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Accumulation of prion protein in muscle fibers of experimental chloroquine myopathy: *in vivo* model for deposition of prion protein in non-neuronal tissues

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Prion protein (PrP) is known to accumulate in some non-neuronal tissues under conditions unrelated to prion diseases. The biochemical and biological nature of such accumulated PrP molecules, however, has not been fully evaluated. In this study, we established experimental myopathy in hamsters by long-term administration of chloroquine, and we examined the nature of the PrP molecules that accumulated. PrP accumulation was immunohistochemically demonstrated in autophagic vacuoles in degenerated muscle fibers, and this was accompanied by the accumulation of other molecules related to the neuropathogenesis of prion diseases such as clathrin, cathepsin B, heparan sulfate, and apolipoprotein J. Accumulated PrP molecules were partially insoluble in detergent solution and were slightly less sensitive to proteinase K digestion than normal cellular PrP. Muscle homogenates containing these PrP molecules did not cause disease in inoculated hamsters. The findings indicate that the PrP molecules that accumulated in muscle fibers have distinct biochemical and biological properties. Therefore, experimental chloroquine myopathy is a novel and useful model to investigate the mechanism of deposition of PrP in non-neuronal tissues and might provide new insights in the pathogenesis of prion diseases.
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Prion diseases such as Creutzfeldt–Jakob disease in humans, and scrapie and bovine spongiform encephalopathy in animals are neurodegenerative disorders characterized by the accumulation in the brain of a protease-resistant, detergent-insoluble abnormal isoform of prion protein (PrP). This abnormal isoform of PrP (PrP^{Sc}) is pathogenic itself and replicates by altering the conformation of a protease-sensitive, detergent-soluble normal cellular isoform of prion protein (PrP^{C}).[1] In addition to the central nervous system, PrP^{Sc} deposition is observed in non-neuronal tissue such as tonsils and skeletal muscles in human prion diseases[2,3] and experimental animals.[4]

PrP is also known to accumulate in non-neuronal tissues under certain pathological conditions unrelated to prion diseases. Frederiske *et al*[5] recently revealed increased PrP immunoreactivity in the regions of fiber-cell degeneration in cataractous lenses in humans. Askanas *et al*[6] reported the accumulation of PrP in vacuolated muscle fibers, in angulated and round atrophic fibers with sarcolemmal enhancement, and in the perivascular inflammatory cells of sporadic inclusion-body myositis in humans.[6,7] It was also reported that the accumulated PrP molecules were sensitive to protease treatment.[7] However, the biochemical and biological characteristics of these PrP molecules have not been fully evaluated.

Chloroquine, a widely used antimalarial agent, is known to be concentrated in lysosomes and to cause elevation of intralysosomal pH.[8] Long-term

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administration of chloroquine sometimes causes myopathy, termed chloroquine myopathy (CM), which is characterized by degenerated muscle fibers with numerous autophagic, rimmed vacuoles.[9] Tsuzuki *et al*[10] established experimental CM in the rat to investigate the mechanism of accumulation of the proteins related to Alzheimer's disease in rimmed vacuoles, because of its histopathological similarity to human myopathies where amyloid β deposition is observed in rimmed vacuoles.

To explore the biochemical and biological properties of PrP molecules that accumulate under pathological conditions unrelated to prion diseases, we established experimental CM in hamsters and characterized the PrP molecules (PrP^{CQ}) that accumulated in affected muscle fibers.

Materials and methods

Animals and Reagents

Female Syrian hamsters, 3–8-week old, were purchased from SLC (Hamamatsu, Japan). Chloroquine diphosphate and Nonidet P-40 (NP-40) were purchased from Sigma Chemical (MO, USA). Proteinase K (PK) and complete mini protease inhibitor cocktail were obtained from Roche Molecular Biochemicals (Germany). Monoclonal antibody 3F4 recognizing hamster PrP109-112 was from Senetek (St Louis, MO, USA). Anti-prion protein polyclonal antibody PrP2B was raised by immunization of rabbits with a hamster PrP89-103 fragment. Polyclonal antibodies for apolipoprotein J (clusterin) and for cathepsin B, and monoclonal antibodies CHC5.9 for clathrin and HepSS-1 for heparan sulfate were purchased from Chemicon (Temecula, CA, USA), Calbiochem (Cambridge, MA, USA), PROGEN Biotechnik GmbH (Germany), and Seikagaku Corporation (Japan), respectively.

