Changes in the Glycosylation Pattern of Prion Protein in Murine Scrapie

IMPLICATIONS FOR THE MECHANISM OF NEURODEGENERATION IN PRION DISEASES*

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In prion diseases, the normal prion protein (PrP\textsuperscript{n}) undergoes a conformational change that results in the abnormal form, named scrapie prion protein (PrP\textsuperscript{scl}). The visual system of rodents provides a good model to study the distribution and glycopattern of PrP in two distinct compartments of a neuron, the cell body and the axon with functional and structural alterations in the expression and trafficking of proteins in different parts of the neurons in physiological or pathological conditions. A preferential location of PrP\textsuperscript{n} in presynaptic membranes has been proposed (9), but it is still a matter of controversy (10). Therefore, while the conversion of PrP\textsuperscript{n} into PrP\textsuperscript{scl} seems to be the central event in TSEs, other aspects such as topology and trafficking of PrP\textsuperscript{sc} might be important in the pathogenesis of these diseases. Alterations in the distribution and/or biochemical pattern of PrP in neurons have clear implications in the pathogenesis of prion diseases and may shed light on the mechanism involved in neurodegeneration in these disorders.

The visual system of rodents provides a good model to study the distribution of PrP in two distinct compartments of a neuron: the cell body and dendrites located in the retina and the axons confined to the optic nerve. The retina and optic nerve are part of the central nervous system and are susceptible to infection and disease caused by prions (11). In rodents, over 95% of the retinal ganglion cells project to the superior colliculus (12). Taking advantage of the relative simplicity of this model, we have studied the distribution and glycosylation pattern of PrP in the retina and optic nerve of Syrian hamsters and C57BL/6J mice. We found that PrP\textsuperscript{n} levels and glycosylation pattern are different in the retina and optic nerve of normal animals. Using the murine scrapie model, we also studied whether PrP changes in these compartments would occur after injection of ME7 or 139A strains in the superior colliculus. Scapie infection caused a progressive accumulation of PrP in the retina and gradual changes in its glycosylation pattern. In the optic nerve, these alterations were observed later in the course of the disease after injection of ME7. The results...
obtained in the visual system were confirmed by studying the PrP profile in the gray and white matter of normal mice and hamsters and in murine scrapie after injection of ME7 and 139A strains in the dorsal hippocampus. Gray matter and white matter are considered parts of the brain enriched in neuronal bodies and axons, respectively. The implications of these findings for the pathogenesis of TSEs are discussed. In this study, PrP is used to refer to the total amount of prion protein in the samples, while PrPc and PrPsc refer to the normal protein and proteinase K (PK)-resistant forms, respectively.

**EXPERIMENTAL PROCEDURES**

**Animals**—Groups of adult Syrian hamsters and C57BL/6J mice were used to study the levels and glycopattern of PrP in different neuronal compartments in normal conditions. Animals were sacrificed by decapitation. Brain, retina, and optic nerves were quickly dissected and frozen in dry ice. The pons and cortex were dissected and taken as representative of the white and gray matter, respectively.

**Stereotaxic Injection**—A group of mice was injected stereotaxically in both right and left superior colliculus or unilaterally in the right dorsal hippocampus with 1 µl of 10% brain homogenate prepared from terminally ill ME7- or 139A-infected mice. Control animals were injected into the right and left superior colliculus with 1 µl of 10% normal brain homogenate. Animals were age-matched at the time of injection.

**Survival Time**—Animals injected with ME7 or 139A in the superior colliculus were sacrificed at 15, 19, and 22 weeks after inoculation. The groups injected into the dorsal hippocampus with ME7 or 139A were sacrificed at 25 and 28 weeks after injection, respectively. Mice injected with normal brain homogenate were sacrificed at 5 weeks. Following the survival time, mice were deeply anesthetized with pentobarbital and sacrificed at 25 and 28 weeks after injection, respectively. Mice injected with normal brain homogenate were sacrificed at 5 weeks. Following the survival time, mice were deeply anesthetized with pentobarbital and perfused with heparinized saline solution to wash the blood out. In addition to the injected animals, a group of age-matched, non-injected control samples were compared, respectively. In murine scrapie, the percentage of the PrP signal obtained in scrapie samples compared with control samples was calculated in each experiment.

