Influence of Mabs on PrP\textsuperscript{Sc} Formation Using \textit{In Vitro} and Cell-Free Systems

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Abstract

PrP\textsuperscript{Sc} is believed to serve as a template for the conversion of PrP\textsuperscript{C} to the abnormal isoform. This process requires contact between the two proteins and implies that there may be critical contact sites that are important for conversion. We hypothesized that antibodies binding to either PrP\textsuperscript{C} or PrP\textsuperscript{Sc} would hinder or prevent the formation of the PrP\textsuperscript{C}–PrP\textsuperscript{Sc} complex and thus slow down or prevent the conversion process. We compared the effect of different antibodies on PrP\textsuperscript{Sc} formation: (i) neuroblastoma cells persistently infected with the 22L mouse-adapted scrapie stain, and (ii) protein misfolding cyclic amplification (PMCA), which uses PrP\textsuperscript{Sc} as a template or seed, and a series of incubations and sonications, to convert PrP\textsuperscript{C} to PrP\textsuperscript{Sc}. The two systems yielded similar results, in most cases, and demonstrate that PrP\textsuperscript{-specific monoclonal antibodies (Mabs) vary in their ability to inhibit the PrP\textsuperscript{C}–PrP\textsuperscript{Sc} conversion process. Based on the numerous and varied Mabs analyzed, the inhibitory effect does not appear to be epitope specific, related to PrP\textsuperscript{C} conformation, or to cell membrane localization, but is influenced by the targeted PrP region (amino vs carboxy).

Introduction

Prion diseases are a group of fatal neurodegenerative disorders that are associated with conformational conversion of the cellular prion protein, PrP\textsuperscript{C}, which is mainly \alpha-helical with very few beta sheets, into a \beta-sheet-rich form, PrP\textsuperscript{Sc} [1–5]. The mechanism by which PrP\textsuperscript{C} is converted to the abnormal isoform is still not clear, but it is presumed to involve a PrP\textsuperscript{C}–PrP\textsuperscript{Sc} complex, with the latter serving as a conformational template [6]. In this model, PrP\textsuperscript{Sc} serves as a template that binds to PrP\textsuperscript{C} and produces a conformational conversion into the abnormal isoform. This raises the issue of whether there are critical contact sites that mediate conversion. If this is the case, interfering with or blocking complex formation should prevent the PrP\textsuperscript{C} to PrP\textsuperscript{Sc} conversion process. Previous reports have described anti-PrP antibodies that can stop or hinder the conversion process add reference 44 and renumber [7–14].

Protein misfolding cyclic amplification (PMCA) is an assay that mimics the PrP\textsuperscript{Sc} propagation process under cell-free conditions. In this method PrP\textsuperscript{Sc} is amplified by converting PrP\textsuperscript{C} to a PrP\textsuperscript{Sc} seed during incubation with periodic sonication [13]. PrP\textsuperscript{Sc} generated by PMCA is infectious in wild-type animals [16] and can be indefinitely propagated while preserving the properties of the original PrP\textsuperscript{Sc} strain [16–18]. Furthermore, PMCA has been quite useful in studying the cofactors that influence PrP conversion [19–26], and in detecting PrP\textsuperscript{Sc} from biological samples of humans and animals [17,27–34].

We hypothesized that antibodies binding to PrP\textsuperscript{C} and/or PrP\textsuperscript{Sc} might hinder or prevent the formation of the PrP\textsuperscript{C}–PrP\textsuperscript{Sc} complex and thus prevent the conversion process. We compared the effect of individual PrP\textsuperscript{-specific monoclonal antibodies (Mabs) on the PrP\textsuperscript{C}–PrP\textsuperscript{Sc} conversion process using both an N2a/22L cell culture model and the test-tube PMCA system. Our results demonstrate that the Mabs have a range of inhibitory effects on the PrP\textsuperscript{C}–PrP\textsuperscript{Sc} conversion process. The degree of inhibition is Mab specific and more dependent on the antibody targeting region than on the specific epitope being recognized. Furthermore, since the PMCA-based method is dose-dependent and rapid, it may serve as an ideal screening assay for potential inhibitors of both PrP\textsuperscript{Sc} accumulation and the progression of prion diseases.