Experimental CM in Hamsters

Hamsters received 50 mg/kg chloroquine diphosphate (10 mg/ml in sterile saline, pH 7.6) as daily intraperitoneal injections for 60 days. Then the hamsters were killed by decapitation under deep anesthesia. Bilateral soleus, tibialis anterior, and quadriceps muscles were removed and immediately snap-frozen in isopentane cooled with liquid nitrogen. Frozen muscles were kept at $-80^{\circ}C$ until analysis.

Immunohistochemical Studies

After the blockage of endogenous peroxidase with 0.3% hydrogen peroxide in methanol, serial 10-μm thick sections of frozen muscle were incubated overnight at $4^{\circ}C$ with the primary antibodies diluted with 10 mM phosphate-buffered saline (PBS) containing 1% normal hamster serum. The sections were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h followed by reactions with 3,3′-diaminobenzidine/H_2O_2 and counterstained with hematoxylin. The serial sections were stained with hematoxylin and eosin (HE), or stained for acid phosphatase or by a modified Gomori-trichrome method.

Brain specimens obtained in some experiments were immersion-fixed in 10% buffered formalin for 24 h at $4^{\circ}C$ and embedded in paraffin for immunohistochemical examination. For detection of abnormal PrP deposition, deparaffinized 8-μm thick sections were treated with hydrolytic autoclaving prior to incubation with 3F4 monoclonal antibody.[11]

Protease Sensitivity Assay of PrP^{CQ}

After confirmation of the histological findings, the remaining frozen muscle was homogenized using Tissue-Tearor (Biospec Products, Oklahoma) in 10 volumes of lysis buffer A (0.5% NP-40, 0.5% sodium deoxycholate in PBS pH 7.4). Homogenates were centrifuged at $3300 \times g$ for 15 min to remove the nuclear fraction and debris. The supernatant was then treated with the indicated amount of PK at $37^{\circ}C$ for 20 min. After stopping the digestion with 4 mM 4-[2-aminoethyl]-benzenesulfonyl fluoride (Pefabloc, Roche, Germany), an aliquot corresponding to 4 mg of muscle tissue was analyzed by Western blotting using polyclonal antibody PrP2B. Labeled PrP was visualized by using CDP-star detection reagent (Amersham, UK).

Detergent Solubility Assay of PrP^{CQ}

The detergent solubility of PrP^{CQ} was determined as described by Lehmann and Harris[12] with minor modification. Briefly, soleus muscle samples from either control or CM hamsters were homogenized in lysis buffer B (15 mM NaCl, 50 mM Tris-HCl pH 7.5, complete-mini protease inhibitor cocktail) containing the designated concentration of NP-40. Homogenate was centrifuged for 5 min at 1600 *g* to remove debris and the nuclear fraction. The supernatant was ultracentrifuged at 265 000 *g* for 40 min at $25^{\circ}C$. Proteins in the supernatant and in the pellet were separately recovered and analyzed by Western blotting using monoclonal antibody 3F4.

Intracerebral Inoculation of PrP^{CQ}

Inoculum was prepared by homogenizing muscular tissue from CM hamsters or the control in 10 volumes of sterile saline, and 20 μl of the inoculum was injected into the brain of 19 3-week-old female hamsters under deep anesthesia. The hamsters were observed for over 2 years and killed to examine PrP molecules in the brain immunohistochemically.

Densitometry and Statistical Analysis

Blots of the gels were scanned with a CanoScan D2400UF (Canon, Japan). Densities of bands were quantified using NIH Image software. Statistical significance of densitometric data was analyzed by repeated measure ANOVA, and statistical comparison at each dose point between groups was made by Student's *t*-test or Welch's *t*-test.

Ethics

Animal handling and killing were in accordance with the nationally prescribed guidelines, with ethical approval for the study granted by the Animal Experiment Committee of Kyushu University.

Results

Histochemical Findings of CM

All the muscle specimens taken from chloroquine-treated hamsters showed various degrees of myopathic changes accompanied with rimmed vacuoles (Figure 1a), and this was consistent with histopathological findings of experimental CM in the rat.[9,10] In addition to the rimmed vacuoles, many muscle fibers contained coarse granular structures that were strongly stained by hematoxylin (Figure 1c) and modified Gomori trichrome stain (Figure 1g), and they showed enhanced acid phosphatase activity (Figure 1h). These abnormal structures were observed in about 10% of muscle fibers in the soleus muscle which was the most affected in all the muscles of chloroquine-treated hamsters. In contrast, muscles of control hamsters did not contain any rimmed vacuoles or coarse granular structures.

