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Research report

Copper binding to PrP^C may inhibit prion disease propagation

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Abstract

Although it has been well established that PrP^C, the normal isoform of PrP^{Sc}, is a copper-binding protein, the role of this metal in the function of PrP^C as well as in prion disease pathology remains unclear. Here, we show that when scrapie-infected neuroblastoma cells were cultured in the presence of copper, the accumulation of PrP^{Sc} in these cells was markedly reduced. In addition, our results indicate that when normal neuroblastoma cells were cultured in the presence of copper ions, they could no longer bind and internalize PrP^{Sc}. In another set of experiments, copper was added to the drinking water of normal and scrapie-infected hamsters. Our results show that administration of copper to normal hamsters induced cerebellar PrP^C accumulation. Most important, a significant delay in prion disease onset was observed when scrapie-infected hamsters were treated with copper. As shown before for neuroblastoma cells, also in vivo most of the copper-induced accumulation of PrP^C was intracellular. We hypothesized that PrP^C internalization by copper may hinder PrP^{Sc} interaction with this molecule, and thereby affect prion disease propagation.

Theme: Disorders of the nervous system
Topic: Degenerative disease: other

Keywords: Copper binding; PrP^C; Prion disease propagation

1. Introduction

PrP^C, a raft-associated GPI-anchored polypeptide, is the normal isoform of PrP^{Sc}, the protein component of the prion [31,32,46]. Prions cause transmissible neurodegenerative diseases such as scrapie and bovine spongiform encephalopathy (BSE) in animals as well as CJD in humans [36]. Both PrP isoforms share the same amino acid sequence but, in contrast to PrP^C, PrP^{Sc} comprises a protease-resistant core peptide denominated PrP 27–30 [25]. It has been postulated that β-sheet rich PrP^{Sc} is produced from mostly α-helical PrP^C by a conformational conversion process, which is not fully understood [12]. Although the molecular genetics, metabolism, biochemistry and structure of the prion proteins, have been mostly elucidated, very little is known about the pathological mechanisms of prion diseases [16,36].

The function of PrP^C has yet to be elucidated albeit the creation and investigation of several lines of $PrP^{0/0}$ mice [6,24,29]. All PrP-ablated mice are immune to infection by prions, and none of them developed a spontaneous prion-like disease [28]. The only clue in the search for the function of PrP^C is the finding that this protein binds copper specifically [4,17,47,50]. Copper was shown to induce conformational changes in PrP^C as well as to enhance its internalization into cells [33]. In addition to copper, Zn^{2+} but not Mn^{2+} stimulated PrP^C endocytosis [34]. Another link between copper binding to PrP^C and its function is the finding that cerebellar cells from $PrP^{0/0}$ mice were more sensitive to copper toxicity and oxidative stress than the comparable cells obtained from wt mice [5]. Similar results were obtained by us in sperm cells [44].

Several reports intended to connect between the copper-binding properties of the isoforms and prion infectivity, sometimes in a contradictory manner. For example, it was suggested that the aggregation of a PrP-derived peptide (106–126), known by its neurotoxicity, was increased by the addition of copper, zinc or iron ions and was reduced upon their removal [19]. In another report, copper was shown to facilitate the conversion of PrP^C into a partially protease-resistant peptide [40,41]. Contrarily, we have recently shown that, as opposed to PrP^C and denatured PrP^{Sc}, native PrP^{Sc} as well as PrP 27–30, were not retained by a

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Cu^{2+}-loaded resin [45]. In another work, a mutant PrP with nine additional octarepeats, which was shown to be associated with familial prion disease, failed to undergo copper-mediated endocytosis [34]. Interestingly, some pathogenic PrP mutations resulted in PrP peptides which accumulate in the endoplasmic reticulum [18] and probably never meet copper ions. It is therefore unclear whether the copper-binding property of PrP^C exerts a beneficial or deleterious effect, if any, in prion disease pathogenesis.

To investigate further the possible connection between copper binding to PrP^C and prion infectivity, we tested the accumulation of both PrP^C and PrP^{Sc} in ScN2a cells, a cell line used to study the metabolism of prion proteins [3,7]. In addition, we set up to investigate whether the interaction of PrP^C with copper ions, in vitro and in vivo, affects the binding of PrP^{Sc} to cells.