Methods

Animals (Ethics Statement)

All procedures involving animals and their care were conducted in accordance with the United States Department of Agriculture Animal Welfare Act and the National Institute of Health policy on Humane Care and Use of Laboratory Animals. Tissue samples from uninfected and prion agent-infected mice and hamsters were obtained using protocols approved by the Institutional Animal Care and Use Committee of the SUNY Downstate Medical Center (protocol #’s 07-250-09 and 07-251-09).
PMCA and Western Blotting

A 10% normal hamster brain homogenate (NBH) was prepared in phosphate buffered saline (PBS) containing 1% Triton X-100, 4mM EDTA and 1% protease inhibitor cocktail (Abcam). PrP-specific Mabs were generated against recombinant (murine or hamster) PrP or brain-derived proteinase K (PK)-resistant purified PrPSc [35] from brains of clinical mice infected with the ME7 mouse-adapted scrapie strain or clinical hamsters infected with the 263K hamster-adapted scrapie strain. The Mabs used in this study were purified (Montage Antibody Purification kit; Millipore, Billerica, CA), isotypep (ELISA Mouse Antibody Isotyping kit; Thermo Fisher, Rockford, IL), and epitope mapped (Table 1). The immunoreactivity of all the Mabs were analyzed on western blots against denatured, PK-digested and undigested PrP derived from uninfected and infected brain homogenates as well as by ELISA against recombinant PrP. With the exception of Mab 3F4, each of the individual Mabs had equivalent immunoreactivity against murine and hamster PrPSc on an immunoglobulin concentration basis. All of the Mabs were highly reactive against both hamster PrPSc and PrPSc isoforms and, for the PMCA studies, were individually added to the 10% NBH at a concentrations of 50 µg/ml. A 10% 263K brain homogenate was prepared in PBS only and diluted to a final concentration of 10−1. A 100 µl aliquot of this homogenate was initially combined with 10 µl of 10% NBH (with or without added Mab). Each sample was sonicated (QSONIC at 480W power, 60 Amplitude, 40,000 J energy, 90 sec process time, 3 sec pulse on−1 sec pulse off), then incubated at 37°C for 1 hr. This was defined as one cycle of serial PMCA (sPMCA). At the completion of each cycle, an additional 10 µl of 10% NBH (with or without Mab) was added. At the end of every five cycles, 100 µl of the total volume was transferred to a new tube containing an equal volume of 10% NBH (with or without Mab) and the cycling reactions continued. At the completion of 40 cycles (sPMCAa), 500 µl from each sample was PK-treated (100 µg/ml final concentration, 50°C, 30 min), followed by the addition of protease inhibitor cocktail. The sample was heated (100°C, 10 min) and then centrifuged at 16,000 x g for 2 min at room temperature. The supernatant was combined with 6X Laemml sample buffer, and 50 µl was electrophoresed in a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to nitrocellulose membrane. The membrane was blocked for 1 hr in PBS containing 0.1% Tween 20 (PBST) with 5% non-fat dry milk and incubated with 2 µg/ml biotinylated Mab 08-6/2F11. The membrane was washed 3 times (10 min each) with PBST, incubated for 60 min in HRP-conjugated streptavidin (Invitrogen) (1:5000 in PBST containing 5% non-fat dry milk) followed by 3 additional PBST washes and detection of proteins with ECL Supersignal West Dura kit (Thermo Fisher). Quantification of PrPSc was performed by densitometric analysis using NIH Image J software.

Epitope Mapping

Cellulose membranes spotted with 99 overlapping 13-mer PrP peptides were produced as previously described [36]. The membranes were blocked with 5% non-fat dry milk/tris-buffered saline containing 0.1% Tween 20 (TBST) probed with antibody diluted 1:5000 in 1% normal goat serum/TBS at 4°C overnight, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary (Cappel 55570) for 2 hours at room temperature, and detected using Millipore Immobilon Western chemiluminescent HRP substrate (Cat WBKLS0500). Membranes were regenerated for re-use by shaking with dimethylformamide for 30 minutes, then 8M urea/50mM Tris-HCl pH 8.0/1% β-mercaptoethanol (β-MC)/1%SDS overnight at 37°C, followed by a 30 min wash in the same buffer, and then twice for 30 minutes each in 50% methanol/glacial acetic acid, and finally three times for 5 minutes each in methanol. After air drying membranes were stored in a sealed container at room temperature.