All of the rimmed vacuoles and the coarsely granular structures in degenerated muscle fibers of CM were positively stained by an anti-PrP monoclonal antibody, 3F4, recognizing hamster PrP109-112 (Figure 1b, d). In control hamster muscles, the 3F4 monoclonal antibody reacted with sarcolemmal membranes (data not shown).

To investigate the possible involvement of some molecules that relate to the metabolism of PrP^{C} or the deposition of PrP^{Sc}, serial sections were immunostained for apolipoprotein J (Figure 1e), clathrin (Figure 1f), cathepsin B (Figure 1i), and heparan sulfate (Figure 1j). Immunoreactivities for these molecules were enhanced in the rimmed vacuoles and coarse granular structures in the affected muscle fibers of CM.

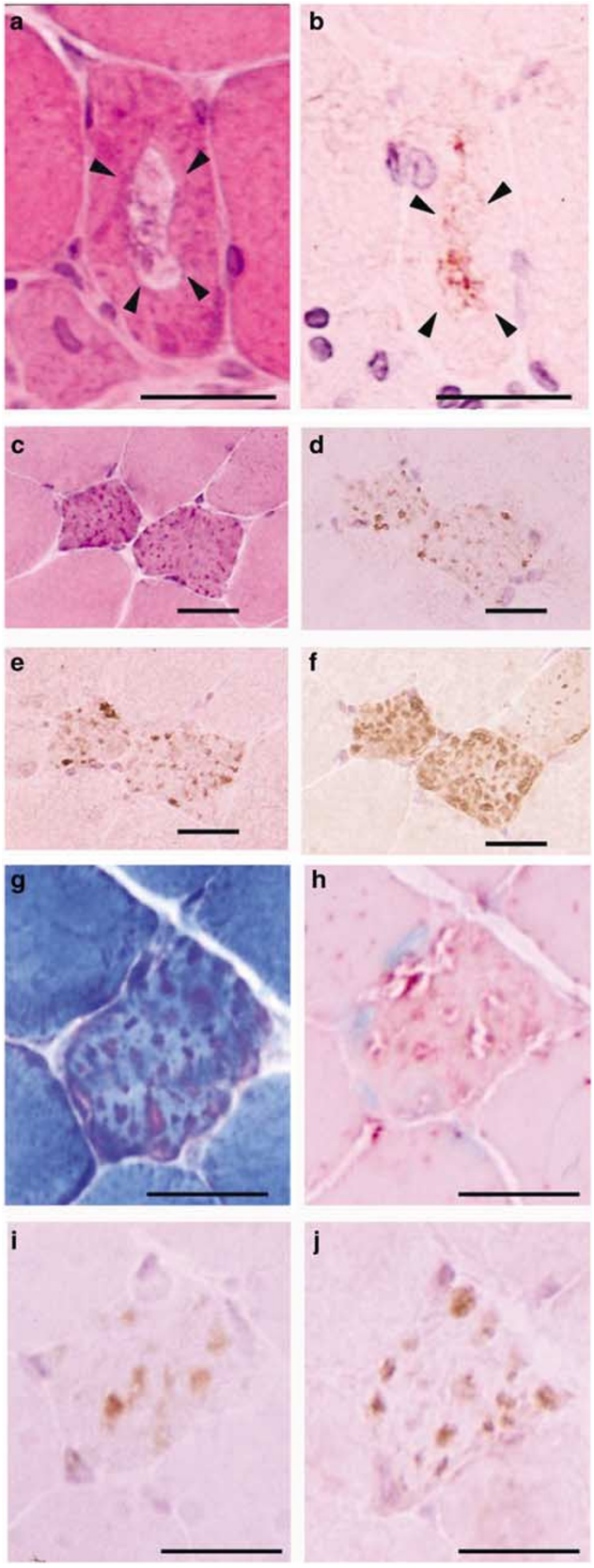

Figure 1 Immunohistochemical findings in CM muscles. Transverse sections of chloroquine-treated (50 mg/kg/day for 60 days) hamster soleus muscles are shown. (**a**) HE stain shows rimmed vacuole formation (arrow heads). (**b**) Immunoreactivity for PrP is detected in a rimmed vacuole (arrow heads) shown in (**a**). (**c–f**), (**g–j**) Serial sections stained with HE (**c**), modified Gomori trichrome (**g**), acid phosphatase (**h**), and immunostained for PrP (**d**), apolipoprotein J (**e**), clathrin (**f**), cathepsin B (**i**), and heparan sulfate (**j**). Scale bars = 20 μm.