Here, we show that the culture of both normal and scrapie-infected neuroblastoma cells (N2a and ScN2a cells, respectively), in the presence of copper, resulted in a large increase in PrP^C concentration. Concomitantly, the accumulation of PrP^{Sc} in the ScN2a cells was reduced. We also show that the binding of PrP^{Sc} to N2a was mostly abolished when these cells were cultured in the presence of copper ions prior to their incubation with scrapie brain homogenates. Consistent with the in vitro results, administration of copper to normal hamsters resulted in a significant augmentation in the PrP^C signal observed in Purkinje cells and in their dendritic trees, comprised in the molecular that copper administration to scrapie-infected hamsters can delay the appearance of disease symptoms.

2. Materials and methods

2.1. Cell culture

N2a and ScN2a were cultured as described [8,51]. These lines are normal and scrapie-infected neuroblastoma cells transfected with 3F4-tagged PrP gene. MHM2 constructs transfected into N2a cells were maintained by the addition of 1 mg/ml geneticin to the cell culture medium. Cells were grown and maintained at 37 °C in minimal essential medium supplemented with 10% fetal bovine serum and 1% glutamine.

2.2. Immunoblotting of cell extracts

At the end of the different experiments, cells (N2a, ScN2a in a T75 flask) were extracted in 3 ml of lysis buffer containing 10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA and 1% Nonidet P-40. Samples were centrifuged at $800 \times g$ (2000 rpm) for 15 min at 4 °C, and the supernatant was concentrated by methanol precipitation. Pellets were resuspended in 2% sarkosyl/STE buffer (10 mM Tris–HCl, pH 7.5, 10 mm NaCl, 1 mM EDTA). Samples were digested in the presence or absence of 40 μg/ml proteinase K (PK) for 30 min at 37 °C and then subjected to immunoblot analysis with αPrP mAb 3F4 [20].

2.3. Preparation of scrapie brain homogenates

Each scrapie sick hamster brain was homogenized in [10% (wt/v)] cold buffer containing 10 mM Tris, 0.3 mM sucrose in 10 ml of phosphate-buffered saline pH 7.4. The homogenate was centrifuged at $800 \times g$ (2000 rpm) 15 min 4 °C, and the supernatant was frozen in aliquots for future experiments. To each T75 flask, 50 μl of homogenate (about 300 μg protein) was added for the designated incubation time.

2.4. Binding experiments

After culturing the cells with the presence or absence of $CuSO_4$ for 24 h, cells were washed, and then incubated with scrapie homogenate suspended in cell culture media for 2 h at 37 °C or 4 °C, as described. After the incubation, the cells were washed once in medium and four times with PBS. The amount of PrP^{Sc} incorporated into the cells was determined by immunoblotting as described above.

2.5. RT-PCR of PrP mRNA

Total RNA was isolated from N2a-C10 and ScN2a-C10 cells using TrireagentTM LS (Molecular Research Center, Cincinnati, OH). RNA was pretreated with DNase I and then amplified by using the RT-PCR method. cDNA was synthesized with PrP-specific primers ATG-ACA-GCA-ACC-AGA-ACA-ACT-T (PrP-599, sense) and C-CAG-GGC-CCA-TCA-GTG-CCA (PrP-995, antisense) to generate a 415-bp fragment. The PCR product was characterized by agarose gel.

2.6. Confocal microscopy and fluorescence staining

Following the designated treatments, N2a-C10 cells were fixed on glass slides by a 4% formaldehyde solution for 10 min at room temperature. Cells were permeabilized with 1% BSA and 0.2% Triton X-100 in PBS solution for 4 min, before blocking with 1% BSA in PBS for 20 min. Subsequently, cells were incubated with αPrP mAb 3F4 in PBS (1/500) for 1 h at room temperature. Cy3 was used as fluorescent anti-mouse antibody. The samples were analyzed by confocal microscopy.

2.7. In vivo infectivity experiments

Groups comprising five male Syrian hamsters, 4 weeks old, were inoculated intracerebrally with 50 μl of 10% scrapie hamster brain homogenate. Animals were observed daily. Four weeks after the brain inoculation, CuSo4 was added to the drinking water for the length of time and concentration

indicated at Fig. 5 and in the text. Prion titers were calculated from disease incubation times as described [39].

2.8. Cu^{2+} administration to normal hamsters

$CuSO_4$, at the concentrations described in the text, was administered to groups of five male Syrian hamsters in the drinking water. Animals were inspected daily for adverse effects. After 30 days of copper administration, all animals were euthanised, and their brains were collected for both Western blotting and immunohistochemistry.