Table 1. Characterization of Mabs.

| Mab       | Isotype | Epitope        | Immunoreactivity |
|-----------|---------|----------------|------------------|
| 08-6/TE4  | (TE4)   | IgG1           | ++               |
| 01-7/10E4 | (10E4)  | IgG1           | ++               |
| 08-1/SD6  | (SD6)   | IgG2b          | ++               |
| 08-1/11F12| (11F12) | IgG2b          | ++               |
| 3F4       |         | IgG2a          | −−               |
| 03-9/EE11 | (EE11)  | IgG2a          | ++               |
| 02-3/3A2  | (3A2)   | IgG1           | ++               |
| 08-1/EE9  | (EE9)   | IgG2b          | ++               |
| 01-16/1B11| (1B11)  | IgG2a          | ++               |
| 01-7/2F7  | (2F7)   | IgG1           | ++               |

Infection and Mab Treatment of N2a Cells

Murine neuroblastoma N2a cells (ATCC line CCL 131) were grown in the Minimal Essential Medium supplemented with 10% FBS, penicillin and streptomycin and infected with 2% 22L brain homogenate as described previously [12]. Following infection, the amount of PrPSc in 200 µg cell lysate aliquots of the N2a/22L cells was determined by PK digestion (1 µg/µl PK for 30 min at 37°C), SDS-PAGE on 12.5% Tris-tricine gels [37] and western blot analysis as previously described [12].

For treatment with cells and NKa cells (from the fifth passage after infection and higher) were plated in six-well plates and incubated for 48 hours at 37°C (uninfected and infected). Each experiment included both a positive control (untreated N2a/22L cells) and a negative control (N2a cells), which were subjected to PK digestion. The level of PK-resistant PrPSc was measured in western blots using HRP-conjugated sheep anti-mouse IgG as the secondary reagent and ECL Supersignal West Dura kit. Membranes were exposed to X-ray film (X-Omat Blue XB-1; Kodak, New Haven, CT) with a constant exposure time of 30 sec. The films were converted into eight-bit grayscale digital files. Quantification of PrPSc was performed by densitometric analysis using NIH Image J software.

Results

The PrP-specific Mabs that were evaluated for their ability to prevent PrPSc to PrPSc conversion have linear epitopes that span the entire prion protein from the amino to the carboxy terminus (Table 1, Fig. 1).
We used N2a cells persistently infected with the 22L mouse-adapted scrapie strain (N2a/22L) to evaluate the effect of each Mab on PrP\textsuperscript{Sc} formation (Fig. 2A and 2B). Treatment with the Mabs did not result in any cytotoxicity to the N2a/22L cells throughout the incubation period. Further, incubation of the N2a/22L cells with 10 μg/ml purified, irrelevant mouse IgG had no effect on PrP\textsuperscript{Sc} formation compared to untreated N2a/22L cultures (Figs. 2A and 2B). Mab 3F4 did not reduce PrP\textsuperscript{Sc} formation compared to control N2a/22L cultures lacking Mab. Mab 3F4 does not react with mouse prion protein so this was not surprising [38]. The ability of a singly added Mab to inhibit PrP\textsuperscript{Sc} formation was not related to a specific epitope since all of the remaining singly added Mabs inhibited PrP\textsuperscript{Sc} formation to varying degrees. Of the individually added Mabs, 5D6 was the most effective at inhibiting PrP\textsuperscript{Sc} formation (95% inhibition) while 3A2 was the least effective (38% inhibition). Targeting the amino terminus with Mab 7E4 was effective at inhibiting 73% PrP\textsuperscript{Sc} formation as was targeting the octapeptide repeat region using Mab 10E4 which resulted in almost 90% inhibition. Strangely, although their epitopes overlap, Mab 11F12 was less effective than Mab 5D6 at inhibiting PrP\textsuperscript{Sc} formation (53% vs 95% inhibition). This is in contrast to Mabs 8E9 and 1B11, which have overlapping epitopes with 8E9 being more expansive, and resulted in 52% and 42% inhibition, respectively. The combination of 5D6 and 11F12 did not result in an additive inhibitory effect and, in fact, resulted in less inhibition than either one alone. This was confirmed in studies where the addition of 8E9 to 5D6 and 11F12 caused a 45% PrP\textsuperscript{Sc} inhibition, which was slightly better than 8E9 alone, although the predicted additive inhibitory effect of 63% for the three Mab combination (48% for 8E9 plus 15% for the 5D6 and 11F12 combination) was not observed.