PrP^{CQ} is Slightly Less Sensitive to PK Digestion

Western blot analysis using a polyclonal antibody PrP2B, raised against hamster PrP89-103, revealed prominent bands of 27 and 30 kDa in either CM muscular homogenates or the control, before digestion with PK. There was no significant difference in the intensity of PrP signals between CM muscles and controls (first lanes, Figure 2a, b). The specificity of these bands was confirmed by absorbing the PrP2B antibody with synthetic peptide PrP89-103 (Figure 2c). Although both of the PrP molecules were completely digested with 50 μg/ml of PK, which was the stringent condition to distinguish PrP^{Sc} from PrP^{C} (data not shown), digestion with a smaller amount of PK revealed different PK sensitivity between the PrP molecules from CM muscles (PrP^{CQ}) and PrP^{C} from the control muscles. PrP^{C} derived from control hamster muscle was apparently digested with 0.375 μg/ml of PK, whereas a considerable amount of PrP^{CQ} of 27 kDa still remained after treatment with 1.0 μg/ml of PK (Figure 2a and b). Statistical analysis of the relative density of the bands revealed a significant difference between PrP^{C} and PrP^{CQ} after treatment with 0.5, 0.75, or 1.0 μg/ml of PK (Figure 2d).

PrP^{CQ} is Partially Insoluble in Detergent

Western blot analysis using a monoclonal antibody 3F4 revealed prominent signals at 35 kDa and additional signals at about 30 kDa in the supernatants from either CM muscle homogenate or the control (first lanes, Figure 3a, b). While PrP^{C} from control muscle was completely solubilized in the lysis buffer containing 0.5% NP-40 (Figure 3a), a considerable amount of PrP^{CQ} remained in the

