Targeting Prion Amyloid Deposits In Vivo

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Abstract. The diagnosis of prion diseases in humans is challenging due to a lack of specific and sensitive non-invasive tests. Many forms of human prion disease including variant Creutzfeldt-Jakob disease (vCJD), Gerstmann-Sträussler-Scheinker (GSS) syndrome, and 10% of sporadic CJD cases are associated with amyloid deposition. Several positron emission tomography (PET) ligands have recently been developed to directly image β-amyloid associated with Alzheimer disease. One of them, methoxy-X04, is a fluorescent derivative of Congo red with high binding affinity toward amyloid fibrils and good blood-brain barrier permeability. Using methoxy-X04, we investigated whether amyloid-targeting ligands can be also employed for direct imaging of amyloid deposits associated with some prion diseases. Such a method could potentially become a novel diagnostic approach for these conditions. Studies were performed on MB mice infected with the 87V mouse-adapted scrapie strain. Labeling of PrP amyloid plaques in brains of presymptomatic and symptomatic mice was demonstrated using in vivo transcranial two-photon microscopy after systemic administration of methoxy-X04. During real-time imaging, PrP amyloid deposits could be clearly distinguished 15 min after intravenous administration of methoxy-X04. The ligand showed rapid clearance from brain areas that did not contain amyloid deposits. PrP amyloid deposits could also be detected by direct application of methoxy-X04 on cerebellar sections from GSS patients. These results suggest that methoxy-X04 or similar derivatives could be used as PET imaging agents to improve the diagnosis of human prion diseases associated with amyloid deposition.

Key Words: Amyloid; Imaging; Methoxy-X04; Prion; Scrapie; Two-photon microscopy.

INTRODUCTION

Prion diseases are invariably fatal neurodegenerative disorders in which the key pathological event is a conversion of prion protein (PrP<sup>C</sup>; C-for cellular) into a highly toxic and infectious conformer PrP<sup>Sc</sup> (Sc-for scrapie) (1, 2). Currently, the diagnosis of prion disease is based primarily on clinical symptoms and the course of disease, as well as the presence of family history when an inherited form is suspected. Magnetic resonance imaging, electroencephalograms, and testing for tau and 14-3-3 proteins in the cerebrospinal fluid have supportive roles, and their individual sensitivity and specificity are limited (3, 4). Confirmation of the ante-mortem diagnosis can only be achieved through brain biopsy (5). Therefore, a non-invasive diagnostic test with high sensitivity and specificity could improve the accuracy of prion disease diagnosis. The urgency for developing reliable diagnostic approaches for these diseases is increased by the transmissibility of bovine spongiform encephalopathy (BSE) to humans (6), resulting in the emergence of variant Creutzfeldt-Jakob disease (vCJD) (7). It is estimated that several thousand individuals are infected with BSE (8) and have become asymptomatic carriers who will likely develop disease in the future (9). These carriers also constitute a reservoir of disease that can spread through blood transfusion and organ transplantation (10).

The conformational transformation of PrP<sup>C</sup> to PrP<sup>Sc</sup> is associated with increased β-sheet content (11), which renders PrP<sup>Sc</sup> highly resistant to degradation. The accumulation of PrP<sup>Sc</sup> on cell membranes (12) and in lysosomes (13, 14) initiates degeneration of central nervous system (CNS) neurons, resulting in spongiform encephalopathy (15). Like β-amyloid protein (Aβ) in Alzheimer disease (AD), partially cleaved and secreted PrP<sup>Sc</sup> fragments may form amyloid deposits in the brain parenchyma (16). Abundant amyloid deposits are a pathological hallmark of vCJD, Gerstmann-Sträussler-Scheinker syndrome (GSS), and kuru. They are also present in a type of sporadic CJD (sCJD) that accounts for about 10% of all CJD cases (12). Although amyloid deposits associated with prion diseases and amyloid deposits in AD are composed of different peptides, they...
Fig. 1. PrP amyloid deposits in MB mice infected with 87V mouse adapted scrapie strain. Numerous deposits in the motor cortex (A) and a giant deposit in the hippocampus (B) are seen detected by anti-PrP immunohistochemistry. PrP amyloid deposits labeled with methoxy-XO4, following in vivo ip injection, are seen in the in the motor cortex (C), the entorhinal/perirhinal cortex region (D), and in the hypothalamus directly under the ependyma of the 3rd ventricle (E). Double immunofluorescent staining of the hypothalamic deposits with anti-PrP mAb and Cy3 is seen in panel (F). Abbreviations: 3rd Vent, the third ventricle; CC, corpus callosum; Ent Ctx, entorhinal cortex; Hyp Th, hypothalamus; PRh Ctx, perirhinal cortex; Rh Sul, rhinal sulcus; Str Or, stratum oriens of the hippocampus. Scale bars: A, C, D = 40 μm; B = 10 μm; E, F = 20 μm.