Studies were performed with PMCA to determine whether a cell-free system can recapitulate the effect of Mabs on PrP\textsuperscript{Sc} conversion observed in infected cells. This system also allowed us to evaluate whether accessibility of Mab to membrane associated PrP\textsuperscript{Sc} in the living cells influences the PrP\textsuperscript{Sc} to PrP\textsuperscript{Sc} conversion process. Mabs (12–50 μg/ml final concentration) were added throughout the sPMCA\textsubscript{40} protocol along with the 10% NBH spiked with a 10\textsuperscript{−4} dilution of 263K-infected hamster brain homogenate as the PrP\textsuperscript{Sc} seed and a 10% normal brain homogenate (NBH) as the source of PrP\textsuperscript{Sc}. We tested the ability of Mabs to inhibit the conversion of PrP\textsuperscript{Sc} to PrP\textsuperscript{Sc}. For each Mab, final concentrations of 1.5–50 μg/ml (lanes 3, 6, 9, 12, 15, 18, 21 and 24), 25 μg/ml (lanes 4, 7, 10, 13, 16, 19, 22 and 25) and 50 μg/ml (lanes 5, 8, 11, 14, 17, 20, 23, and 26) were prepared in hamster NBH and used in the sPMCA reactions. Compared to sPMCA\textsubscript{40}, which contained no Mab (lane 1) and with the exception of 02–3/3A2, the majority of the PrP-specific Mabs inhibited the conversion process in a dose-related manner although some were more effective than others. Mabs 7E4, 10E4, 11F12, 8E11, and 8E9 completely inhibited the conversion process at 50 μg/ml while Mabs 1B11 and 2F7 inhibited the conversion process to a lesser degree. The inhibition caused by the Mabs was a specific response since sPMCA\textsubscript{40} studies replacing Mabs with purified normal mouse IgG (at 12–50 μg/ml in the 10% NBH did not cause any inhibition of PrP\textsuperscript{Sc} formation (data not shown). It is interesting to note that, with the exception of only 8E9, the epitopes for all the Mabs that caused complete inhibition are located in the amino half of the PrP while those that caused incomplete inhibition are located in the carboxy half of PrP. There was good correlation between the extent of PrP\textsuperscript{Sc} inhibition when 10 μg/ml Mab in cell culture was compared to 12 μg/ml Mab with sPMCA\textsubscript{40}. A separate study using sPMCA\textsubscript{40} demonstrated that Mabs 3F4 and 5D6 caused complete inhibition of PrP\textsuperscript{Sc} formation at 12–50 μg/ml (Fig. 4). Therefore we extended those studies and evaluated the effects of Mabs 3F4 and 5D6 using a wider range of Mab concentrations (1.5–50 μg/ml). Compared to the other antibodies in this study, Mabs 3F4 and 5D6 had the most pronounced effects on PrP\textsuperscript{Sc} formation as demonstrated by the low concentrations of 3 and 6 μg/ml, respectively, causing complete inhibition (Fig. 4). The potent inhibitory effect of 5D6 on PrP\textsuperscript{Sc} observed using sPMCA\textsubscript{40} coincides with its dramatic effect in the N2a/22L culture model. Furthermore, the poor PrP\textsuperscript{Sc} inhibition by 3A2 with sPMCA\textsubscript{40} (Fig. 3B) corresponded well with the poor inhibition (only 32% reduction compared to negative control) observed in the cell culture system (Fig. 2A and 2B).
Discussion

Currently, there is no effective treatment for prion diseases. To date, hundreds of chemical compounds have been identified that antagonize prion propagation \textit{in vitro} in cell culture-based assays and/or \textit{in vivo} in animal studies [39–43]. Unfortunately, many compounds efficient \textit{in vitro} studies were only effective in animal models if treatment was begun before or close to the time of inoculation with the infectious agent [44]. Furthermore, many of the candidate compounds have limited usefulness clinically due to toxicity or their inability to cross the blood-brain barrier [e.g. Congo red [45], iododoxorubicin, β-sheet breakers].

Additional therapeutic and/or prophylactic strategies have been and continue to be pursued. Vaccination with recombinant mouse PrP delays the onset of prion disease in mice [46]. Passive immunization with anti-PrP antibodies was shown not only to inhibit formation of PrPSc in a cell-free system [47], but was also shown to prevent infection of susceptible N2a cells [7] and to inhibit prion replication in infected cells [8,47,48]. The effectiveness of these treatments were also dependent on when they were administered relative to the time of infection.

In an initial passive immunization study using wild-type CD1 mice, Mabs 8B4 (to mouse PrP residues 34–52) and 8H4 (to mouse PrP residues 175–185) given immediately after challenge with 139A scrapie by intraperitoneal (IP) injection (50 μg/week), resulted in a significant prolongation of the incubation period with 10% of the 8B4 treated animals remaining disease free in the group challenged with a lower dose of PrPSc [10]. In another study using higher antibody doses (4000 μg/week IP) of either ICSM 18 (to mouse PrP residues 146–158) or ICSM 35 (to mouse residues 95 to 105), prion infection from a peripheral source was completely prevented if treatment was continued for 7 or 30 days immediately following PrPSc challenge [9]. Furthermore, a transgenic mouse model that expresses Mab 6H4 is resistant to prion infection via IP injection by a mechanism that involves either perturbation of cellular PrP trafficking/PrP degradation or disruption of the PrPSc-PrP interaction [49].