2.9. Immunohistochemistry

Glass slides carrying 8-μm-thick cryostat sections were fixed in formalin 4% in PBS for 30 min. Samples were rinsed with PBS and then immersed for 1 h in blocking solution (10% normal goat serum in BSA 1%/PBS solution) supplemented with 1% Triton X-100. This was followed by incubation over night with the anti-PrP mAb-3F4 (1:50 in blocking solution). Samples were then washed in PBS and incubated with fluorescein (FITC)-conjugated affinipure goat anti-mouse IgM (Jackson Immunoresearch Laboratories, PA, USA), before washing and examination in a Zeiss 410 Confocal laser scanning microscope.

2.10. Atomic absorption

Samples from brain homogenates, either all brain or cerebellum, were precipitated by TCA to separate proteins from other components. Nitric acid was added to both the protein and the soluble fraction and copper concentration measured by a Varian Spectra 300 Zeeman atomic absorption spectrometer.

2.11. Statistical analysis

To compare the study groups, ANOVA and the nonparametric Kruskal–Wallis test were applied.

3. Results

3.1. Copper inhibits the accumulation of PrP^{Sc} in ScN2a cells

N2a and ScN2a [7] cells were cultured in the presence or absence of copper as described in Section 2 and were subsequently extracted and immunoblotted with αPrP mAb 3F4. ScN2a cells comprise both PrP^C and PrP^{Sc}, and it is believed that an active PrP^C to PrP^{Sc} conversion process occurs in these cells [3,7,10]. As opposed to PrP^{Sc}, which comprises a PK-resistant core-denominated PrP27–30, PrP^C is highly sensitive to PK digestion [25]. As can be seen in Fig. 1A, culturing of both N2a and ScN2a cells in the presence of copper resulted in increased accumulation of total PrP. Since

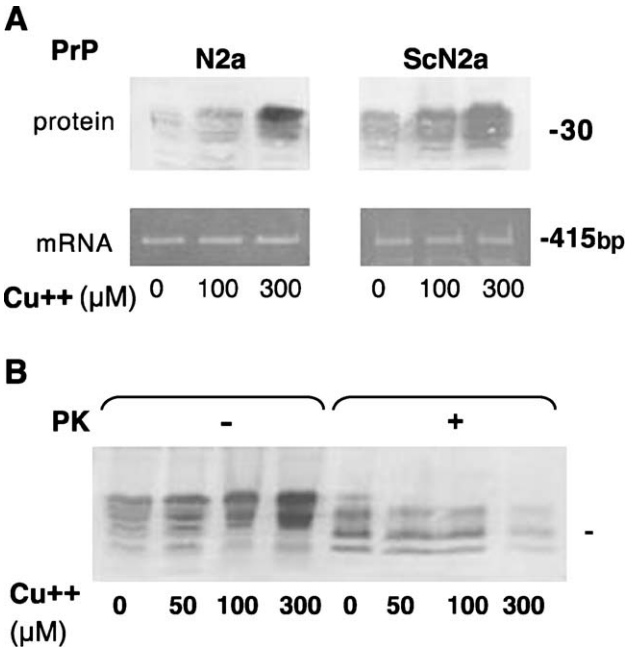

Fig. 1. Copper ions increase accumulation of total PrP and inhibit the accumulation of PrP^{Sc}. (A) N2a and ScN2a were cultured in the presence or absence of increasing concentrations of Cu^{2+} for 48 h. Subsequently, the cells were tested for the presence of either PrP protein with αPrP mAb 3F4, or for PrP mRNA expression by RT-PCR. (B) Scrapie-infected neuroblastoma cells (ScN2a-C10) were incubated in the presence of increasing concentrations of Cu^{2+} for 48 h. Subsequently, the cells were extracted as described in the methods and digested in the presence or absence of PK before immunoblotting with αPrP mAb 3F4.

this accumulation was not the result of increased PrP RNA expression (Fig. 1A), we assume that PrP^C degradation in copper-cultured cells may be delayed. We attribute this effect on PrP^C to the copper-induced internalization of this protein, which was previously reported [23,33]. As opposed to the increased concentration observed for total PrP, the concentration of protease-resistant PrP^{Sc} in ScN2a cells remained constant and even decreased significantly at high copper concentrations (Fig. 1B). We suggest that the binding of PrP^C to copper, which has been shown to result in its internalization at these copper concentrations [33], can also interfere with the conversion of PrP^C to PrP^{Sc}. Such conversion is believed to occur on membrane rafts [11,48,49].