have ultrastructural morphological similarities and share common physical-chemical properties (17). Due to their high β-sheet secondary structure, amyloid plaques in both diseases bind Thioflavin-S and show apple-green birefringence when stained with Congo red and examined under polarized light (18).

Recently, several derivatives of Thioflavin-S and Congo red have been developed as positron emission tomography (PET) ligands to directly image β-amyloid in AD (19, 20). We suggest that a similar concept can be applied for prion diseases associated with amyloid deposition. An example of such newly developed conformation specific ligands designed for PET imaging is methoxy-X04 (1,4-bis(4′-hydroxystyryl)-2-methoxybenzene), a derivative of Congo red and Chrysamine-G that has a reduced molecular weight and contains no acid groups, making it more lipophilic and blood-brain barrier (BBB) permeable (20, 21). Methoxy-X04 retains a high affinity toward β-pleated sheet amyloid fibrils and can be easily synthesized with carbon-11 (11C), making this compound a viable probe for PET studies (21). We used methoxy-X04 as a representative of BBB-permeable, conformation-specific ligands to demonstrate the feasibility of in vivo targeting of amyloid plaques composed of PrPSc. Methoxy-X04 is fluorescent, allowing for direct detection of deposits by in vivo transcranial two-photon microscopy. Following systemic injection of this ligand, numerous cortical plaques were visualized in the cortex of living, pre-symptomatic and symptomatic MB mice infected with the 87V mouse-adapted scrapie strain. The specificity of methoxy-X04 binding to PrP amyloid plaques was confirmed by triple fluorescent staining using anti-PrP monoclonal antibodies (mAb) and Thioflavin-S. In addition, numerous multicentric PrP amyloid deposits in cerebellar sections of GSS patients could be labeled by direct application of methoxy-X04. These observations may lead to the development a new sensitive and reliable ante-mortem diagnostic test for prion diseases associated with amyloid deposition.
Fig. 2. Shows characterization of methoxy-X04 labeling of PrP amyloid deposits using multi-channel fluorescent microscopy (A, D, G, J: filter set A; B, E, H, K: filter set I3; C, F, I, L: filter set Y3. Scale bar = 25 μm). A–C: Section from a control 87V-infected MB mouse, which did not receive a methoxy-X04 injection, was immunostained with anti-PrP mAb/Cy3. No amyloid deposits were seen using filter sets A (A) or I3 (B). Plaque immunostaining is seen only using filter set Y3, showing immunoreactivity with anti-PrP mAb (C). D–F: Section from an 87V-infected MB mouse following ip methoxy-X04 injection. PrP amyloid plaques are detected using filter set A (D). No staining is seen with filter set I3 (E) or with filter set Y3 (F), since Thioflavin staining and anti-PrP mAb immunolabeling were not done on this section. G–I: Section from an 87V-infected MB mouse following ip methoxy-X04 injection. Double immunofluorescent staining demonstrates colocalization of methoxy-X04 labeling (G, filter set A) with anti-PrP immunostaining (I, filter set Y3). No labeling is seen in panel (H) since Thioflavin staining was not done on this section. J–L: Section from an 87V-infected MB mouse following ip methoxy-X04 injection. Triple fluorescent staining shows the same plaque stained with methoxy-XO4 (J, filter set A), Thioflavin-S (K, filter set I3), and anti-PrP mAb/Cy3 (L, filter set Y3).