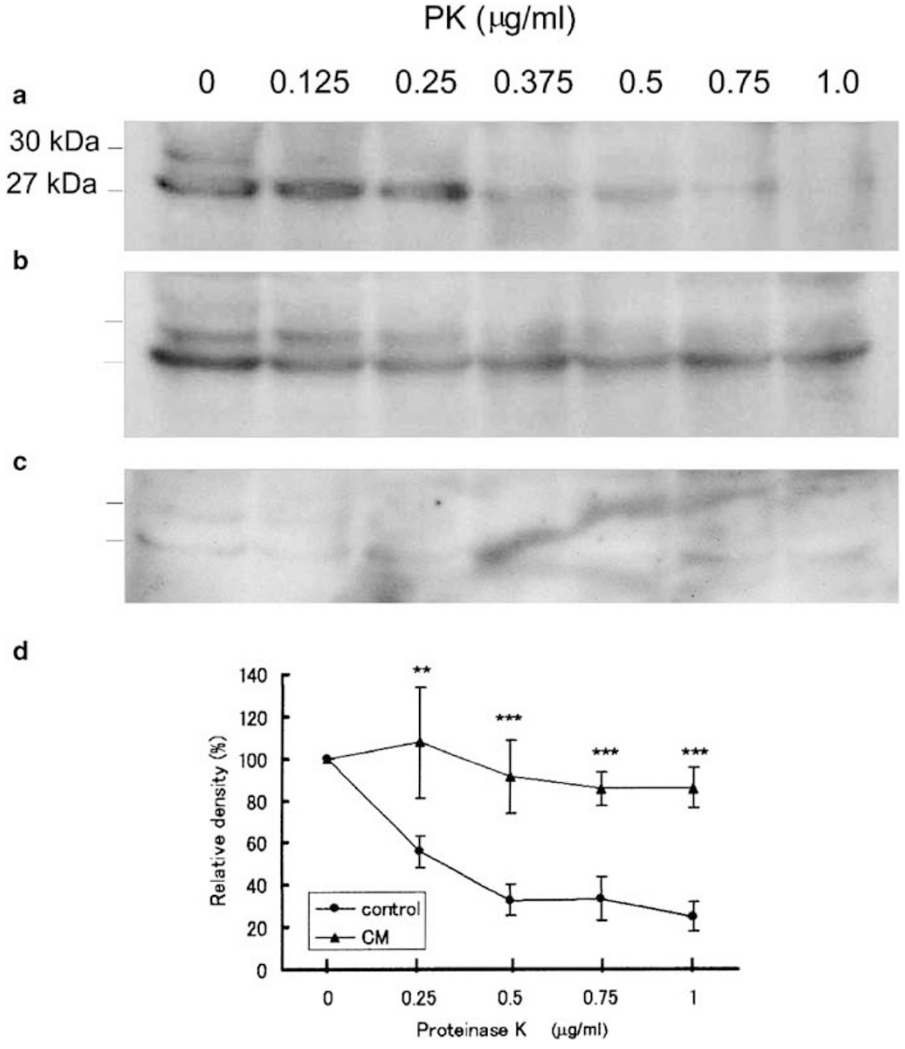

Figure 2 PK sensitivity of PrP molecules in CM muscles. (**a–c**) PrP molecules in the homogenate of control hamsters (**a**) or CM hamsters (**b**) were detected with PrP2B antibody after digestion with a designated amounts of PK at 37°C for 20 min. Prominent bands of 27 kDa and 30 kDa were diminished after the antibody had been absorbed by a synthetic polypeptide used for immunization (**c**). (**d**) Densitometric analysis of 27 and 30 kDa bands. Data from three independent experiments are indicated. $^{**}P<0.05$, $^{***}P<0.01$.

