Title: Bone marrow stroma cells are susceptible to prion infection

Yuka Takakura[1, 2], Naohiro Yamaguchi[1], Takehiro Nakagaki[1], Katsuya Satoh[3], Jun-ichi Kira[2] and Noriyuki Nishida[1*]

[1]Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences

[2]Department of Neurology, Kyushu University Graduate School of Medicine

[3]Department of Clinical Neurology, Nagasaki University Hospital Sciences

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* Corresponding author. Address: Sakamoto 1-12-4, Nagasaki, 852-8523 Japan.

noribaci@net.nagasaki-u.ac.jp Tel +81-95819 7059, Fax +81-95819 7060

Abstract

Abnormal protease-resistant prion protein (PrP-res) is the only surrogate biochemical marker for prion diseases, and a sensitive technique to detect PrP-res in blood or tissues is urgently needed. Primary cultured bone marrow stromal cells (MSCs) expressed PrP and were capable of supporting stable human prion infection. Using a mouse-adapted BSE strain, we demonstrated that PrP-res can be detected in expanded MSCs. We then analyzed the bone marrow cells collected at autopsy from two individuals with sporadic Creutzfeldt-Jakob disease (CJD), and, in both cases, cultured MSCs were positive for PrP-res. These data would suggest that ex vivo MSC expansion accompanied by PrP-res analysis could be a helpful tool in the definitive diagnosis of prion disease at an earlier stage in the disease process than is currently possible, and with considerably less distress to the patient. (134 words)

Key words: prion protein, CJD, BSE, bone marrow stromal cell, MSC

Introduction

Creutzfeldt-Jakob disease (CJD) in man is thought to be caused by an "infectious protein particle", termed prion[1]. Accumulation of the disease-associated form of prion protein (PrP^{Sc}) and infectivity are seen mainly in the central nervous system (CNS), but are not limited to the CNS. Infectivity in the blood of sporadic CJD patients and experimentally infected animals has been reported[2], and recently it has been shown that variant CJD (vCJD) can be transmitted by transfusion[3,4]. The problem, however, is that detection of PrP^{Sc} in blood is extremely difficult and no reliable test exists[5]. For this reason, we instead focused on bone marrow stroma cells (MSCs)[6,7], which possess multipotential stem cell-like characteristics, and investigated whether or not they were susceptible to TSE agents. Ex vivo cultured MSCs expressed PrP^{C} and were susceptible to a CJD agent. In addition, we were able to detect PrP^{Sc} in MSCs isolated from both infected animals and sporadic CJD patients. These results suggest that bone marrow biopsy followed by ex vivo expansion of MSCs could form the basis of a new diagnostic test for TSEs.

Materials and Methods

Isolation and culture of MSCs

Adult male Wistar rats, 8 weeks old, were killed and the femurs and tibias were dissected out. Isolation of the bone marrow was performed according to the method described by Azizi et al[7]. The ends of the bones were cut and the marrow was extruded with 5 ml of alpha-MEM (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml kanamycin, or 100 U/ml penicillin and 100μg /ml streptomycin. The bone marrow cells were incubated at 37 ºC with 95% humidity and 5% CO_2 for 48h, and the non-adherent cells removed by replacing the medium. Adherent cells were subcultured several times, and used for the transmission studies. The cells were subjected to a neuronal differentiation study to confirm that they were stromal cells (MSC), according to Dezawa's method[8,9]. Additionally, bone marrow stromal cells isolated from mice, hamsters and cows were cultured in the same way as for rat MSCs. Normal human MSCs were purchased from Cambrex Bio Science Walkersville, Inc. In the case of CJD patients, informed consent was obtained from the patient's family, and the investigation protocol was approved by the Ethics Committee of Nagasaki University Hospital (ID 06012755).

Animal TSE models

To establish a rat TSE model, 10% (w/w) homogenates of a brain taken from a Gerstmann-Sträussler-Scheinker syndrome (GSS) patient carrying the P102L mutation in PRNP[10] were prepared with sterile PBS. We first inoculated the homogenate into Wistar rats (3 weeks old) and NZW mice (4 weeks old). Although all the rats (n=6) remained free of any neurological signs and were healthy until 2 years post inoculation, the NZW mice developed typical mouse TSE after around 230 days. The mouse brain homogenate was then inoculated into Wistar rats, and those rats developed disease at about 330 days. Accumulation of PrP^{Sc} in the affected brains was confirmed by Western blotting (data not shown), and by histology, in which common characteristics of prion disease such as spongiform change, neuronal loss, and gliosis were observed, and accumulated PrP^{Sc} was stained diffusely by 6H4 antibody. The agent isolated was designated Nagasaki University-1 isolate (Nu-1).