**Western Blot Assays**—Tissues were homogenized at 4 °C in phosphate-buffered saline containing protease inhibitors (Roche Molecular Biochemicals), 0.5% Triton X-100, and 0.05% SDS. The total amount of protein in each homogenate was estimated using the Micro BCA Protein Assay kit (Pierce). Membranes were stained after the Western blot procedure with Protogold (BBI International). In some cases, gels were stained with Silver Express (Amersham Biosciences) to confirm that an equivalent amount of proteins was loaded from each sample. The total amount of protein was estimated by densitometric analysis of bands stained in the membranes and gels. Analysis was performed only when no significant differences were found between the samples. To identify PrPc and PrPsc, PK (Roche Molecular Biochemicals) digestion was performed on samples from animals infected with scrapie. Digestion was performed at 37 °C for 30 min using 0.5 µg of PK/7.5 µg of total protein.

Proteins from brain, optic nerve, and retina homogenates were separated by SDS-PAGE (precast 10% NuPage bis-Tris gel, Invitrogen). Proteins were transferred onto nitrocellulose membranes after which the membranes were stained with a Reversible Protein kit (Sigma). Membranes were blocked in 5% nonfat milk for 1 h at room temperature and then incubated in phosphate-buffered saline containing 0.3% Tween 20 and the primary antibodies. 3F4 (1:50,000, epitope MKHM, a kind gift from Dr. Richard J. Kascsak) and 6H4 (1:10,000, epitope DYEDRYYRE, Prionics) were used as primary antibodies to detect PrP in hamster and mouse samples, respectively. Membranes were incubated with the primary antibody for 2 h at room temperature or overnight at 4 °C. Immunoreactivity was revealed with a peroxidase-conjugated secondary antibody (Amersham Biosciences, 1:5,000) and ECL (Amersham Biosciences). In some cases, PNGase treatment was performed according to the manufacturer’s instructions (PerkinElmer Life Sciences).

**Densitometric Analysis**—The proportion of PrP and its different isoforms were quantified by densitometry. In normal animals, the PrP signal in blots containing different dilutions of brain, retina, and optic nerve homogenates were compared against standards, i.e., signals, of known amounts of hamster and mouse recombinant PrP (Prionics). Results obtained within optic nerve/white matter and retina/gray matter samples were compared, respectively. In murine scrape, the percentage of the PrP signal obtained in scrape samples compared with control samples was calculated in each experiment.

The signal of the three different isoforms of PrP, di-, mono-, and non-glycosylated, was also estimated by densitometry. The percentage of the signal of each PrP isoform compared with the signal of the total amount of PrP was calculated in normal and scrape mice at different survival times. Comparisons between the gray/white matter and retina/optic nerve in normal animals and between control and scrape mice were performed using Student’s t test, and p < 0.05 was taken as significant.

**RESULTS**

**Profile and Distribution of PrPc in Normal C57BL/6J Mice and Syrian Hamsters**—The PrPc pattern observed by immunoblotting from normal hamster and mouse is shown in Fig. 1. In brain homogenates, two major bands corresponding to the di- (33–35 kDa) and monoglycosylated (30 kDa) forms and one minor representing the non-glycosylated band (27 kDa) of PrP were observed in hamster (Fig. 1A) and mouse (Fig. 1B). In
optic nerve, the three bands were detected in a similar profile. In contrast, only the di- and monoglycosylated bands were detected in retina. The proportion of each glycosylated isoform of PrP in the brain, optic nerve, and retina samples was calculated by densitometric analysis of the blots and is shown in the graphs in Fig. 1. The profile of the different isoforms in the two species is very similar. The non-glycosylated band of PrP was not detected in the retina of both hamster and mice (Fig. 1, A and B).