MATERIAL AND METHODS

Animals

MB mice infected with 87V mouse adapted scrapie strain are a well-established model of transmissible prion disease associated with the formation of amyloid plaques composed of PrP fragments (22–24). Infection invariably leads to death in all animals if the disease is allowed to progress (25–27). Six-week-old MB mice were inoculated by intra-cerebral injection of 25 μl, 1% (w/v) brain homogenate from a terminally...
sick 87V-infected MB mouse as previously described (25). All animals were housed in an animal colony with 12/12 hours light/dark cycle and ad libitum access to food and water. Starting from week 35 post-inoculation, mice were evaluated weekly for the first symptoms of prion infection using an apparatus containing a series of parallel bars (3 mm in diameter) placed 7 mm apart. The earliest detectable clinical symptoms of CNS involvement included an impaired activity level and competency when mice attempt to cross a series of parallel bars. Testing was performed by an observer blinded to the animal’s experimental status. An animal was considered clinically symptomatic if it scored positive for disease for 3 weeks in a row. This method of early detection of symptomatic prion infection in mice has been validated and widely used by the authors previously (28–31). For 87V-infected MB mice, the incubation period (i.e., time from inoculation until a mouse scored positive for disease) ranged from 43 to 45 weeks (25).

Human Subjects

Eight-µm-thick paraffin cerebellar sections from 4 fully characterized GSS cases were obtained from the National Prion Disease Pathology Surveillance Center (Case Western Reserve University, Cleveland, OH) for use in this study.

Preparation of Methoxy-XO4

Methoxy-XO4 was synthesized as previously described (21), and the purity was determined by high pressure liquid chromatography (HPLC) to be >90%. For direct tissue staining, a 100-µM solution of methoxy-XO4 in 40% ethanol and 60% H2O (pH 10 adjusted with NaOH) was prepared. For in vivo imaging studies, methoxy-XO4 was diluted in a mixture of 10% DMSO, 45% propylene glycol, and 45% phosphate-buffered saline (pH 7.5; 0.01 M; 5 mg/ml) for intra-peritoneal (ip) administration, or in physiological saline (pH 12 adjusted with NaOH; 1 mg/ml) for intravenous (iv) administration.

Tissue Processing and Staining

Four symptomatic 87V-infected MB mice and 4 non-infected MB mice received ip injections of methoxy-XO4 (10 µg/gram). They were killed 24 hours later by ip injection of sodium pentobarbital (150 mg/kg). An additional 4 symptomatic and 4 non-infected mice were killed without injection of ligand. All animals were transcardially perfused with 20 ml of 0.1 M phosphate buffered saline (pH 7.4) with the addition of heparin followed by 80 ml of 4% formaldehyde in 0.1 M phosphate buffer. The brain was removed from the skull, cryoprotected using increasing concentrations of sucrose, and cut into serial, 40-µm-thick coronal sections on a freezing microtome (Leica SM2400, Nussloch, Germany). Anti-PrP immunohistochemistry was performed on free-floating sections. Endogenous peroxidase activity was quenched in a bath with 0.3% H2O2 for 30 min. Non-specific staining was blocked by incubation in the blocking solution provided as a part of the Vector Elite staining kit (“Mouse on Mouse” kit; Vector Laboratories, Burlingame, CA). Since the amyloid in 87V-infected MB mice consists of a variety of PrP fragments, the primary antibody solution contained a mixture of mAbs raised against synthetic peptides corresponding to different fragments of murine PrP: 8B4 (residues 34–45), 11G5 (residues 115–130), 8H4 (residues 175–185), and 8F9 (residues 220–231) (32–34). The concentration of all mAbs was 0.25 mg/ml, with the total concentration of protein in a stock solution of 1 mg/ml as assessed by a Coomassie colorimetric assay. A 1:2,000 dilution of the stock solution was used. The mixture of primary antibodies was applied for 30 min, followed by thorough washing and the anti-mouse IgG biotinylated secondary antibody according to the manufacturer’s instructions. For fluorescent microscopy, sections were incubated with streptavidin conjugated with Cy3 (1:400; Sigma, St. Louis MO), whereas for light microscopy they were incubated with avidin-horseradish peroxidase complex and developed using 3,3′-diaminobenzidine kit with nickel ammonium sulfate (Vector Laboratories).