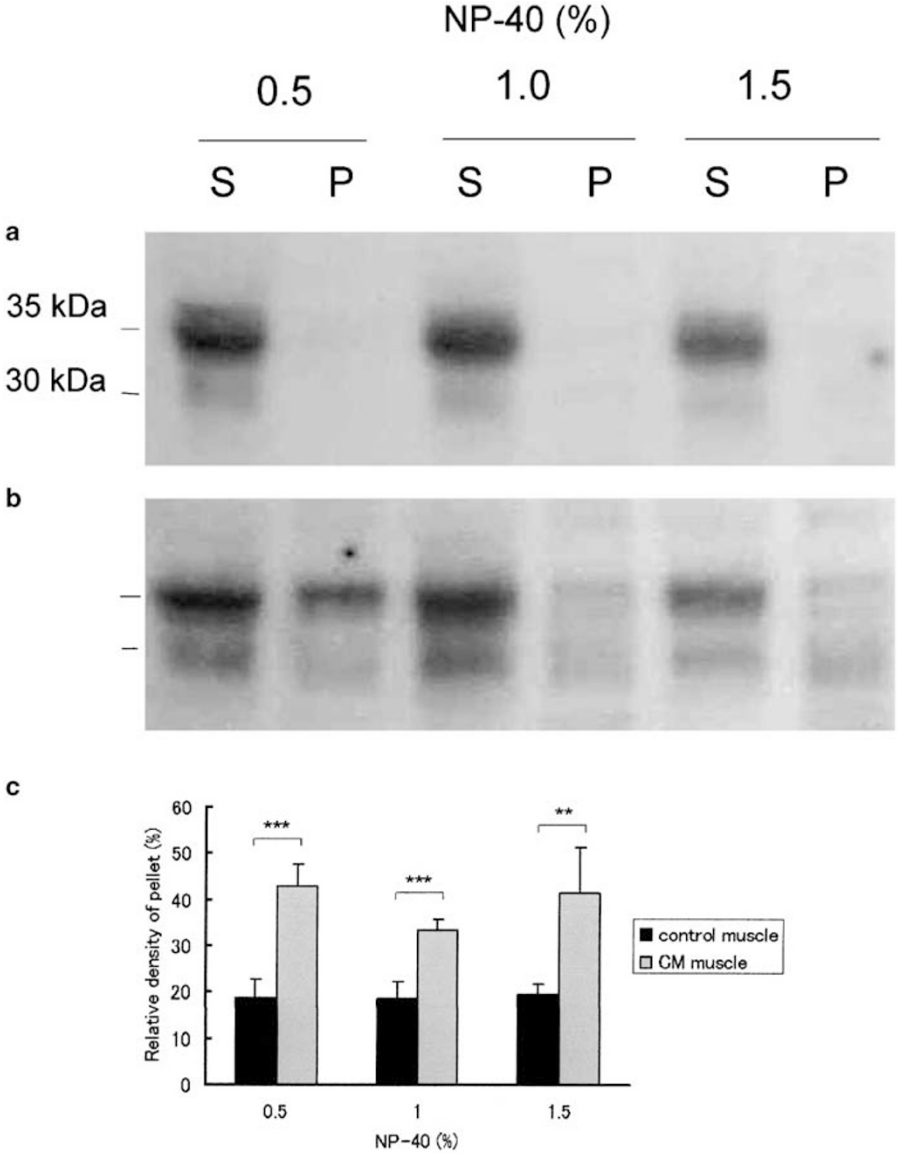

Figure 3 Solubility in NP-40 of PrP molecules in CM muscles. (**a**, **b**) Muscles from control hamsters (**a**) or CM hamsters (**b**) were homogenized in the lysis buffer containing designated amount of NP-40 and subsequently ultracentrifuged at 265 000 *g* to separate detergent-soluble PrP molecules in the supernatant (S) and detergent-insoluble molecules in the pellet (P). PrP molecules were labeled with 3F4 monoclonal antibody. (**c**) Relative PrP amount in the pellet fraction. Percentage of PrP signals (30 and 35 kDa) of the pellet fraction in the sum of those of the pellet and the supernatant is shown. Data from three independent experiments. $^{**}P<0.05$, $^{***}P<0.01$.

insoluble fraction in the presence of 0.5, 1.0, or 1.5% NP-40 (Figure 3b, c).

PrP^{CQ} is not Pathogenic

To investigate whether PrP^{CQ} is able to cause pathological changes characteristic of prion diseases *in vivo*, 10% muscular homogenates containing PrP^{CQ} were injected into the brain of Syrian hamsters. The hamsters were observed over 2 years after the inoculation had been given, and none of them developed any signs of prion diseases nor muscle disorders (data not shown). Histological examination of the brain revealed no significant pathological findings or abnormal PrP deposition (Figure 4).

Discussion

In this study, we have demonstrated that slightly less PK-sensitive and partially detergent-insoluble PrP^{CQ} accumulated in affected muscle fibers of experimental CM in hamsters. While PrP molecules from control muscle were sensitive to PK digestion at 0.375 μg/ml and fully soluble in buffer containing 0.5% NP-40, PrP^{CQ} molecules were less sensitive to PK digestion up to 1.0 μg/ml, and a considerable portion of them was insoluble in buffer containing