To assure that the pattern observed in the immunoblots was due to the presence of carbohydrate motifs linked to PrP, PNGase treatment was performed in the samples. In brain, optic nerve, and retinas, a single band of ~27 kDa was observed after PNGase treatment of hamsters and mice samples confirming that the pattern observed in the immunoblots was given by the three isoforms of PrP (Fig. 1, A and B).

To investigate the proportion of PrP in the retina, optic nerve, and brain homogenates, we calculated the percentage of PrP relative to the total amount of proteins in the samples (Table I). The proportion of PrP detected in the optic nerve was significantly higher than in the retina in both hamster and mouse (Student’s t test, p < 0.05). We extended our analysis to the gray and white matter of both species, and again a higher percentage of PrP in the brain samples enriched in axons (white matter) was observed.

**Distribution and Profile of PrP after Injection of ME7 or 139A in the Superior Colliculus**—We have studied the profile and distribution of PrP in the optic nerve and retina of mice injected with ME7 or 139A in the superior colliculus at different times postinoculation. After injection of ME7, the first clinical signs of scrapie characterized by ruffled fur, hunched posture, and reduction of activity were observed at 19 weeks. Advanced clinical signs were observed at 22 weeks after injection of ME7 and were characterized by an increase in the severity of early clinical signs and severe loss of weight and piosis. ME7-injected mice showed a distended bladder detected at post-mortem examination from 19 weeks onwards. Animals injected with 139A in the superior colliculus showed similar first clinical signs only at 22 weeks. Therefore, 139A-injected mice showed a ~3-week delay in the evolution and onset of scrapie compared with ME7-injected mice. Analysis was performed at 15, 19, and 22 weeks after injection of both strains.

The levels of PrP in scrapie-infected mice samples were compared with those obtained from age-matched normal mice (Fig. 2). After injection of ME7, a highly significant increase of PrP was observed from 19 weeks onward (Student’s t test, p < 0.05) in retinas (Fig. 2). Following the injection of 139A, an increase of ~4-fold PrP was observed in retinas at 22 weeks (Fig. 2). No changes in the levels of PrP were detected in the optic nerve of mice injected with ME7 or 139A (data not shown). Therefore, an increase in PrP levels following the progression of the disease occurred in retinas after the injection of ME7 and 139A in the superior colliculus.

The glycosylation pattern of PrP also changed gradually during the course of the disease after injection of ME7 and 139A (Fig. 3). In mice infected with ME7 the non-glycosylated band was clearly visible in retinas 15 weeks after injection of ME7, while it was not detected in retinas from control mice (Fig. 3A). This occurred concomitantly with a significant decrease of the diglycosylated band in ME7 retinas compared with the control retinas (Student’s t test, p < 0.05). These changes were progressive toward the end of the disease, and by 19 weeks, the increase in the non-glycosylated band of PrP in retinas was significantly higher compared with retinas at 15 weeks postinjection. In the optic nerve (Fig. 3B), a significant increase in the non-glycosylated band was observed at 22 weeks after injection of ME7 (Student’s t test, p < 0.05). Similar changes in the PrP glycopattern were observed in retina (Fig. 3C) and optic nerve (Fig. 3D) 22 weeks after injection of 139A strain. Thus, an accumulation of the non-glycosylated isoform of PrP occurred in the retina and in the optic nerve following the progression of the disease after injection of ME7 and 139A in the superior colliculus.

To verify whether the changes described in scrapie were caused by the lesion produced by the injection of the homogenate per se, a group of mice was injected with 10% normal brain homogenate into the superior colliculus. The response to the injection of 1 μl of 10% normal brain homogenate in the brain parenchyma, characterized by microglial activation, T-cell infiltration, and neuronal death, shows a peak between 2 and 5 days, and it is resolved by 4 weeks after injection (3). We looked at the PrP pattern 5 weeks after the injection of a 10% normal brain homogenate, and no changes in the amount or glycopattern of PrP were observed in retina and optic nerve compared with control, non-injected mice (Fig. 3E). Thus, PrP-altered distribution and glycosylation is not caused by the lesion initiated by the surgery or reaction to the injection.