3.2. Copper ions inhibit the binding of PrP^{Sc} to N2a cells

In order to commence the conversion of PrP^C to PrP^{Sc}, prion infection must include an early step where PrP^{Sc}, either directly or through its cofactors in prion propagation, encounters PrP^C on membranal rafts [23,31]. Since copper was shown to induce the internalization of PrP^C from these membrane micro-environments [23,33], we decided to investigate whether PrP^{Sc} can still bind to copper-treated N2a cells. To this aim, we cultured N2a cells comprising 3F4-tagged PrP [9,51], in the presence or absence of copper for 24 h, washed them with PBS to discard unbound copper ions and

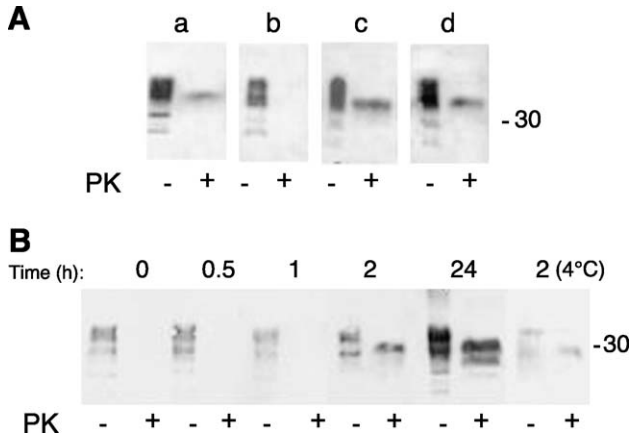

Fig. 2. Copper inhibits the binding of PrP^{Sc} to N2a-C10. (A) (a) N2a-C10 cells were incubated with scrapie-infected hamster brain homogenate for 2 h, washed, extracted and subsequently digested in the presence or absence of PK and immunoblotted with mAb 3F4. (b) Cells were cultured for 24 h with 500 μM $CuSO_4$, and washed before incubation with the scrapie brain homogenate as above. (c) Cells were incubated with 500 μM $CuSO_4$ for 1 h before incubation with the scrapie brain homogenate as above. (d) Scrapie brain homogenate was incubated with 500 μM $CuSO_4$ for 2 h, prior to its addition to cells. (B) N2a-C10 cells were cultured in the presence of scrapie hamster brain homogenate for different periods of time, as described in the figure. All the incubations were performed at 37 °C, except the last one, performed for 2 h at 4 °C. Subsequently, the cells were washed, digested in the presence or absence of 40 μg/ml PK and immunoblotted with αPrP mAb 3F4.

subsequently incubated the cells with scrapie hamster brain homogenate. We used total brain homogenates instead of partially purified PrP^{Sc} since the prion protein purification includes a detergent induced aggregation step [25], which may change the native PrP^{Sc}–cell interactions. Following 2 h of incubation with the homogenate, the cells were washed and subsequently extracted as described in the methods. Cell extracts were digested in the presence or absence of PK, before immunoblotting with αPrP mAb 3F4. Our results show (Fig. 2a) that while considerable amounts of PrP^{Sc} remained attached to the cells after 2 h of incubation with the scrapie brain homogenate, binding of PrP^{Sc} to N2a cells was mostly abolished when these cells were previously cultured in the presence of 500 μM copper. This was also true for the binding of PrP from PK digested scrapie brain homogenate (not shown), suggesting that the binding of PrP^{Sc} and that of its protease-resistant core, PrP 27–30, to normal cells is independent from other protease sensitive molecules present in scrapie brain homogenates. Interestingly, the binding of PrP^{Sc} to N2a cells was not affected when copper was incubated either with the scrapie brain homogenate, or with the N2a-C10 cells for only 1 h before the binding experiment (Fig. 2a, c and d, respectively), suggesting that copper does not inhibit the binding of PrP^{Sc} to cells by blocking specific binding sites. PrP^{Sc} binding to cells was inhibited only when the cells were cultured in the presence of copper for more than 24 h, inferring that the metal ions generated metabolic changes in the N2a cells, which subsequently hindered the binding of PrP^{Sc} to them.

The concentration of copper ions in the N2a culture medium required for the inhibition of PrP^{Sc} incorporation was similar to those used before for PrP^{C} internalization [33]. When the N2a cells were incubated with copper for 24 h, the concentration of copper that was effective for PrP^{Sc} binding inhibition was between 200 and 500 μM. For a longer culture time (48–72 h), inhibition occurs also at 100–200 μM (not shown). In all cases, no cell toxicity, as measured by cell death, could be detected.