Mouse BSE model: Primary infection of a BSE agent isolated from a cow in the UK was done using RIII mice (killed at 412 days) and the affected brains were kindly provided to us by Dr. Yokoyama (Tsukuba). We then passed this mouse-adapted BSE

into ddY mice twice. Mice intracerebrally inoculated with brain homogenate all developed disease at around 150 days, with infectivity in the brain reaching around $10^{8.9}$ LD_{50}/g. At the third passage, 100 µl of a 10% brain homogenate were inoculated into the peritoneal cavity, and samples of bone marrow, spleen and brain were collected every 4 weeks. Infected mice showed abnormal gait and other neurological signs at around 20 weeks post inoculation and died at 28 wpi. In some experiments, mouse-adapted GSS agent, Fukuoka-1 strain[11], was also inoculated into ddY mice.

Ex vivo infection (Prion infection to the MSC)

Normal rat MSCs (passage 5) growing in a T25 flask were exposed to 0.2 % of Nu-1 rat brain homogenate for 24 h, after which an equal volume of fresh medium was added and the incubation was continued for another 48 h. Then, after washing the cells several times with PBS, we split the cells at a ratio of 1:3. At every passage, confluent cells were lysed and subjected to Western blotting for PrP^{Sc} as described below.

PrP^{Sc} detection

Brain, spleen and lymph node were homogenized at 10% in phosphate buffered saline. Total proteins were extracted by mixing with 1% Triton X-100/DOC buffer (1% Triton

X-100, 1% Deoxycholic acid, 300 mM NaCl, 50 mM Tris-HCl pH 7.5) and the mixture was centrifuged at 500 x g at 4 ºC for 15 min to remove cell debris. The supernatants were digested with Proteinase K (20 μg/mg protein, 37 ºC, 30 min) and mixed with sodium dodecyl sulfate (SDS) loading buffer (50mM Tris-HCl pH 6.8, containing 5% glycerol, 1.6% SDS, and 100mM dithiothreitol) and boiled for 10 min. Confluent cell cultures were lysed for 30 min at 4 ºC in 0.5% Triton X-100/DOC buffer (0.5% Triton X-100, 0.5% Deoxycholic acid, 150mM NaCl, 50mM Tris-HCl pH7.5). The supernatant was collected after a short centrifugation at 10,000 x g, and the total protein concentration was measured using the BCA protein assay (Pierce). The protein concentration was adjusted to 1.0 mg/ml, and digested with proteinase K (PK) (40 μg/mg protein, 37 ºC, 30 min), and then PK-resistant and insoluble PrP was concentrated by centrifugation at 19,000 x g for 45 minutes at 4 ºC. The pellet was dissolved in 1 x SDS loading buffer and boiled for 10 min.

In the cell samples as well as the tissues samples, PrP^{Sc} was detected by Western blotting. SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 1.5mm, 12% acrylamide gels. The proteins were transferred onto an Immobilon-P membrane

(Millipore) in transfer buffer containing 20% methanol, and the membrane was blocked with 5% nonfat dry milk in TBST (10mM Tris-HCl pH 7.8, 100mM NaCl, 0.1% Tween 20) for 60 min at room temperature. Blocked membranes were reacted with anti-PrP antibodies diluted 1:1000 in 1% nonfat dry milk/TBST overnight at 4 ºC. The membranes were then washed 3 times for 5 min in TBST, reacted with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted 1:5000 in 1% nonfat dry milk/TBST for 60 min at room temperature and washed again 5 times for 5 min in TBST. Immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech). The anti-PrP polyclonal mouse antiserum (SS#28) used has been described previously. The M20 goat antibody to C-terminal PrP peptides was purchased from Santa-Cruz Biotech (Santa Cruz, CA)[12]. Horseradish peroxidase (HRP)-conjugated anti-goat immunoglobulin G antibodies were purchased from Santa-Cruz Biotech.

