and CD105, distinct stem cells in the mesenchymal cell population, show triploblastic differentiation for all three germ layers [42]. Thus, the identification of MSC sub-populations that have a higher neuroprotective potency will be of interest to enhance the protective effects against prion diseases.

In prion-infected mice, microglial activation is often spatially associated with the brain regions where PrPSc accumulates before clinical onset [43–45]. However, it is

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<td>CB-MSCs</td>
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<td>152, 160, 161, 162, 164, 167, 168, 176</td>
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<td>PBS</td>
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Fig. 3. Effect of CB-MSCs on the survival of mice infected with the Chandler prion strain. (a) Survival curves and survival periods for individual mice. CB-MSCs (1×10⁵ cells in 2 µl PBS) were transplanted into Chandler strain-infected mice at 120 days p.i. (n=9, closed square). As a sham-operation group, the same volume of PBS was injected into Chandler strain-infected mice at 120 days p.i. (n=7, open square). The x-axis indicates the survival time after prion inoculation, and the y-axis indicates the survival rate (%). The table on the right shows the survival times for individual mice in each group. **, P<0.01 (Kaplan–Meier survival estimate, generalized Wilcoxon test). (b) Changes in body weight. After the transplantation of CB-MSCs, mice were weighed weekly up to the terminal stage of the disease. The graph shows mean weight±SD. *, P<0.05; **, P<0.01, [one way analysis of variance (ANOVA) with post hoc Bonferroni test].
controversial whether activated microglia have neuroprotective or neurotoxic functions. The blockade of colony-stimulating factor receptor 1 signalling prolonged the survival of prion-infected mice, which was accompanied by reduced microglial activation [46]. However, knockout of the CD40 ligand reduced the survival of mice infected with prions with enhanced microglial activation [47]. These findings suggest a detrimental effect of activated microglia in prion diseases. By contrast, the depletion of microglia in cerebellar slice culture increased the accumulation of PrP\textsuperscript{Sc} and severe neuronal loss occurred as a consequence [48, 49]. Recently, we reported that prion-infected CD14-deficient mice exhibited prolonged survival compared with wild-type mice [50]. These findings suggest that microglia have neuroprotective roles. In the present study, prion-infected mice transplanted with CB-MSCs survived longer than mice in the sham-operated group and they exhibited increased microglial activation without any reduction in the PrP\textsuperscript{Sc} level (Figs 4 and 5). These results were repeated independently and the data were quantified at each time point based on a total of four mice. The mean levels±SD "were indicated."
In the present study, we detected greater up-regulation of IL-1β gene expression in CB-MSC-transplanted prion-infected mice (Fig. 6). Thus, it is conceivable that the microglia being activated further by CB-MSC-transplantation had little effect on the clearance of PrPSc. The intracerebral injection of lipopolysaccharide induced microglial activation, with marked production of IL-1β but little clearance of PrPSc [57], which supports our hypothesis.

MSCs produce various cytokines, chemokines and inflammation mediators, depending on the microenvironments, and pleiotropically modulate the activities of immune cells such as T-cells, NK cells, monocytes/macrophages and microglia [58, 59]. Immunomodulation is believed to be one of the mechanisms that mediate the neuroprotective effects of MSCs in neurodegenerative diseases and traumatic injuries [21, 60]. The enhancement of microglial activation in CB-MSC-transplanted prion-infected mice appeared to be an immunomodulatory effect of CB-MSCs. In addition to greater microglial activation, the expression of pro-inflammatory cytokine genes, i.e. IL-1β and TNF-α, was up-regulated significantly in CB-MSC-transplanted prion-infected mice compared with sham-operated prion-infected mice. Interestingly, the gene expression levels of IL-10, an anti-inflammatory cytokine that stimulates microglia to shift into the M2-type activation state, and the gene expression levels of Chil3 and Retnla, well-known M2-type microglia activation marker genes, were also up-regulated in CB-MSC-transplanted prion-infected mice (Fig. 6). These results suggest that CB-MSCs influenced the microglial activation state and that the microglia polarized more into the M2-type activation state, and the gene expression of cytokines genes, IL-1β and TNF-α, were up-regulated [11, 61, 62]. It is not known whether the differences in the pro-inflammatory cytokine gene expressions may be attributed to differences in the origin of the MSCs, i.e. isolated from...
compact bone in the current study and from bone marrow in the previous studies, or differences in the pathobiology of the prion diseases and AD. Interestingly, the transplantation of BM-MSCs into a rat model of traumatic brain injury reduced the number of microglia as well as down-regulating the expression levels of some pro-inflammatory cytokine genes and up-regulating the expression levels of some anti-inflammatory cytokine genes [63]. This also suggests a shift