Fig. 2b shows that PrP^{Sc} binds to the cells in a time-dependent manner. PrP^{Sc} could be detected on the cells after at least 2 h of incubation with the brain homogenate, suggesting that the interaction of PrP^{Sc} with these cells is likely to be specific, and may even include internalization of PrP^{Sc} following the binding of the protein to cell targets. Consistent with that, the fact that PrP^{Sc} binding to N2a cells was significantly more intense at 37 °C than

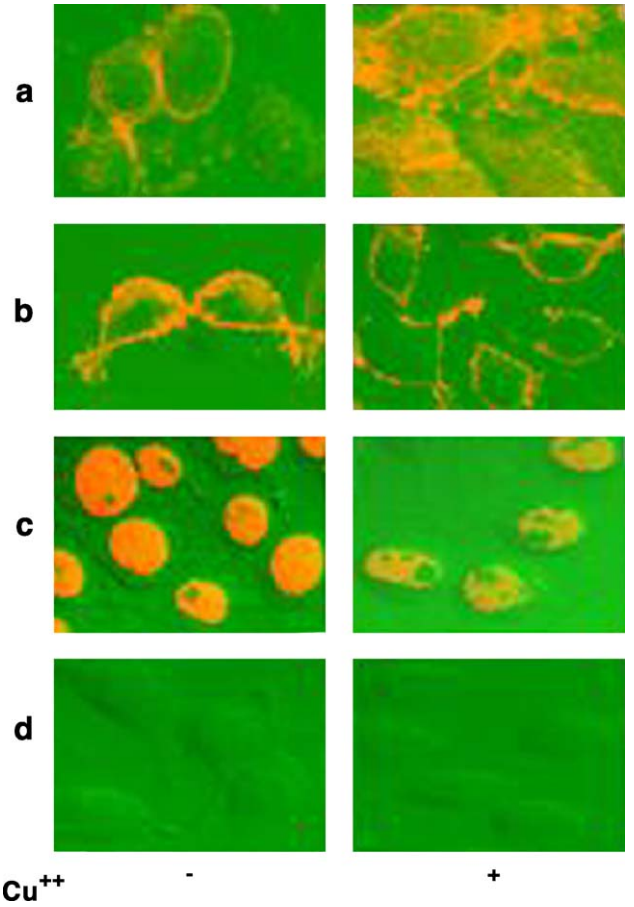

Fig. 3. Copper inhibits the internalization of PrP^{Sc} to N2a cells. N2a cells, cultured in the presence or absence of 200 μM $CuSO_4$ for 48 h, were incubated with scrapie hamster brain homogenate for 2 h. Subsequently, cells were fixed, immunostained with αPrP mAb 3F4 and analyzed by confocal microscopy. Except panel b, all experiments were performed after permeabilization of the cells. (a) N2a cells before incubation with brain homogenate. (b) N2a cells after incubation with brain homogenate, without permeabilization during immunostaining. (c) N2a cells after incubation. (d) Secondary antibody alone.

at 4 °C also suggests, as was shown for other systems, that most of the PrP^{Sc} may have been internalized into the cells [1,13].

3.3. Copper inhibits the internalization of PrP^{Sc} to N2a cells

To investigate whether copper inhibits not only the binding but also the internalization of PrP^{Sc} to N2a cells, we immunostained N2a cells cultured in the presence or absence of copper prior to their incubation with scrapie brain homogenate, with αPrP mAb 3F4. Fixed cells were observed and analyzed by confocal microscopy.

Fig. 3 shows the results of such an experiment. As expected, in the absence of scrapie brain homogenate, copper addition to N2a cells resulted in an increased signal for total PrP as well as in PrP internalization into the cells (Fig. 3a). The addition of brain homogenate caused a dramatic change in the signal of total PrP in the cells. Without cell permeabilization, when only PrP on the cells membrane could be observed (Fig. 3b), the concentration of PrP was moderately reduced by the addition of copper. However, when the cells were permeabilized in the presence of Triton X-100 (see Section 2), a large amount of intracellular PrP was detected, mostly close or even inside the cell nucleus. This signal was markedly reduced when the cells were cultured in the presence of copper (Fig. 3c). The fact that almost no signal could be observed on the cell membrane after permeabilization is due to the limitations of confocal microscopy. A very strong signal, such as the internalized PrP in this case, quenches much weaker signal, such as the membrane signal observed in Fig. 3b,