Some sections were optionally stained with Thioflavin-S, allowing for simultaneous demonstration of triple labeling that included methoxy-XO4, Cy3, and Thioflavin-S using a fluorescent microscope with different filter combinations.

Sections from animals that did not receive ip injections of methoxy-XO4 were immunostained with anti-PrP mAb and DAB for light microscopy, or double stained with anti-PrP mAb and Cy3, followed by either Thioflavin-S or methoxy-XO4, for multiple fluorescent detection. Direct staining with methoxy-XO4 was performed by immersion of slides in an ethanol solution of this dye for 10 min and differentiation using 80% ethanol in 0.2% NaOH for 2 minutes. A separate set of sections stained only with Thioflavin-S or methoxy-XO4 with or without 30-min pretreatment with 80% formic acid.

Sections from GSS patients were deparaffinized and stained with Thioflavin-S, methoxy-XO4, or immunostained. For immunohistochemistry, sections were pretreated with 80% formic acid for 15 min (35). Endogenous peroxidase activity was quenched by 30-min treatment with 0.3% H2O2 in methanol. Sections were then washed and blocked with 10% fetal bovine serum for 30 min. PrP plaques were detected using a 1:1,000 dilution of 78295 rabbit polyclonal anti-PrP antibody (provided by Dr. R. Kascak, New York State Institute for Basic Research, Staten Island, NY), followed by biotinylated anti-rabbit secondary antibody (1:2,000; Vector Elite staining kit, Vector Laboratories) and avidin-horseradish peroxidase complex. The color reaction was developed using a 3,3′-diaminobenzidine kit with nickel ammonium sulfate (Vector Laboratories).

Fluorescent detection was performed using a Leica DMLB fluorescence microscope. Staining with methoxy-XO4 was detected using filter set A (excitation 340–380 nm, dichroic mirror 400 nm, 425 nm long pass emission filter). Thioflavin-S with filter set I (excitation 450–490 nm, dichroic mirror 510 nm, and 515 nm long pass emission filter), and anti-PrP immunohistochemistry with Cy3 fluorochrome using a filter set Y3 (excitation 535/50, dichroic mirror 565 nm, and 610/74 emission filter).

In Vivo Detection of Prion Amyloid Plaques Using Transcranial Two-Photon Microscopy

