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Identification of the pathological prion protein allotypes in scrapie-infected heterozygous bank voles (*Clethrionomys glareolus*) by high-performance liquid chromatography–mass spectrometry

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Abstract

Cerebral formation of the pathological isoform of the prion protein (PrP) is a crucial molecular event in prion diseases. The bank vole (*Clethrionomys glareolus*) is a rodent species highly susceptible to natural scrapie. The PrP gene of bank vole is polymorphic (Met/Ile) at codon 109. Here we show that homozygous $109^{Met/Met}$ voles have incubation times shorter than heterozygous $109^{Met/Ile}$ voles after experimental challenge with three different scrapie isolates. An HPLC–MS/MS method was optimized and applied to investigate whether in heterozygous animals both PrP allotypes are able to undergo pathological conversion. The results demonstrate that both allotypes of the prion protein participate to pathological deposition.

Keywords: Mass spectrometry; Prion protein; Reporter peptides; Transmissible spongiform encephalopathies

1. Introduction

Transmissible spongiform encephalopathy (TSE) diseases or prion diseases are a group of fatal neurodegenerative disorders affecting animals, e.g. bovine spongiform encephalopathy (BSE) and scrapie in sheep, and humans, e.g. Creutzfeldt-Jakob disease (CJD). Human TSE diseases can occur either as sporadic or genetic forms, the latter being always associated with mutations of the PrP gene. Acquired forms have been reported as deriving from accidental iatrogenic transmission or from ingestion of BSE contaminated food [1].

TSE diseases are usually characterized by the formation and accumulation in the brain of a partially protease resistant protein (PrP^{res}) [2,3], which derives from an host-encoded soluble protein (PrP^{C}). In humans, the prion protein consists of 253 amino acid residues, with 8, 9 amino acids long tandem repeats at the amino-terminus, two N-glycosylation sites (residues 181 and 197), one disulfide bond (Cys179–Cys214) and a glycosylphosphatidylinositol (GPI) membrane anchor at the carboxy-terminal residue [2]. The amino acid sequences of the cellular and pathological protein isoforms are identical [4] and they differ by conformational properties. PrP^{res} is characterized by a high β-sheet content, insolubility in detergents, and a partial resistance to proteolytic treatment that trim the N-terminal end of the protein yielding a protease resistant core, called PrP27-30; conversely, PrP^{C} is a soluble protein with a high content of α-helices and high susceptibility to proteolytic digestion [2,5–7].

The mechanism of the pathological conversion of the prion protein is unknown, but the primary structure of the protein may facilitate this transition and the development of the disease. Polymorphisms of the PrP gene influence the susceptibility of humans and animals to prion diseases. In humans,

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the Met/Val polymorphism at codon 129 is critical for the susceptibility and for the clinico-pathological phenotype of the disease [8–10]. In sheep, polymorphisms at codons 136, 154 and 171 of the PrP gene codons are known to affect the susceptibility to natural scrapie and to experimentally transmitted scrapie and BSE [11,12]. In experimentally infected mice as well, the incubation time of the disease depends on the PrP gene, which can be either 108^{Leu} and 189^{Thr} (*prnp* allele *a*) or 108^{Phe} and 189^{Val} (*prnp* allele *b*) [13]. Heterozygous mice ($prnp^{a/b}$) injected with some mouse-adapted scrapie strains show an incubation time that is longer than in $prnp^{a}$ and $prnp^{b}$ homozygous subjects [14]. A similar phenomenon, described as "overdominance of heterozygotes", may occur in sporadic CJD, where heterozygous $129^{Met/Val}$ individuals show longer survival times [15].

The bank vole (*Clethrionomys glareolus*) is a wild rodent highly susceptible to natural scrapie whose PrP gene is polymorphic at codon 109, coding either for isoleucine or methionine (unpublished data). Here we show that scrapie infected heterozygous $109^{Met/Ile}$ animals have longer survival times than homozygous $109^{Met/Met}$ voles injected with the same material. It is possible that 109^{Ile} PrP allotype does not participate to the pathological conversion of PrP and, as a consequence, delays the progression of the disease. To test this hypothesis we used a previously developed HPLC–MS/MS protocol [16,17] which was here optimized to selectively monitor reporter peptides of PrP^{res} allotypes 109^{Met} and 109^{Ile} in heterozygous bank voles.