Immunohistochemistry

Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned into 5 μm slices. The tissue sections were stained with hematoxylin and eosin (HE), or subjected to immunological staining of PrP, using the hydrolytic autoclaving

and formic acid method prior to incubating with primary antibodies such as SAF32 (SPI bio, France) and 6H4 (Prionics AG, Switzerland) overnight, and then the sections were reacted with envision polymer horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G antibodies (vectastain ABS Kit, Dako Cytomation) for 60 min. Immunostaining was visualized by 3, 3'-diaminobenzidine chromogen via a horseradish peroxidase reaction.

Results and discussion

MSCs have unique characteristics. They grow well ex vivo without transformation and are multipotential progenitor cells which can be used for auto-cell transplant therapy[8]. We isolated and subcultured bone marrow cells, and were able to obtain relatively uniform cell types from human, rat and bovine samples, but not from those of mouse and hamster. (Fig.1a). The rat bone marrow cells grew for more than 2 years (100 passages), but the human cells stopped dividing at about 15 passages, and the bovine, mouse and hamster cells continued to grow for only a few passages. To confirm that the cells isolated from rat bone marrow were indeed MSCs, we tried to differentiate them into neurons, according to the method described by Dezawa et al[9] (data not shown).

To examine the expression of PrP^{C}, MSCs were analyzed by Western blotting. Anti-PrP monoclonal antibody (SAF32) raised against N-terminal octapeptide repeats of PrP could detect normal PrP in the MSCs from all species (Fig 1b), and its expression on the cell surface was also confirmed by immunostaining (Fig 1c).

Isolated MSCs from uninfected rat bone marrows were subjected to an ex vivo transmission study using a rat-adapted GSS strain, Nagasaki isolate (Nu-1). Primary cultured rat MSCs (P3) were incubated with 0.2 % brain homogenate and passaged for more than 2 years. In the early phase, little or no PrP^{Sc} could be detected in the cultured MSCs, but with repetition of passages, the amount increased (Fig. 2) and multinuclear cells were observed. The ex vivo infected MSCs initially produced only small amounts of PrP^{Sc}, which increased with passaging, and stable PrP^{Sc} production was confirmed after 50 passages, suggesting that the infection spread only gradually. A similar observation has been reported in mouse neuronal cells[12]. To confirm that the MSC/Nu-1 cells were indeed infected with the agent, we inoculated the cell lysates (P25 and P50) intracerebrally into Wistar rats (4 weeks old). After one year, the rats all showed signs such as reflection abnormality, and Western blotting of the brains

confirmed the presence of PrP^{Sc} (data not shown). These results provide evidence that the MSCs express PrP^{C} and are susceptible to TSE agents, and are capable of supporting stable infection.

To elucidate whether or not MSCs can be infected with the TSE agent in vivo, we isolated MSCs from terminally sick animals. The MSCs from Nu-1 Wistar rat at 300 dpi grew slowly compared with those from young rats, and we were able to detect PrP^{Sc} (data not shown). In addition, MSCs from terminally sick mice infected with mouse adapted BSE or Fukuoka-1 agents were also positive for PrP^{Sc} (Fig 3a). Next, we inoculated MSCs from four terminal BSE-infected mice into the brains of normal ddY mice (five each). After 20 weeks, all exhibited a yellowish pubic region and abnormal hind limb reflex, but after that, clinical signs worsened only slowly. After 25 weeks, we sacrificed some of the mice and were able to confirm accumulation of abnormal PrP in the spleens (Fig 3b, arrows). This result provides evidence that MSCs in vivo do indeed carry the agent.

We next asked how soon the PrP^{Sc} could be seen in MSCs from infected mice. After intraperitoneal inoculation of 100µl of the BSE mouse 10% brain homogenate, brains,

spleens and bone marrows were collected from 4 or 5 animals every 4 weeks. PrP^{Sc} was first detected in spleen at 4 wpi and in brains at 24 wpi. MSCs from bone marrow were isolated and expanded (passaged 3 times) in order to harvest enough cellular proteins, and showed weak PrP^{Sc} signals at 4 wpi, which disappeared at 8 wpi, and were again detected at 16 to 28 wpi. (Fig 3c). This indicates that infection of the MSCs in vivo could precede the accumulation of PrP^{Sc} in brain.