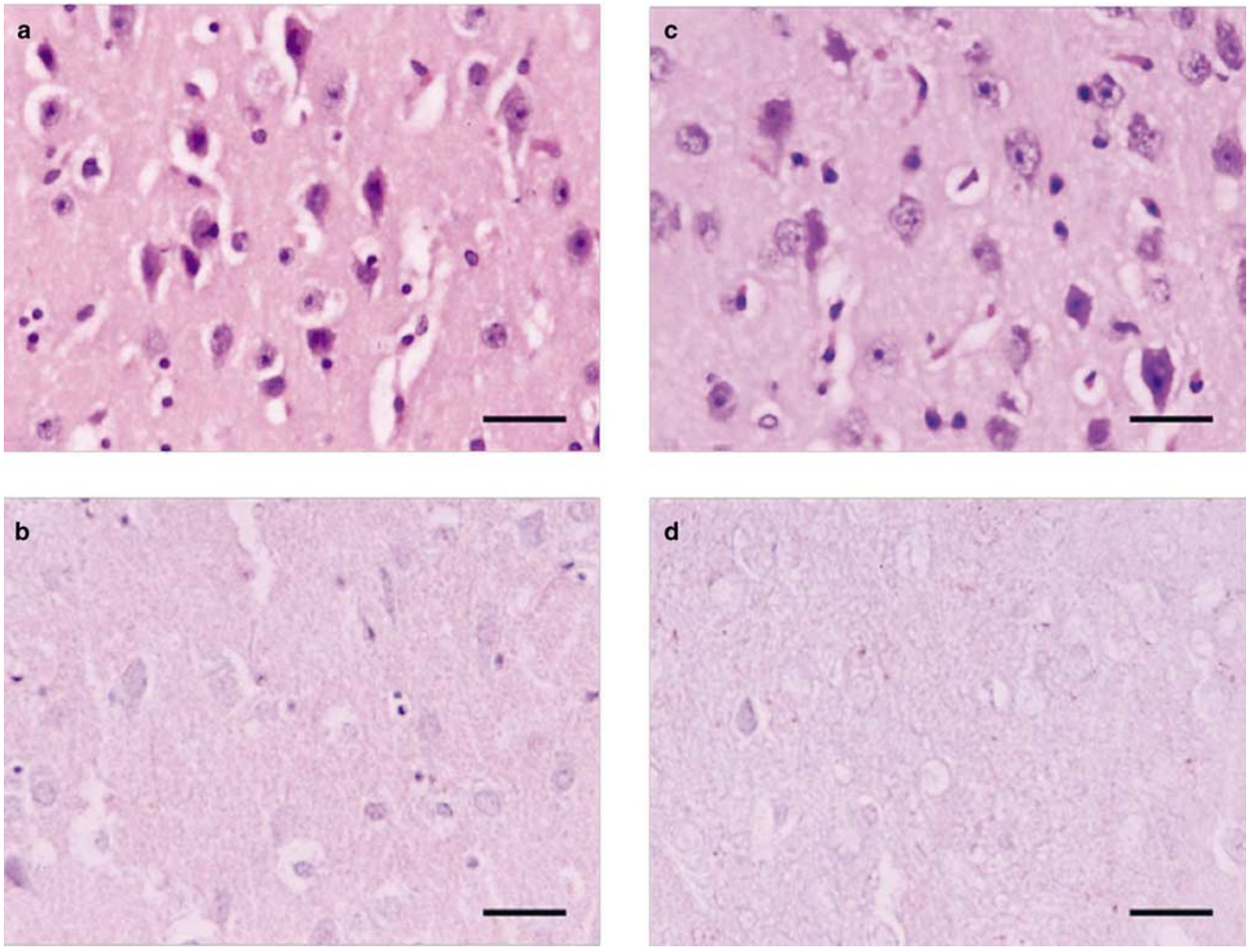

Figure 4 Histological analysis of hamster brain inoculated with CM muscle homogenate. Sections of the brain inoculated with PrP^{C}-containing muscle homogenate (**a**, **b**) or PrP^{CQ}-containing muscle homogenate (**c**, **d**) were stained with HE (**a**, **c**) or immunostained using 3F4 monoclonal antibody (**b**, **d**). Neither neurodegenerative change nor abnormal PrP deposition was revealed. Scale bars = 30 μm.

1.5% NP-40. These biochemical properties of PrP^{CQ} molecules are distinct from PrP^{C}.

There have been several attempts to create *de novo* PrP^{Sc}-like molecules *in vitro*. Using a cell-free conversion system, Horiuchi *et al*[13] were able to convert PrP^{C} into a protease-resistant isoform by the addition of PrP^{Sc} under physiological conditions. Even in the absence of PrP^{Sc}, an acidic buffer can give a β-sheet-dominant conformation and PK resistance to PrP^{C} *in vitro*.[14,15] Nevertheless, no *de novo* PrP^{Sc}-like molecules succeeded to reproduce the disease *in vivo*.[16] In the present study, PrP^{CQ} molecules were not capable of causing any pathological changes in the central nervous system. PrP^{CQ} molecules seem to be distinct from both of these *de novo* PrP^{Sc}-like molecules and PrP^{Sc}, because PrP^{CQ} molecules possess neither marked resistance to PK and detergent insolubility nor transmissibility of the disease conditions.

The CM model reported in this study is different from other PrP-conversion models previously reported, in that PrP^{CQ} molecule was generated *in vivo* in the absence of exogenous input of PrP^{Sc} or PrP^{Sc}-like molecules. Instead, the microenvironment in the lysosome was altered in hamsters by the injection of chloroquine to produce this distinct PrP molecule. Lysosomes are acidic compartments that have been reported to play an important role in the conformational conversion of PrP in prion diseases.[17,18] Chloroquine raises intralysosomal pH to as high as 6.0–6.5, causing marked changes in intracellular protein processing and trafficking.[8] As a consequence of the long-term administration of chloroquine, skeletal muscle fibers degenerate, with numerous autophagic vacuoles.[9] In the process of forming autophagic vacuoles, endogenous muscular PrP^{C} could acquire the properties of PrP^{CQ}.