Fig. 6. Effect of CB-MSCs on gene expression in the brains of prion-infected mice. CB-MSCs (1×10^5 cells in 2 µl PBS) were transplanted into Chandler strain-infected mice (n=3, black bars, Chandler MSCs) and mock-infected mice (n=3, white bars, Mock MSCs) at 120 days p.i. The same volume of PBS was injected into Chandler strain-infected mice (n=3, grey bars, Chandler PBS) and mock-infected mice (n=3, red bars, Mock PBS) as a sham-operation group. At 145 days p.i., 21 days after transplantation, mice were sacrificed and their hippocampi were collected for total RNA isolation. qRT-PCR was performed as described in the Methods section. Gene expression levels relative to the mock-infected/sham-operation group (Mock PBS group) are shown (mean±SD). Significant differences between the CB-MSC-transplanted group and the PBS-injected group are indicated by asterisks. *, P<0.05; **, P<0.01; one-way ANOVA with Tukey’s post hoc tests.
of microglia into the M2-type activation state in the presence of MSCs in response to acute traumatic injury. It is expected that the neuropathobiology of acute traumatic injury and slow progressive encephalopathies such as AD and prion diseases will differ; however, these findings suggest that MSCs could modulate the microglial activation state into the M2-type by responding to each disease condition in a different manner to exert neuroprotective functions. The M2-type of microglia can produce anti-inflammatory cytokines and neurotrophic factors [64], so it is possible that alternatively activated microglia may facilitate neuroprotection and regeneration.

Our previous study showed that the immortalized human BM-MSCs transplanted to prion-infected mice produced various neuroprotective factors, such as NGF, BDNF, NT3/4 and VEGF [29]. However, in the current study we used primary CB-MSCs without any genetic modification or labelling in order to exclude any clonal or gene modification effects. Thus, we could not assess the other neuroprotective mechanisms of MSCs, such as neuronal differentiation, stimulation of the differentiation of endogenous neural stem cells, neurovasculatization or decrease of oxidative stress, or assess the distribution of the transplanted CB-MSCs.

In this study, we showed that the autologous transplantation of CB-MSCs mitigated the disease progression of prion-infected mice. The CB-MSCs transplantation did not influence the accumulation of PrPSc, but it did enhance microglial activation, which appeared to be polarized into the M2-type activation state. It remains to be elucidated whether the M2-type polarized microglia have neuroprotective roles against prion propagation. However, the fact that CB-MSC transplantation partly prevents disease progression even after the clinical onset will encourage further studies on the application of regenerative medicine in the treatment of prion diseases.

METHODS

Animals and prion inoculation

All of the procedures for animal experiments were conducted according to protocols approved by the Institutional Committee for Animal Experiments at Hokkaido University. The mouse-adopted scrapie Chandler and Obihiro strains was used in this study. Four-week-old female ICR mice (CLEA, Japan) were inoculated intracerebrally (into the left hemisphere) with 20 µl of 2.5 % brain homogenate of the Chandler strain-infected, Obihiro strain-infected, or mock-infected mice.

Preparation of CB-MSCs from mouse compact bone by MACS

Six-week-old Jcl:ICR female mice were euthanized under anaesthesia with Sevoflurane (Maruishi Pharmaceutical Co. Ltd., Japan). The femur and tibia were obtained to isolate CB-MSCs [65]. Both ends of the femur and tibia were cut by scissors and the bone marrow was washed out with Hanks’ balanced salt solution (HBSS, Sigma, USA) by inserting a 27 G needle (Terumo, Japan) into the cavity. The femur and tibia were then cut into small pieces using bone scissors. After washing with HBSS, the bone fragments were digested with 1 mg collagenase II ml⁻¹ (Sigma) in HBSS with constant shaking at 220 r.p.m. for 2 h at 37 °C. The digest was filtered through a 100 µm cell strainer (BD Falcon, USA) and the bone fragments remaining on the cell strainer were washed three times with HBSS. The bone fragments were cultured with Dulbecco’s modified Eagle’s medium (DMEM; Sigma) containing 10 % FBS (Gibco, USA), 10 % horse serum (Gibco), 2 mM l-glutamine (Wako, Japan), 10 mM HEPES (Gibco) and 100 U penicillin–streptomycin ml⁻¹ (Gibco) (FBS-HS-DMEM) in 10 cm plates. The filtered cells were also cultured with FBS-HS-DMEM in 10 cm plates at 37 °C under 5 % CO₂ and 5 % O₂. The cells were freshly fed every day for the first 3 days and passaged every 3 to 4 days.