PrP is expressed mainly in the brain but also in many other organs, including the lymphoreticular system tissues (LRS)[13,14]. Although the role of PrP^{C} in blood cells remains to be established, PrP^{C} is known to be expressed by hematopoietic stem cells (HSCs)[15], immature and mature T cells, B cells, monocytes and dendritic cells (DCs)[16]. CD43+ Gr-1+ granulocyte precursors in bone marrow have also been shown to express PrP[17]. When we analyzed murine bone marrow by in situ hybridization, PrP^{C} mRNA stained in HSCs but not MSCs (data not shown). Therefore, although we cannot exclude the possibility that MSCs start expressing PrP when the cells are cultured ex vivo, our data would suggest that MSCs could express PrP and be a long-term reservoir for TSE agents in vivo, in which case circulating white blood cells

could also become infected by contact with the MSCs.

To determine whether human MSCs are also susceptible to TSE agents, we examined bone marrows obtained at autopsy from two CJD patients. The first case was a 66-year-old female who had been clinically diagnosed as having sporadic CJD. The patient had died 27 months after the onset. An autopsy was held 7 hours after her death, and we collected bone marrow from the sternum. Because coagulation of the blood had already advanced, we were able to collect only an extremely small amount of bone marrow cells and it took about 2 months to grow sufficient MSCs for the experiment. One mg of extracted protein was digested and concentrated at 10,000 x g for 1h, then subjected to Western blotting for PrP^{Sc} using 3F4 antibody. A relatively weak but clear signal was seen in this patient's MSCs (Fig.4a). The second case was a 70 year-old male who also had been diagnosed with sporadic CJD. Abnormally elevated signals in the brain had been observed during screening with diffusion NMR, at which time the patient was free of symptoms. A few months later, he presented with mild dementia and neurological abnormalities. As the clinical manifestations progressed, CSF analysis showed that 14-3-3 was elevated and CJD was diagnosed. The patient died about a year

after the onset. This time, we were able to collect bone marrow immediately after death. A good amount of adherent cells was observed in the primary culture dishes, and after expanding them we were able to recover the cellular proteins and perform Western blotting analysis. PrP^{Sc} was detected even in the P1 cell lysate (Fig 4b). These findings suggest that the ex vivo expansion of MSCs could be an alternative diagnostic method for CJD.

Hadlow et al. were the first to report that infectivity could be reached at 10^5 LD_{50} in the bone marrow (whole femur) of Swiss mice infected with a mouse-adapted scrapie strain, Chandler isolate[18, 19]. Others, however, subsequently failed to detect PrP^{Sc} in bone marrow by Western Blotting[20]. Brown et al. inoculated the bone marrow from CJD patients into two normal primates, neither of which went on to develop TSE[21]. These results might mean that there was little infectivity in the samples used, or accumulation of PrP^{Sc} and infectivity in bone marrow might depend on either the strain of agent or the host species. How real the risk of infectivity is in the bone marrow of CJD patients remains to be assessed using sensitive models. At this point, it is too early to state that CJD could be transmitted during bone marrow transplantation, as no accidental

transmission has been reported to date.

Because the clinical features of CJD vary, definitive diagnosis relies on typical brain pathology and the post mortem detection of the pathological form of prion protein in the brain tissue. Protein analysis of the cerebrospinal fluid (CSF) and diffusion NMR are useful tools for differential diagnosis of neurodegenerative disorders and contribute to the clinical diagnosis of typical CJD[22]. Recently, a method for the amplification of abnormal PrP in vitro (PMCA) from biological samples has been developed and has great potential for application to clinical practice[23,24], but it still remains to be approved for use on human blood samples. The histological examination of brain biopsy specimens is currently the only way to confirm the clinical diagnosis of TSE before death. Bone marrow biopsy could thus lead to an early definitive diagnosis, enhancing the possibility of successful treatment.

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Figure legends

Figure 1. Bone marrow stromal cells express PrP^{C}. a. Phase-contrast pictures of human, mouse, and rat MSCs. b. Western blotting shows PrP^{C} expression in these cells. All MSCs isolated from different animals were positive for PrP^{C}. c. Immunostaining for PrP on rat MSCs. Typical punctuated staining was seen on the cell surface.

Figure 2. Ex vivo transmission of Nu-1 agent on rat MSCs. PrP signals for the Proteinase-K resistant form of PrP (PrP^{Sc}) were seen in the culture. N: uninfected normal control. P: Nu-1 rat brain homogenate sample. Numbers indicate times of passages from infection.