This experiment was performed on symptomatic and pre-symptomatic 87V-infected MB mice. Pre-symptomatic mice were between 35 and 38 weeks following inoculation and did not display any neurological signs of disease. Three symptomatic and 3 pre-symptomatic mice received an ip injection of methoxy-XO4 (10 µg/gram of body weight) 24 hours prior to
imaging. Controls consisted of 2 non-infected MB mice that had received an injection of the ligand and 2 infected and symptomatic MB mice that did not receive the ligand. For in vivo transcranial two-photon microscopy imaging, animals were anesthetized by ip injection of Ketamine HCl 0.12 mg/gram of body weight and Xylazine 0.016 mg/gram diluted in distilled water (36). Under aseptic conditions, the skin over the skull was opened with a midline excision and the periosteum was removed. A 2-mm window in the skull was opened at a point located 1 mm posterior to the bregma and 1 mm laterally from the midline suture. Bone was carefully thinned using a high-speed drill (Fine Science Tools, Foster City, CA). To avoid damaging the underlying cortex by friction-induced heat, a cold sterile saline solution was administered to the skull periodically and drilling was intermittently halted to permit heat dissipation. Using a micro-surgical blade, the skull thickness was further reduced to approximately less than 30 μm. Respiration-induced movements during imaging were reduced by fixing the skull with a cyanoacrylic glue (no. KG-585; Elmer Products, Inc., Columbus, OH) to a custom-made, 400-μm-thick stainless steel plate with a central opening for skull access. The plate was screwed to 2 lateral bars located on both sides of the mouse head and fixed to a metal base (36). In vivo imaging was performed using an MRC-1024m Bio-Rad Multiphoton system (Bio-Rad, Hercules, CA) equipped with long working distance objectives ×10 and ×60. Two-photon fluorescence was generated with 750 nm excitation from a mode-locked Ti:Sapphire laser (Tunami, Spectra-Physics, Mountain View, CA). Light waves emitted by methoxy-XO4 were collected in the range of 380 to 480 nm. Three-dimensional volumes were acquired by a stack of x-y sections starting at the surface of the thinned skull to a 200-μm depth inside the cortex (21, 36, 37). Following in vivo two-photon imaging, animals were killed by ip injection of sodium pentobarbital and transcardiac perfusion as described above.

Real-time observations of methoxy-XO4 brain entry and labeling of plaques was performed in 3 symptomatic and 3 presymptomatic prion-inoculated mice. In addition to making a bone window, these mice had their femoral vein cannulated with P-10 polyethylene tubing (Warner Instruments Inc., Hamden, CT). Mice were placed on a microscope stage in a prone position and imaged prior to ligand administration (T = −1 min). Methoxy-XO4 (5 μg/gram) was administered through an extension attached to the intravenous cannula. Images were acquired at T = 0, 1, 4, 10, 15, 25, 35, 45, and 60 min. The technical details of imaging were as described above. Following imaging, animals were killed by ip injection of sodium pentobarbital and transcardiac perfusion as described above.

RESULTS

Labeling PrP Amyloid Deposits in Mouse Tissue

Numerous plaques immunoreacting with anti-PrP mAb could be demonstrated in the cortex and in the hippocampus of MB mice infected with the 87V scrapie strain (Fig. 1A, B). Plaques were detected in all neurologically symptomatic mice and in pre-symptomatic mice killed after 35 weeks post-inoculation. The density of plaques was significantly greater in symptomatic than in pre-symptomatic animals. The greatest density of plaques was observed at the junction of the circular and motor cortical areas (Fig. 1A), as well as on the rostral frontal cross-sections through the brain, and at the junction of the retrosplenial and somatosensory cortical areas on more caudal cross-sections. The largest (giant) plaques were found in the hippocampus lined along the stratum oriens of the CA1 sector and the corpus callosum/alveus (Fig. 1B). Large aggregates of plaques making nearly continuous linear amyloid deposits were observed in the hypothalamus directly under the ependyma of the third ventricle (Fig. 1E, F). These observations are consistent with the previously described distribution of PrP amyloid plaques in 87V-infected MB mice (23, 24). In addition, diffuse staining in the neuropil, especially in the hippocampus, septum, and in poorly delineated patchy areas of the cortex, was seen with immunohistochemistry, especially on light microscopy preparations stained with DAB. This diffuse staining most likely represents high levels of PrPSc in the interneuronal space, which did not form amyloid fibrils since these areas did not show increased staining with Thioflavin-S (24). Such focal accumulation of PrPSc in the hippocampus, the septum, and in the cortex is typical for accumulation of PrPSc in mouse models of prion diseases (38).