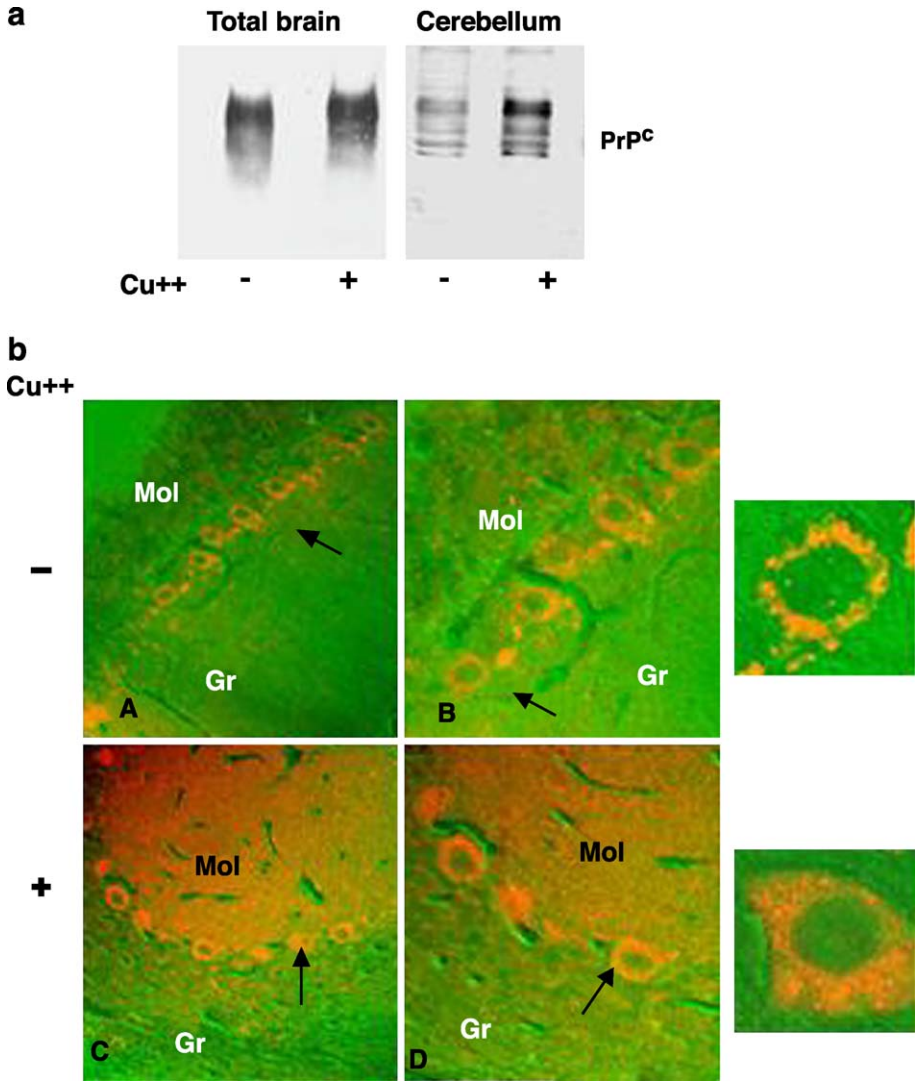

Fig. 4. Accumulation of PrP^{C} in the cerebellum of copper-treated hamsters. Copper (24 ppm) was administered to normal hamsters for 1 month. Following this period of time, these and control untreated hamsters were euthanised. (a) Brains were homogenized (total brain or cerebellum only), and immunoblotted with αPrP mAb 3F4. (b) Brains were sliced following perfusion for immunocytochemistry with αPrP mAb 3F4. (A, B) Control hamsters; (C, D) after treatment with 24 ppm copper. Inserts: Enlarged Purkinje cell; Mol: Molecular layer; Gr: Granular Layer; arrow: Purkinje cell layer.

and renders it almost invisible. These experiments therefore indicate that culturing of N2a in the presence of copper ions inhibit both the binding as well as the internalization of PrP^{Sc} to these cells.

3.4. Accumulation of PrP^{C} in the cerebellum of copper-treated hamsters

To study whether the effects of copper ions on the metabolism of the PrP isoforms can also be observed in vivo, and thereby affect prion disease pathogenesis, we first administered CuSO4 to normal hamsters for 30 days by adding the metal salt to their drinking water. A wide range of copper concentrations was used; from 150 μM (24 ppm), which is an average concentration used in our cell culture experiments to 2 mM (300 ppm). These copper concentrations were shown previously to be relatively safe for long term administration [15,35]. In total, copper was administered for 1 month to four groups (each comprising five hamsters), at 24, 48, 160 and 300 ppm. No adverse effects were observed. Subsequently, the hamsters were sacrificed, and the concentration of brain PrP^{C} was investigated both by immunoblotting and immunocytochemistry with αPrP mAb 3F4. While a PrP immunoblot of whole brain homogenates did not indicate a significant difference in PrP^{C} concentration between copper-treated and -untreated animals, the concentration of PrP^{C} increased considerably in the cerebellum of the treated animals. Similar results could be appreciated from the immunocytochemistry experiments (Fig. 4b). Following copper administration to the hamsters, the concentration of PrP^{C} in Purkinje cells as well as in the molecular layer of the cerebellum comprising the Purkinje cells dendritic tree, not only increased significantly, but was now also cytosolic in addition to membranal. All results in Fig. 4 are shown for the lowest concentration of copper used (24 ppm), since all others were very similar.