Figure 3. PrP^{Sc} was detected in MSC culture from terminally sick animals and was infectious. a. MSCs were isolated from mouse-BSE infected and Fukuoka-1 infected ddY mice. Both cultures were positive for PrP^{Sc}. b. Immunostaining for PrP^{Sc} in spleen obtained from a mouse inoculated with BSE-MSCs, indicating the cells were truly carrying infectivity. c. Time-course analysis of PrP^{Sc} in BSE-inoculated mice. MSCs were isolated from intraperitoneally infected mice

and PrP^{Sc} was detected. Note that PrP^{Sc} was seen in MSCs as early as 16 wpi.

Figure 4. a. *Ex vivo* expanded MSCs from CJD patients were positive for PrP^{Sc}. a. Bone marrow cells were isolated at autopsy of a sporadic CJD patient. After expansion of MSCs for a month, Western blotting for human PrP could detect PrP^{Sc} in the cell lysate. b. MSCs from another sporadic CJD patient. Bone marrow was obtained immediately after death. PrP^{Sc} was seen in the culture even at P1. PK: Proteinase-K digested sample.

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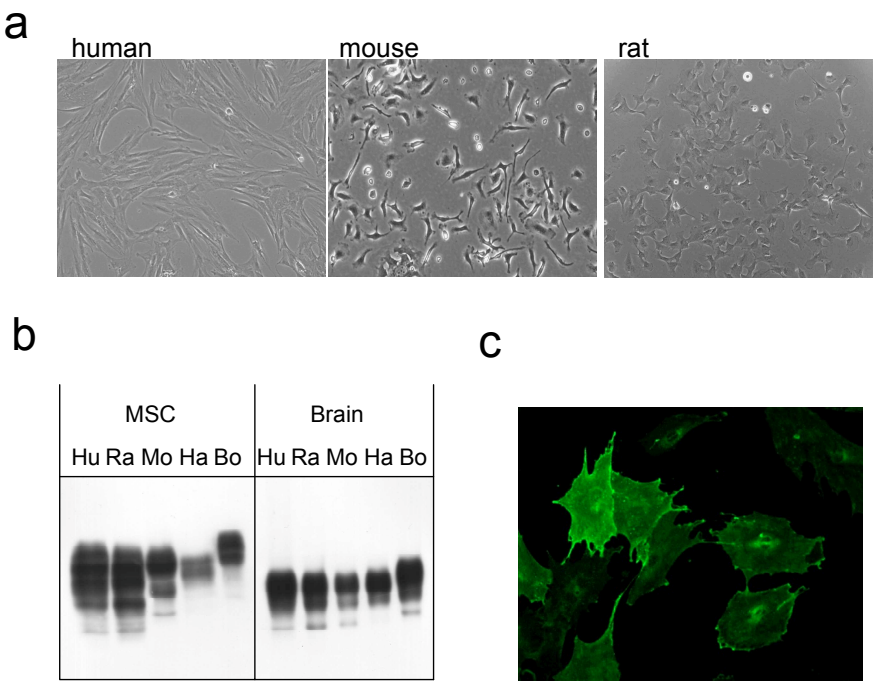
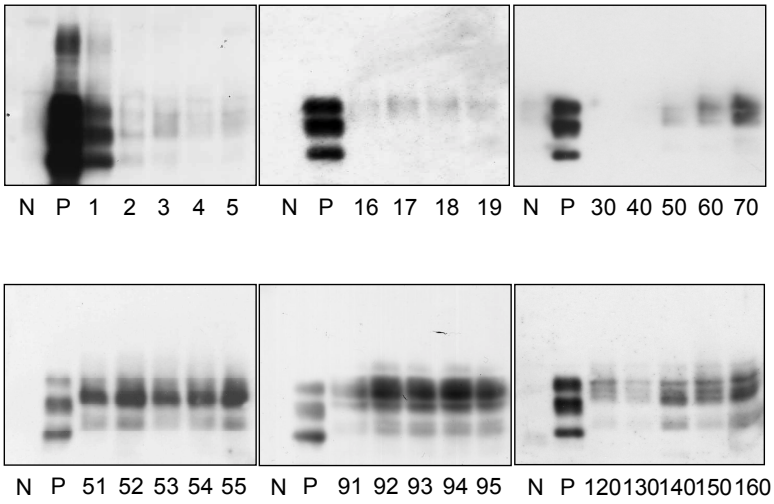