Following direct application of either methoxy-X04 or Thioflavin-S PrP amyloid plaques could be detected with fluorescent microscope using filter sets A or I3, respectively. PrP amyloid plaques did not show auto-fluorescence with these filter sets (Fig. 2A, B). No staining with either methoxy-X04 or Thioflavin-S was observed if sections were pretreated with formic acid, which destroys the β-sheet structure of amyloid fibrils that binds both dyes. Because methoxy-X04 is BBB permeable, labeling of plaques could be obtained by injecting the ligand into living animals. In mice killed 24 hours following ip injection of methoxy-X04, numerous amyloid plaques were labeled in the cortex, the hippocampus, and in the hypothalamus (Fig. 1C–E). Like Thioflavin-S, methoxy-X04 did not stain diffuse amorphous PrP deposits, which were seen only with anti-PrP immunohistochemistry. The specificity and sensitivity of plaque labeling by methoxy-X04 could be demonstrated using multiple fluorescent labeling (Fig. 2). Sections from the brain where plaques were labeled with ip methoxy-X04 injection were subsequently stained with anti-PrP mAB/Cy3. Double staining of plaques with methoxy-X04 and anti-PrP mAB could be confirmed using multichannel fluorescent microscopy. Virtually all plaques labeled with methoxy-X04 also immunoreacted with anti-PrP mAB (Fig. 2G–I). Conversely, all clearly defined PrP amyloid deposits (but not...
diffuse parenchymal staining) that were detected by immunohistochemistry were also labeled with methoxy-X04, indicating the high sensitivity of this ligand. In triple-stained sections, deposits already labeled with anti-PrP mAbs and methoxy-X04 were also stained with Thioflavin-S, confirming their amyloid composition (Fig. 2J–L).

Staining of PrP Amyloid Deposits in Human Tissue

The affinity of methoxy-X04 to amyloid deposits composed of human PrPSc fragments was examined on cerebellar sections from GSS patients. Directly applied methoxy-X04 labeled numerous amyloid deposits in all layers of the cerebellar cortex (Fig. 3). Giant, multicentric amyloid plaques typical for GSS were seen in the molecular layer (Fig. 3A, B). These multicentric plaques were also stained with Thioflavin-S or with anti-PrP immunohistochemistry. Because immunostaining of amyloid associated with human paraffin embedded prion diseases requires pretreatment with formic acid to unmask epitopes, double immunofluorescent staining could not be performed on the sections from GSS patients.

In Vivo Targeting of PrP Amyloid Plaques Using Transcranial Two-Photon Microscopy

PrP amyloid deposits labeled with methoxy-X04 could be directly imaged in vivo using transcranial two-photon microscopy. Two types of experiments were performed: 1) imaging of plaques 24 h after ip administration of the ligand, and 2) real-time observation of plaque labeling following iv ligand administration. Both in symptomatic and in pre-symptomatic 87V-infected MB mice that received ip injection of methoxy-X04 24 h before imaging, numerous plaques could be seen in the motor cortex through the thinned skull bone (Fig. 4A). There were no other structures labeled with methoxy-X04. Topical administration of a 0.005% solution of Thioflavin-S (applied by gently elevating the bone on one side of the imaging window) resulted in increased signal intensity of already labeled plaques, but not in the appearance of new lesions. No plaques were seen in 87V-infected MB mice that did not receive the ligand, or in non-infected MB mice that did receive the ligand. All animals subjected to in vivo imaging were killed, their brains were cut, and sections were stained with Thioflavin-S and anti-PrP mAb/Cy3. These were analyzed using a multichannel fluorescent microscope as described above. Triple fluorescent staining demonstrated that these same plaques detected with methoxy-X04 also immunoreacted with anti-PrP mAb and were stained with Thioflavin-S.

Real-time imaging experiments were performed to demonstrate the dynamics of plaque labeling with methoxy-X04 and clearance of the non-specific distribution of the ligand. Prior to iv ligand administration, no deposits could be detected by transcranial two-photon microscopy (T = −1 min). Images taken 1 min after ligand administration showed strong fluorescence in the brain’s arterial vessels followed by a blush of non-specific staining of brain parenchyma, which had a peak intensity at T = 4 min. Although fluorescence in large vessels remained strong, the background fluorescence started to decrease over the following minutes and the first amyloid deposits could be clearly imaged at T = 15 min (Fig. 4B). The observations were repeated at T = 25 min, 35 min, 45 min, and 60 min. A further decrease in background intensity was observed over this time. The intensity of plaques labeling remained strong. Similar results were obtained in both symptomatic and pre-symptomatic 87V-infected MB mice. Following real-time imaging animals were killed and brain sections were stained and analyzed as described above.