The copper concentration was measured in the treated and control hamster's brains (24 ppm). Extracts of total brains as well as of cerebellum were precipitated by TCA, and subsequently copper was measured by atomic absorption at both the protein fraction and the soluble fraction. No significant difference was found between the samples in the TCA soluble fraction (about 3.5 μg copper/ml), or in the total brain protein fraction (about 12.5 μg copper/g protein). Interestingly, there was a significant increase in the copper concentration in the cerebellum of the copper-treated animals: 31.6 μg copper/g protein, as opposed to 14.1 μg copper/g protein in the control cerebellum. It was previously shown that copper administration at this concentration and even much higher (up to 300 ppm) had no effect on blood copper concentration [15].

Purkinje cells and the molecular layer are the cerebellar areas known to comprise PrP^{C} [21,43], mainly in the cell membrane [22]. The fact that copper administration resulted in increase copper binding to cerebellar proteins constitutes

We speculate that, as in the N2a cells, copper administration to animals did not result in de novo expression of PrP^{C} but probably in the delayed degradation of the protein in specific brain cells, due to the binding of copper to the cell surface. Indeed, copper treatment induced PrP^{C} internalization into Purkinje cells, as was shown before for N2a cells.

3.5. Copper administration to scrapie-infected hamsters delays the onset of disease

To investigate whether copper administration to scrapie-infected hamsters may interfere with disease onset, we added copper to the drinking water of hamsters infected with scrapie hamster brain homogenate. Since the highest concentration used in the previous experiment was not toxic, we used 300 and 750 ppm for long term (30 days following inoculation to disease symptoms) and 3000 ppm for treatment pulses of 10 days every 30 days. No adverse effects were observed in the infected animals treated with copper ions, nor in normal controls treated similarly. As can be seen in Fig. 5, copper administration delayed disease onset in all groups. Interestingly, the most encouraging results were observed in the groups treated at high copper concentrations for short periods of time. We speculate that administration of copper at high concentrations at a time point in disease incubation when PrP^{Sc} is still present at very low concentrations may delay the binding of PrP^{Sc} to new cells, at least for the time required to restore PrP^{C} molecules to the appropriate cell targets. Interestingly, the concentration of PrP^{Sc} at the time animals were sacrificed (when they showed difficulties getting water and food) was the same for all groups (not shown).

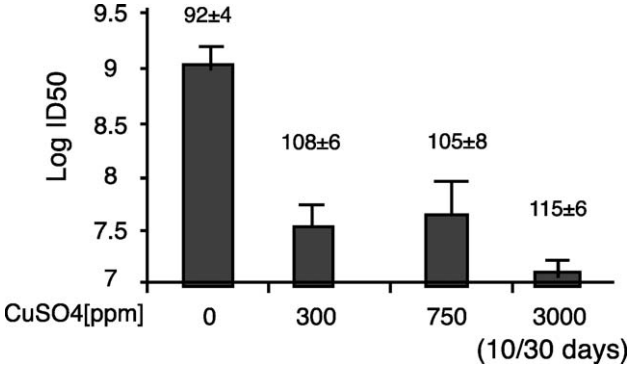

Fig. 5. Copper administration to scrapie-infected hamsters delays the onset of disease. Several groups of hamsters were inoculated i.c. with scrapie brain homogenate. Copper ions were added to the drinking water of designated groups, as described in the text. All groups were observed daily until the appearance of disease symptoms in each individual animal. Infectivity titers were determined based on the incubation period determined for each group [38]. The copper concentration added to the drinking water of each group is stated in the figure. For 3000 ppm copper, the metal salt was administered to the hamsters 10 days of each 30 days, starting as for all the groups 30 days after the inoculation.

4. Discussion

We have shown in this work that when ScN2a, a cell line permanently infected with prions, was cultured in the presence of copper ions, the accumulation of PrP^{Sc} was reduced. In addition, the binding and internalization of PrP^{Sc} to N2a, was inhibited by pre-culturing of these cells in the presence of copper. Concomitantly, such copper treatment enhanced the accumulation of PrP^{C} molecules, probably as a result of the internalization of the normal prion protein into the cells. We suggest that the internalization of PrP^{C} by copper, which removes the normal prion protein from its membrane cell location, may interfere with the binding of PrP^{Sc} to the cells as well as with the conversion of PrP^{C} into PrP^{Sc}. Consistent with these observations, copper administration both increased the accumulation of PrP^{C} in specific brain cells of normal hamsters as well as delayed the onset of prion disease in scrapie-infected hamsters.