**Detection of PrPsc after Injection of ME7 or 139A in the Superior Colliculus**—PK digestion was performed to identify PrPsc known to be partially resistant to proteolysis. PrPsc was detected in both optic nerve and retina from 15 weeks onward after injection of ME7 or 139A in the superior colliculus (Fig. 3). In retinas, PrPsc presented two major bands: the di- and monoglycosylated bands. In optic nerves, PrPsc showed three bands corresponding to the di-, mono-, and non-glycosylated isoforms. PrPsc glycopattern was similar after injection of ME7 or 139A and remained relatively unchanged toward the end of the disease. No PrPsc was detected in control mice.

**Distribution and Profile of PrP after Injection of ME7 and 139A in the Dorsal Hippocampus**—Clinical signs of scrapie after injection in the right dorsal hippocampus were similar to those described after injection in the superior colliculus in the previous section. First clinical signs were observed at 22 and 25 weeks postinjection.
FIG. 3. Glycosylation changes in retina and optic nerve after the injection of ME7 or 139A in the superior colliculus. In A and B, Western blots and graphs show the increase in the non-glycosylated band in retina (A) and optic nerve (B) after injection of ME7. Note the decrease in the diglycosylated band from 15 weeks onward in retinas. In C and D, Western blots and graphs show the increase in the non-glycosylated band 22 weeks after injection of 139A in retina (C) and optic nerve (D). In E, Western blots from retinas and optic nerve samples of mice 5 weeks after injection of 10% normal brain homogenate in the superior colliculus. Each line represents a sample of one animal in a total of five in this group. D, M, and N correspond to the di-, mono-, and non-glycosylated PrP, respectively. ct, control; wpi, weeks postinjection. Student’s t test, p < 0.05 (**).
weeks after injection of ME7 and 139A, respectively. Animals were sacrificed by the time they showed advanced clinical signs at 25 (ME7) and 28 (139A) weeks after inoculation.

We studied glycosylation changes in the gray and white matter of mice at terminal stage of the disease after injection of ME7 and 139A. Analysis of glycopattern of PrP in control mice (Fig. 5) showed that the non-glycosylated band is present in a significantly higher proportion in the white matter compared with the gray matter (Student’s t test, \( p < 0.05 \)), supporting the results described above in the visual system. After scrapie infection, a significant increase in the non-glycosylated band of PrP was seen in the gray (Fig. 5A) and white matter (Fig. 5B) concomitant with a decrease of its diglycosylated band. Therefore, the changes described in the visual system of scrapie since early stages of the disease are also present in the gray and white matter at advanced stage of the disease.

**DISCUSSION**

The visual system of rodents provides a useful model to study differences between distinct compartments of neurons. The retina contains the cell body and dendrites of neurons, while the axons of the retinal ganglion cells constitute the optic nerve. In rodents, virtually all the retinal ganglion cells project to the superior colliculus (12). Using the visual system of rodents as a model, it is possible to follow the disease in a single neuronal pathway increasing the possibilities to detect changes related specifically to cell bodies and axons at early stages of the disease. The retina and optic nerve are part of the central nervous system, and hence the results may be relevant for other areas of the brain in which such analysis is a rather difficult, if not impossible, task. Finally, the pathogenesis of prion diseases is fully reproducible in this system (11). Using this model, we were able to study the distribution and glycopattern of PrP in two defined neuronal compartments in normal hamster and mice and in murine scrapie.