Previous studies have reported that the 132–140 portion of PrP [8] or the 132–156 region of PrP [50–53] are important for the generation of PrPSc. Rigter et al. [54] found two high affinity binding regions for protein-protein interactions using ovine peptide-arrays: (i) sheep-PrP peptides 43–102, including the amino-terminal octarepeats, and (ii) sheep-PrP peptides 134–177.
which encompasses most of the scrapie susceptibility-associated polymorphisms in sheep. Moroncini et al. [55] found that residues within the 89–112 and 136–158 segments of PrP^C are key components of the PrP^C–PrP^Sc complex. Beringue et al. [11] reported that antibodies exclusively binding PrP^C were relatively inefficient inhibitors of PrP^Sc accumulation compared with antibodies that additionally recognize disease-associated PrP isoforms. Féraudet et al. [56] screened 145 anti-PrP Mabs for their capacity to inhibit PrP^Sc replication in infected N2a or Rov9 cells. They identified four different linear epitopes that hindered the PrP^C to PrP^Sc conversion: the amino terminal region 26–35, the octarepeat region 59–89, the intermediate region 97–102, and the central region 130–160. The observation that antibodies that bind to the amino terminus of the prion protein are capable of inhibiting conversion suggests that the

Figure 3. A. Western blot of 263K brain homogenate that was used as the seed for PMCA. Dilutions of the brain homogenate were prepared and either untreated (lanes 1 and 2) or PK-treated (lanes 3 and 4) prior to SDS-PAGE and western blotting. A 10−3 dilution prior to and after PK demonstrates the three protein banding pattern typical for 263K brain homogenate whereas no bands are visible at a 10−4 dilution of the same homogenate. B. Western blotting of the PMCA products following sPMCA40 in the absence and presence of PrP-Specific Mabs. Forty cycles of serial PMCA was carried out in the absence or presence of Mabs as described in the text. Each Mab was added at a final concentration of 12, 25, and 50 μg/ml. Following PK treatment, the PMCA products were subjected to SDS-PAGE, western blotted and immunostained for PrP^Sc. The protein bands were quantitated and the level of PrP^Sc inhibition, relative to the no Mab and normal mouse IgG controls, were determined.

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Figure 4. Influence of Mabs 3F4 and 5D6 on PrP^Sc formation following sPMCA40. Mabs 3F4 and 5D6 were added to sPMCA40 at final concentrations of 0–50 μg/ml. The PMCA products were PK treated and western blotted. The PrP^Sc was quantitated and the level of PrP^Sc inhibition was determined relative to control reactions.

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endogenous proteolytic cleavage occurs after the site of conversion.

To more completely explore the possible therapeutic effect of anti-PrP antibodies, and to establish another system to analyze the influence of Abs on the conversion process, we screened Mabs prion-resistant on our laboratory for their capacity to inhibit PrP^Sc formation. This screening was performed using N2a/22L cells and cell-free sPMCA. In N2a/22L cultures, all Mabs that react with mouse PrP reduce PrP^Sc formation although with varying efficiency. Thus, similar to previous results [55], we found that the ability to inhibit PrP^G to PrP^Sc conversion was not restricted to a single epitope or limited to a specific region of the protein. However, the greatest inhibition was observed with Mabs that targeted epitopes in the amino terminal, unstructured region of the PrP. The greatest inhibition in the N2a/22L cells was with Mab 5D6. This is consistent with a prior study using Mab 6D11 (anti-PrP residues 95–105) which in a screen of multiple Mabs, only one produced the greatest inhibition (~100%) [12]. Mab 6D11 has also been shown to have some efficacy in vivo prolonging the pre-symptomatic incubation period [57]. The Mab inhibition results obtained using PMCA were similar to that found in the cell culture system. PMCA has the advantages over the cell culture model of being cost-effective, simple, rapid, sensitive, and more amenable to studies of dose dependence. For identification of potential candidate Mabs that might have in vivo activity it is likely that such Mabs would have to produce 90 to 100% inhibition in the much simpler in vitro systems.

The interaction of PrP^G to PrP^Sc is critically dependent on the structural compatibility of the molecules as supported by the existence of a species barrier for prion infection, related to minor structural compatibility of the molecules as supported by the.

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