Compact bone-derived cells that adhered to plastic plate were harvested with 0.1 % collagenase I (Wako, Japan) in PBS (pH 7.2) when the cells reached about 70 % confluence. The cells were collected by centrifugation and incubated for 15 min on ice with 200 µl CD11b microbeads (Miltenyi Biotec, USA) diluted at 1 : 10 with HBSS containing 0.5 % FBS (0.5 % FBS-HBSS). The CD11b-positive cells were then removed by being passed through an MS column set on MACS separators (Miltenyi Biotec), and the pass-through fraction was collected. The collected cells were subsequently incubated for 15 min with 200 µl CD 45 microbeads (Miltenyi Biotec) and the pass-through fraction was collected. The collected cells were analysed using the FACSVerse flow cytometer (BD Biosciences).

Flow cytometric analysis

The CB-MSCs were harvested by collagenase treatment and suspended with 0.5 % FBS-HBSS. The CB-MSCs were added to 96-well plates (1 × 10⁶ cells per well). After centrifugation, the cells were incubated for 30 min on ice with 100 µl primary rat antibodies against mouse CD11b, CD45, CD29, CD44, CD73, CD90.2, CD105, CD106 and Sca-1 at a 1 : 200 dilution. Except for anti-CD73 antibody, all of the antibodies were purchased from Biolegend (USA). Anti-CD73 antibody was purchased from BD Bioscience (USA). Rat IgG2a kappa and IgG2b kappa, both from Biolegend, were used as isotype controls. The cells were washed three times with 0.5 % FBS-HBSS and incubated for 30 min on ice with anti-rat Alexa Fluor 488 (Molecular Probes, USA) at a dilution of 1 : 1000. After being washed three times with 0.5 % FBS-HBSS, the cells were stained with 5 µg propidium iodide ml⁻¹ (Molecular Probes) in HBSS for 5 min. The cells were analysed using the FACSVers flow cytometer (BD Biosciences).

Transplantation of CB-MSCs

To transplant CB-MSCs into the hippocampus, mice were anaesthetized by intramuscular injection with xylazine.
(10 mg kg\(^{-1}\)) and ketamine (50 mg kg\(^{-1}\)) and placed on a stereotaxic apparatus (Narishige, Japan). After making a linear scalp incision, burr holes were drilled to accommodate stereotaxic placement into the left hippocampus (2.0 mm caudal; 2.0 mm lateral to bregma; depth, 2 mm). CB-MSCs (1 × 10\(^5\) cells in 2 µL PBS) were transplanted over a period of 15 min using a Hamilton syringe with a 31-gauge needle set in a micromanipulator. In the sham operation, 2 µL of PBS was injected into the same position.

**Immunoblotting**

Brains were semi-sectioned sagittally and homogenized in 20 % (w/v) TMS buffer [10 mM Tris-HCl (pH 7.5), 5 mM MgCl\(_2\), 5 % glucose]. The 20 % brain homogenate (250 µl) was mixed with an equal volume of a detergent buffer [8 % Zwittergent 3–14, 1 % Sarcosyl, 100 mM NaCl and 50 mM Tris-HCl (pH 7.5)] and digested with collagenase I at 0.5 mg ml\(^{-1}\) for 15 min at 37 °C in a water bath. To detect proteinase K (PK)-resistant PrP\(^\text{Sc}\), the samples were digested with PK (Roche Diagnostics, Germany) at 20 µg ml\(^{-1}\) for 30 min at 37 °C in a water bath. After stopping PK digestion by adding Pefabloc (Roche Diagnostics) to 2 mM, DNase I was added to the samples at 40 µg ml\(^{-1}\) and incubated for 5 min at room temperature. A half volume of butanol–methanol solution (2:butanol: methanol=5:1) was added and PK-resistant PrP\(^\text{Sc}\) was recovered by centrifugation at 20 000 g for 10 min at 20 °C. The resulting pellet was dissolved in 1 × SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 5 % glycerol, 3 mM EDTA, 5 % SDS, 4 M urea, 0.04 % bromophenol blue, 4 % β-mercaptoethanol] by boiling for 5 min. To detect the total PrP, PK digestion and Pefabloc treatment were omitted. SDS polyacrylamide gel electrophoresis and immunoblotting for the detection of PrP were performed as described previously [66]. The specific bands were visualized using ECL Western blotting detection reagents (GE Healthcare, UK) and a LAS-3000 chemiluminescence image analyser (Fujifilm, Japan). Quantitative analysis was performed with Image Reader LAS-3000 version 1.11 (Fujifilm).