Although animals in our experiment received only a single dose of methoxy-X04 and survival was limited to 24 h, they were monitored for possible signs and symptoms of ligand toxicity (39, 40). Within the 24-h observation period (from injection until the moment they were killed) no changes were noted in physical appearance, unprovoked behavior or response to external stimuli in animals. In addition, hematoxylin and eosin-stained sections of the heart, liver, spleen, or kidneys did not reveal any pathology.

DISCUSSION

The BBB-permeable derivatives of Congo-red (19, 21), such as methoxy-X04 and the Thioflavin derivative, Pittsburgh Compound B (PIB) (41, 42), were developed to target Aβ deposits with PET and improve the diagnostic accuracy of AD (20, 43, 44). In this paper we have demonstrated that this concept can be extended to prion diseases associated with PrPSc amyloid deposition. Following peripheral administration of methoxy-X04, numerous plaques were labeled and could be imaged in vivo in 87V-infected MB mice using transcranial two-photon microscopy. Deposits were detected both in symptomatic and presymptomatic prion-infected mice. The real-time imaging experiments demonstrated that methoxy-X04 rapidly enters the brain of prion-infected mice and has rapid clearance from brain areas that do not contain the targeted binding site, which are PrP amyloid deposits. Multiple fluorescent staining with anti-PrP mAb and Thioflavin-S specifically confirmed that methoxy-X04-labeled lesions contained PrP protein organized in amyloid fibrils. This demonstrates the feasibility of using BBB-permeable, conformation-specific ligands as imaging agents to improve the ante-mortem diagnosis of prion diseases associated with amyloid deposition.

Due to the occurrence of BSE in Europe, Asia, and North America, a significant portion of the human population are at risk of contracting vCJD. Although, so far...
Fig. 3. Shows staining of PrP amyloid deposits in cerebellar sections of GSS patients with methoxy-X04. A: Numerous deposits could be visualized in the cerebellar cortex following direct application of methoxy-X04. Arrows indicate autofluorescence of 2 remaining Purkinje cells. High magnification of classical multicentric GSS amyloid plaque stained with methoxy-X04 (B), Thioflavin-S (C), and anti-PrP immunohistochemistry (D). Abbreviations for layers of the cerebellar cortex: gr, granular layer; mo, molecular layer; pu, Purkinje cell layer. Scale bars: A = 100 μm; B–D = 10 μm.

only about 150 vCJD cases have been reported, it is estimated that at least several thousand Britons could be infected with BSE (8). Human susceptibility to prion infection is determined by multiple genes, resulting in variable incubation times; hence, asymptomatic carriers of vCJD with greater resistance to infection may develop symptoms of the disease in the future (9). An additional concern is that these carriers constitute a reservoir of disease that can be further spread through organ transplant or blood transfusion. The first possible transmission of vCJD by blood transfusion was recently reported in the UK (10). Therefore, the incidence of human prion disease may increase in the future.