The inhibition of PrP^{Sc} binding by copper occurred only at specific conditions. Copper had no effect when it was added to the scrapie brain homogenate, nor when it was added to the cells 1 h before the binding experiment. Inhibition of PrP^{Sc} binding to cells by copper required the culture of the cells in the presence of copper for a considerable length of time and at the concentrations known to induce internalization of PrP^{C}. Interestingly, copper was shown to induce the reversible internalization of PrP^{C} after less then 1 h of cell treatment [33,34]. The discrepancy between this time frame and our experiments can be explained in several ways. It is possible that after 1 h, only part of the PrP^{C} was internalized, as was indeed shown by Pauly and Harris. Since this process was reversible, PrP^{C} may have done his way back to the cell surface during the incubation of the cells with the scrapie brain homogenate. In our experiments, which include 24 h of incubation with copper ions, PrP^{C} could have been completely or even irreversibly internalized due to metabolic changes that occurred in the cell. It is, of course, also possible that the inhibition of PrP^{Sc} binding to the cells, as well as copper inhibition of disease propagation, was not a direct result of the copper-induced internalization of PrP^{C}, but rather a more general effect of copper on cell metabolism. It should always be remembered that although copper may be very important for the function and metabolism of PrP^{C}, for copper, PrP^{C} is just another protein out of many with which it may interact.

Our in vivo results are also consistent with the data obtained by cell culture experiments. Administration of copper to normal hamsters resulted in increased accumulation of PrP^{C} in Purkinje cells and in the cerebellar molecular layer, brain areas that are known to express PrP^{C} [21,43]. In addition, copper administration to scrapie-infected hamsters, even for short periods of time, resulted in a significant delay in prion disease onset. Although high copper concentrations have been shown to affect the immune system [35], more experiments will be required to assess whether such effect also interferes with prion disease pathogenesis under copper administration.

Other investigators have also tried to connect between the binding of copper to PrP^{C} and prion infectivity. Jobling et al. [19] reported that aggregation of a neurotoxic PrP-derived peptide (106–126), is increased by the binding of copper ions. It is clear that the conclusions from our work and that of Jobling et al. are not in accordance. While we suggest that copper exerts a protective effect from prion infection, probably because of the rearrangement in PrP^{C} location, the other report suggests that copper enhances aggregation of PrP peptides and thereby the development of the disease. It is important to stress that we are using different systems and that although some PrP peptides have been shown to be neurotoxic, it is most probable that the mechanism of their deleterious effect does not resemble prion disease pathology.

The typical purification of PrP^{Sc} includes a detergent induced aggregation step, which subsequently renders the prion protein insoluble [37]. These insoluble aggregates have been shown repeatedly to require denaturing treatments for their solubilization [2,26,42]. To this aim, and to avoid non specific binding of aggregates to cells, we have used in this work enriched scrapie brain homogenates for the binding experiments with cells, as opposed to aggregated PrP^{Sc}. The rational for this strategy was that in brain homogenate, PrP^{Sc} is present in its normal environment, i.e., cholesterol-rich membrane microdomains [30]. In rafts, PrP^{Sc} is probably much less aggregated than in its purified form, as can be seen by the fact that it does not precipitate to the lower fractions in floatation gradients [30]. It is, however, conceivable that PrP^{Sc} was internalized into the cell together with other raft components, which may facilitate or hinder the pathogenic process.

It is important to point out that our results, although very suggestive, do not prove the existence of a direct or unique interaction between PrP^{Sc} and PrP^{C}. Other cell, or raft components may and probably do participate in the binding of PrP^{Sc} and in its internalization process. In addition, copper interacts with many cell proteins and is a cofactor in many cell functions [14,27]. More work will be required to identify such molecules and elucidate their role in prion pathogenesis. The more cell components identified, the more ways may be designed to inhibit prion propagation.

Although results presented here suggest that copper administration to rodents can hinder prion propagation in vivo, we should remember that long term administration of copper at high concentrations is too toxic to be used in the human prion diseases for patients or, more important, as a prophylactic treatment for at risk individuals. Even though, it is important to elucidate the mechanism by which copper interferes with prion disease pathogenesis, and in the future design non-toxic reagents that can also inhibit such pathway.

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