**Histopathology and IHC**

Mice were dissected under anaesthesia and their brains were fixed with 10 % formalin (Wako), soaked in 60 % formic acid (Wako) for 1 h and then kept in 70 % ethanol (Wako). After embedding in paraffin, the samples were sectioned coronally at a thickness of 3 µm. The sections were deparaffinized, rehydrated and subjected to haematoxylin-eosin (HE) staining or IHC. For HE staining, sections were stained in haematoxylin (Wako) for 3 min, washed with tap water for 5 min and then washed again with deionized water. After pretreatment with 95 % ethanol, the sections were stained with 0.5 % eosin (Wako) for 2 min and dehydrated using an ethanol series. The sections were permeabilized against xylene (Wako) and enclosed with a cover glass using Mount-Quick (Daido Sangyo, Japan).

To detect PrP\(^\text{Sc}\) by immunohistochemistry, sections were autoclaved for 20 min at 135 °C [67]. The sections were treated with 3 % H\(_2\)O\(_2\) in methanol for 15 min, blocked with 5 % FBS in PBS for 30 min and then incubated with mAb 31C6 [68] (0.5 µg ml\(^{-1}\)) for 1 h at 37 °C. After being washed with PBS containing 0.1 % Tween 20 (PBST), the sections were incubated with Envision + system HRP-labelled polymer conjugated to goat anti-mouse immunoglobulins (Dako, Denmark) for 1 h at 37 °C. The sections were washed with PBST and developed with DAB peroxidase (Vector Laboratories, USA), followed by counterstaining with Mayer’s haematoxylin.

To detect GFAP and Iba-1, markers for activated astrocytes and microglia, respectively, the sections were treated twice in a 500 W microwave with citric acid buffer (0.01M citric acid and 0.01M sodium citrate) for 5 min for antigen retrieval [67]. Next, the sections were treated with 0.3 % H\(_2\)O\(_2\) and blocked with FBS as described above. After blocking, the sections were incubated with anti-GFAP antibodies (Dako) at 1 : 2000 or anti-Iba-1 antibodies (Wako) at 1 : 200 dilution for 1 h at 37 °C. After washing with PBST, the sections were incubated with EnVision + system HRP-labelled polymer conjugated to goat anti-rabbit immunoglobulins (Dako) for 1 h at 37 °C. The sections were then developed and counterstained as described above.

**In vitro migration assay**

Brains from Chandler strain-infected mice at 120 days p.i., or age-matched mock-infected mice, were homogenized to 20 % (w/w) in DMEM. Two brains were pooled for each homogenate. The homogenates were centrifuged at 10 000 g for 10 min at 4 °C, and the resulting supernatants were passed through a 0.22 µm pore-size filter. Aliquots of the brain extracts were stored at –80 °C until use. CB-MSCs starved in serum-free DMEM for 24 h were harvested with 0.1 % collagenase I. Wells in the 24-well plate were supplied with serum-free DMEM containing brain extracts, and CB-MSC suspensions (5 × 10\(^4\) cells with 400 µl of DMEM) were then added to the insert well with a polycarbonate membrane (24-well Millicell hanging cell culture inserts; pore size, 8.0 µm; Millipore, USA). The 24-well plate containing the insert wells was incubated for 16 h at 37 °C. The CB-MSCs on the polycarbonate membrane were stained for 1 h on ice with 1 % crystal violet in methanol. After washing with deionized water, non-migratory cells that stayed on the upper side of the polycarbonate membrane were removed using a cotton swab. The migrated CB-MSCs that passed through the pores and clung to the underside of the membrane were observed using a BIOREVO BZ-9000 microscope (Keyence, Japan), and the cell numbers were counted using the NIH Image J Program.

**qRT-PCR**

After the mouse brains were collected, the hippocampi were isolated under the microscope and total RNA was extracted using TRIzol reagent (Life Technologies, USA). First-strand cDNA was synthesized from 1 µg of the total RNA using a First Strand cDNA synthesis kit (GE Healthcare). The qRT-PCR reaction mixtures contained the diluted cDNA...
Statistical analysis

The results were analysed using the comparative cycle threshold (2-ΔΔct) method to calculate fold changes.

Statistical analysis

The statistical analyses were performed with JMP Pro 12.2.0 statistical software (SAS Institute).

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

All procedures for animal experiments were conducted according to protocols approved by the Institutional Committee for Animal Experiments at Hokkaido University (protocol no. 13-0143, 03-0144).

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