The first part of this study aimed to characterize the distribution and glycosylation pattern of PrP\(^\text{c}\) in retina and optic nerve of normal animals. We found that the proportion of PrP\(^\text{c}\) with respect to the total protein is significantly higher in the optic nerve than in the retina. In addition, the non-glycosylated band of PrP was also present in higher proportion in the optic nerve than in the retina. These differences can be extended to other neurons in the central nervous system as the analysis of the gray and white matter showed similar results. A more disperse distribution of PrP in dendrites concomitant with a compact and conspicuous immunostaining of axons has been described in axons from cerebellar neurons (10). In addition, a high level of infectivity has been described in the optic nerve of variant Creutzfeldt-Jacob disease patients (14). Since infectivity has been so far correlated to the presence of PrP\(^\text{sc}\) our...
findings provide a possible explanation for these results. Several pieces of evidence have suggested that PrP glycosylation may play a critical role in the pathogenesis of prion diseases (15). It has been proposed that the differential targeting of neurons by prion strains is due to the presence of distinct glycoforms of PrPc in distinct brain regions (16). The differences in distribution of PrP glycoforms in distinct compartments of neurons may influence the targeting of neurons in prion diseases as well as the physiological function of PrP in neurons.

We also analyzed the influence of scrapie infection caused by the injection of ME7 and 139A in the distribution and glycopattern of PrP in the cell bodies (retina) and axons (optic nerve). We have shown for the first time in vivo that an accumulation of PrP concomitant with an increase in its non-glycosylated isoform occurs initially in the cell bodies of retinas early in the course of the disease. The magnitude of these alterations depends on the time at which the analysis was done after the inoculation and followed the evolution of the disease. In the optic nerve, these changes appeared later in the incubation period. These results were found after the injection of either ME7 or 139A suggesting that they are not strain-specific.

It has been shown that PrPsc is produced more rapidly in scrapie-infected cells when PrPc is non-glycosylated (17). Therefore, an increase in PrP and the changes in its glycopattern in the retina could be explained by a preferential accumulation of some glycoforms of PrPsc in the cell body. However, PrPsc isoforms in retinas showed predominantly the monoglycosylated band and not the non-glycosylated one as would be expected in this hypothesis. Following tunicamycin treatment, glycosylated protease-resistant PrP is formed in cell culture (18). In this study it was suggested that the high proportion of the non-glycosylated isoform of PrP favors the conversion of the diglycosylated isoform of PrP into the abnormal PrPsc. Alternatively, a preferential accumulation of non-glycosylated forms of PrP was observed in cell culture after proteasome inhibition (19). A deficit in the quality control machinery due to an excess of unfolded or misfolded PrP, not necessarily protease-resistant, was proposed to explain these results. It is possible that similar mechanisms operate in vivo. Our findings show glycosylation changes occur in murine scrapie, and they follow the evolution of the disease caused by ME7 and 139A.

An abnormal axonal transport of some glycoforms of PrP during scrapie could cause changes in the glycopattern and accumulation of PrP in retina. PrPsc is transported by fast axonal transport in neurons (8), but no changes in the distribution of neuronal PrP have been conclusively shown in scrapie-infected mouse. In transgenic mice, mutation of the first glycosylated site caused an accumulation of PrP in neuronal cell bodies supporting the view that changes in glycosylation are linked to impaired transport of PrP in neurons (20). Moreover, disturbance in the glycosylation process that could cause alterations in the transport and distribution of PrP has been described during PrPsc formation in cell culture experiments (13). Alterations in the trafficking of PrP have direct implications in the pathogenesis of prion diseases. An impaired axonal transport might cause, or be a consequence of, the degeneration of synaptic terminals, which is detected early in the disease (5). A putative linkage between impaired transport of PrP and the slow progression of prion diseases through neuroanatomical pathways is tempting. It remains to be determined whether or not abnormal distribution of other neuronal proteins and general impairment of the axonal transport system occur during scrapie incubation.

In summary, we have shown that the glycopattern and distribution of PrP differ in particular regions of the neuron in normal rodents. In murine scrapie, we found that an accumulation of PrP concomitant with changes in its glycopattern occurs in neuronal cell bodies in murine scrapie caused by ME7 and 139A. We propose that these changes are part of the pathogenesis of neuronal death in prion diseases.

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