In the present study, it remains unclear whether chloroquine modifies PrP^{C} molecules directly or indirectly. A previous study revealed that chloroquine does not directly interact with PrP molecules in scrapie-infected neuroblastoma cells.[19]

Furthermore, it has been reported that unfolded recombinant human prion protein PrP90-231 forms a stable protein folding intermediate rich in β-sheet at pH lower than 4.[14] Matsunaga *et al*[20] reported that pH is a crucial factor in determining the conformational state of some amyloidgenic proteins. They found that synthetic Aβ42 and stefin B peptides, showing similar amino-acid alignment to PrP90-144, tend to form amyloid fibrils at acidic pH. Considering these observations, it is unlikely that chloroquine directly interacts with PrP^{C} molecules. An increase in lysosomal pH due to chloroquine, and subsequent metabolic changes in lysosomal systems might be responsible for the biosynthesis of PrP^{CQ} molecules.

Besides experimental CM, there are a few experimental models in which PrP molecules of skeletal muscle are rendered partially PK-resistant and detergent-insoluble. Chiesa *et al*[21] established transgenic mice expressing PrP molecules with nine-octapeptide insertional mutation. Mutated PrP molecules obtained PrP^{Sc}-like properties in the brain and the periphery, producing neurodegeneration similar to an inherited prion disease in humans. In their model, the primary structure of PrP molecules was changed and the mutated PrP was overexpressed not only in the brain but also in the skeletal muscle and heart. This model is quite different from our CM model, in that the primary structure of PrP molecules was not manipulated.

The other experimental model is a transgenic mouse harboring high copy numbers of wild-type PrP transgenes, which spontaneously exhibited necrotizing myopathy, demyelinating polyneuropathy, and focal vacuolation of the central nervous system without apparent deposition of PrP^{Sc}.[22] In spite of severe neurodegeneration and neuromyopathy, only small amount of PK-resistant PrP was detected in affected muscles and brains. They concluded that low level of PK-resistant PrP might reflect aggregation of PrP^{C} and was not correlated with neuropathological changes in these transgenic mice. In our study, PrP^{CQ} after PK digestion did not show molecular characteristics of PrP^{Sc} in prion diseases, suggesting that PrP^{CQ} acquires less PK sensitivity through a different mechanism from that of PrP^{Sc}. Although the expression level of PrP was not increased in CM (data not shown), it is possible that distinct biochemical properties of PrP^{CQ} might simply be due to protein aggregation or alteration in PK–protein ratio, not to the conformational change of monomeric PrP^{C} molecules.

PrP^{CQ} in the affected muscles of the present model was accompanied by the accumulation not only of lysosomal markers but also of those molecules known to be involved in prion disease pathogenesis, such as clathrin, heparan sulfate proteoglycan, and apolipoprotein J.[23–25] It is known that certain sulfated glycans, such as heparan sulfate and pentosan polysulfate, stimulate PrP conversion *in vitro*.[26] Then, it might be possible that accumulated heparan sulfate in the CM muscles contribute to the acquisition of altered PK sensitivity and partial detergent insolubility of the PrP molecules.

Experimental CM in the rat has been established previously as a model of myopathies with rimmed vacuoles, including distal myopathy with rimmed vacuole formation and inclusion-body myositis. Owing to of the deposition of amyloid β in inclusion-body myositis,[27] experimental CM has been utilized by several groups as a peripheral model to investigate the pathogenesis of Alzheimer's disease.[10,28] The precise mechanism of rimmed vacuole formation in CM is still unknown; however, it has been reported that chloroquine causes an increase in endogenous autophagosomes in mammalian cells.[29] Similar mechanism(s) might be shared between amyloid β deposition in the CM rat model and PrP^{CQ} accumulation in our CM hamster model.

The PrP2B polyclonal antibody revealed prominent 27 kDa signals with additional 30 kDa signals (Figure 2), while the 3F4 monoclonal antibody reacted with dominant 35 kDa signals and additional 30 kDa signals (Figure 3), which were similar to Cp33-37 signal in skeletal muscle of hamster.[30] The common signals of 30 kDa were detected by both of the two antibodies. Minor epitope differences between the two antibodies might account for such a diversity of PrP signals, but it remains to be elucidated.

Finally, together with the biochemical and biological properties of PrP^{CQ}, the immunohistochemical findings in CM muscles of the molecules known to be involved in prion disease pathogenesis indicate that experimental CM in hamsters is a useful *in vivo* model to investigate the mechanism of PrP accumulation in the pathogenesis of PrP-related diseases.

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