Currently, no specific and sensitive non-invasive tests for prion disease exist. The clinical diagnosis in symptomatic patients is made based on disease course and the combined results of electrophysiological, biochemical, and MRI studies, which individually have modest sensitivity and specificity (4, 45). A combination of all this information leads to an accurate diagnosis in the advanced stages of the disease in a majority of cases; however, only brain biopsy achieves a 95% level of ante-mortem diagnostic accuracy (5). On the other hand, a correct diagnosis in the early stages is often missed. Direct imaging of amyloid could provide an opportunity to improve the diagnostic accuracy of prion diseases associated with amyloid deposits. In fact, abundant PrP amyloid deposits are a pathological hallmark of virtually all cases of vCJD (16) and GSS, especially in kindreds associated with the most common P102L PrP mutation (46, 47). Making the correct diagnosis in both diseases is challenging and very often symptomatic subjects are either undiagnosed or may bear the wrong diagnosis for a long period. vCJD has an extended prodromal phase when patients routinely report non-specific sensory symptoms and experience psychiatric problems, including depression, mood instability, psychosis, and discrete cognitive symptoms (48). These signs and symptoms with an otherwise
normal neurological examination, normal spinal tap, and MRI often lead to an erroneous psychiatric diagnosis (48). The suspicion of vCJD is raised when the picture of more profound cognitive impairment associated with ataxia and myoclonus emerges. In GSS, where the disease course is dominated by progressive cerebellar dysfunction (47), patients may undergo extensive and expensive genetic workup for various genetic defects related to a broad category of the spinocerebellar ataxias before they are properly diagnosed. If a brain biopsy or autopsy is not performed, some individuals may not be properly diagnosed at all. Therefore, amyloid-specific PET ligands may offer a substantial improvement in the diagnostic accuracy and sensitivity for these forms of prion diseases. The sensitivity of detection would be related to the level of PrP amyloid accumulation. Typically the amyloid burden in both diseases is large and numerous plaques can be detected. The specificity of the test will depend upon the absence of other structures that methoxy-X04 and similar ligands could bind, such as AD β-amyloid and neurofibrillary tangles. Taking into account that the average age of vCJD patients is 28 years (49) and the average age of GSS patients is between 40 and 50 years (5), any binding to PrP amyloid found using conformation-specific ligands is not likely to be confused with binding to AD amyloid, where the patients are typically older than 70 years. In addition, the regional distribution of amyloid in prion disease and AD is very distinct. Therefore, PET imaging using amyloid targeting ligands, such as methoxy-X04, will likely be a highly specific and sensitive diagnostic method for these forms of prion disease. However, this kind of diagnostic approach may not be as useful in sCJD, since PrP amyloid plaques are found in only 10% of cases and the average age of sCJD patient is 67 years, that is, when many subjects also develop Aβ plaques.

Recently, the first clinical reports describing the detection of AD β-amyloid in humans using PET and amyloid-specific ligands have been published (50). These studies were done using PIB, which was chosen from several similar compounds. Its pharmacokinetic properties appear to be slightly superior to those of methoxy-X04, although methoxy-X04 meets the requirements of a successful PET ligand candidate (21). Importantly, PIB binding to postmortem brain homogenates is dominated by the amyloid component (51), whereas methoxy-X04 retains some affinity toward neurofibrillary pathology (21). This feature favored PIB for selective targeting of amyloid in AD, but is less important for prion amyloid imaging. PIB underwent extensive toxicity testing prior to entering human trials (50). Such detailed studies have not yet been performed for methoxy-X04, but no apparent toxicity has been noted during our animal experiments or in other laboratories.

Clinical PET trials in AD using ligands for Aβ imaging are ongoing (50). Our study demonstrates that a similar approach is feasible for in vivo imaging of amyloid deposits associated with prion diseases. Therefore, this approach could improve the diagnostic accuracy of GSS or vCJD. Using in vivo transcranial two-photon microscopy we were able to demonstrate PrP amyloid deposits in infected but still presymptomatic mice. Since subjects with vCJD and GSS accumulate PrP amyloid in the initial stages of symptomatic disease, it is possible that PET ligands targeting amyloid deposits would provide a method for making an accurate diagnosis early in the disease or even in the preclinical stages.

Fig. 4. In vivo detection of PrP amyloid deposits in presymptomatic 87V-infected MB mice using transcranial two-photon microscopy. A: Numerous plaques are seen in the motor-somatosensory cortex of an 87V-infected MB mouse that received methoxy-X04 ip 24 hours prior to imaging. Arrow points to a plaque that is enlarged in the insert (×6 linear magnification). B: Shows an image from a real-time imaging experiment 15 min following iv methoxy-X04 administration; PrP amyloid plaques could be clearly distinguished (arrowhead). Fluorescence in vessels (v) was also seen. Scale bars: A = 40 μm; B = 30 μm.
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