Figure 1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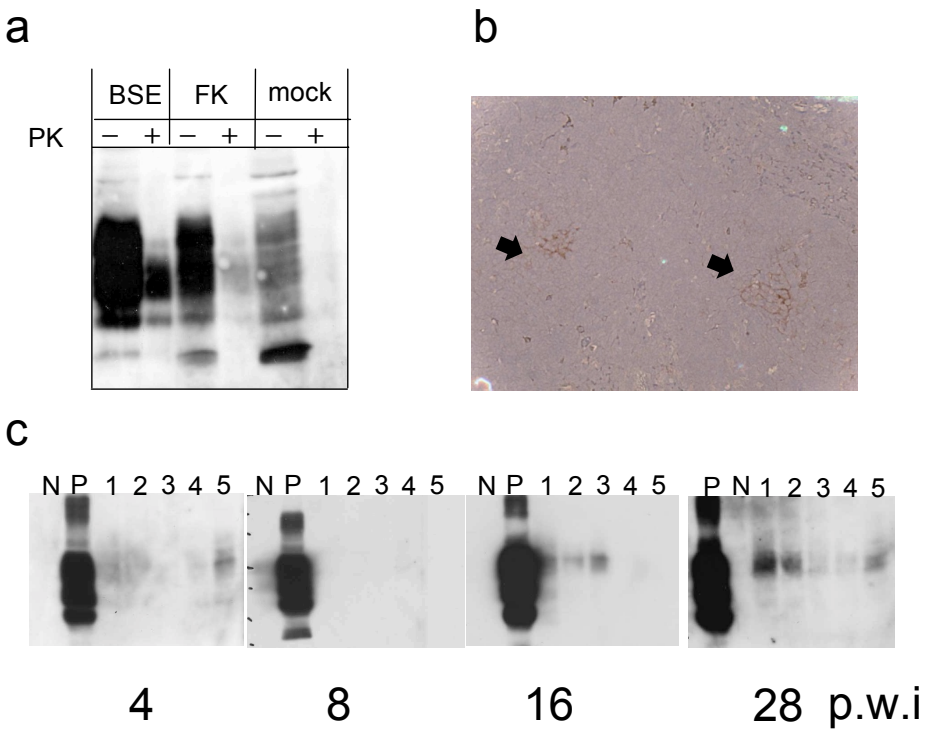
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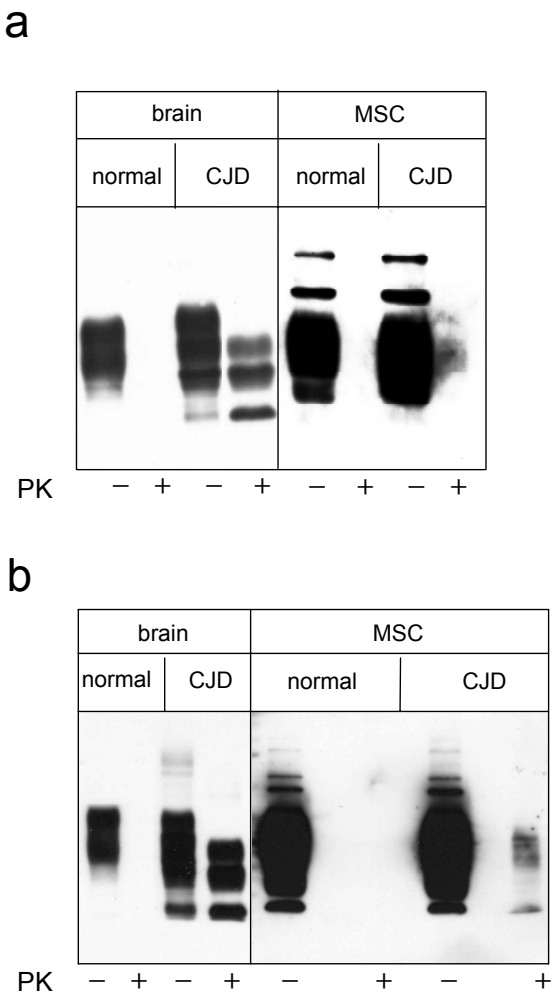
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Figure 4