2. Experimental

2.1. Chemicals

Trifluoroacetic acid and acetonitrile were purchased from Fluka (St. Louis, MO, USA) and were chromatography grade. All other reagents were of analytical-grade available from Sigma–Aldrich (St. Louis, MO, USA).

Water was either purified in the laboratory using a Millipore Milli-Q water purification system (Volketswil, Switzerland) or was HPLC grade (Fluka).

2.2. Sample preparation

Homozygous $109^{Met/Met}$ ($n = 14$) and heterozygous $109^{Met/Ile}$ ($n = 9$) bank voles were inoculated with two natural sheep (SS3 and SS5) and one natural goat (SG1) scrapie isolates. Beginning one month after inoculation, the voles were examined for neurological symptoms, and once the first clinical signs of disease were detected, the voles were examined daily. The first signs consisted of hyperactivity/excitability along with the absence of the typical hiding behavior under the sawdust's cage; neurological symptoms were represented by upwards movements of the head (headbobbing), disturbed balance and impaired motor coordination. The animals were kept alive until the terminal stage of the disease, which was characterized by pronounced ataxia, kyphosis and severe hypo-activity and -reactivity. At this stage the animals were sacrificed with carbon dioxide. Survival time was calculated as the interval between inoculation and terminal disease. Brains were collected and immediately frozen. To obtain suitable amount of tissue for optimizing the HPLC–MS/MS method, brains from heterozygous voles were pooled.

PrP27-30 was extracted from diseased brains and purified according to a published protocol [18], comprising sequential ultracentrifugation steps in detergents and a proteinase K treatment. Briefly, the brains were homogenised in 9 volumes of a 10% solution of sarcosyl (N-lauroylsarcosine sodium salt) pH 7.4. The homogenates were clarified in a Beckman rotor 100.3 at $16{,}000 \times g$ for 15 min. Supernatants were treated with 0.1 M EDTA pH 7.6 and centrifuged at $145{,}000 \times g$ for 90 min. The pellets were resuspended in 0.02 M Tris–HCl, pH 8.5, 0.1% Zwittergent 3,14 (*N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulphonate). The samples were then centrifuged at $145{,}000 \times g$ (58,000 rpm) for 60 min on a cushion of 20% sucrose. The pellets were resuspended and centrifuged as above. The resulting pellets were stirred overnight at room temperature in a solution of 0.2 M Tris–HCl pH 8.5, supplemented with 1 M $CaCl_2$ and 0.2 μg/ml micrococcal nuclease. Subsequently, the samples were incubated with 0.4 μg/ml proteinase K for 30 min at 37 °C. After the addition of 0.1M EDTA pH 7.6, the mixture was centrifuged at $200{,}000 \times g$ for 2 h through a cushion of 20% sucrose. Purified PrP27-30 samples were inactivated with 80% formic acid for 3 h at 4 °C and centrifuged at $14{,}000 \times g$ for 30 min at 4 °C. The supernatant was neutralized with 0.5 M ammonium bicarbonate (pH 7.8). PrP27-30 suspension was desiccated in speed vacuum (Speed Vac Sc 110; Savant), resuspended in 50 μL of digestion buffer (1 mM EDTA, 25 mM Tris–HCl pH 8.25 and 1% octyl-beta-glucopyranoside) and incubated with 2.5 μg of bovine trypsin (Roche, Basel, Switzerland) at 37 °C for 24 h. Enzymatic digestion was stopped by diluting with 50 μL of 20% trifluoroacetic acid.

2.3. PrP^{res} allotypes profiling by LC–MS

Allotype profiling was carried out by the reporter peptide approach described in Schininà et al. [17]. Tryptic peptides were fractionated by HPLC, on a LabService Analytica apparatus (model LabFlow 4000, Bologna, Italy), which was connected to an ion trap mass spectrometer equipped with an electrospray ion source (ES-IT, mod. LCQ, ThermoElectron, San Jose, CA, USA). The peptide mixture was injected onto a reverse phase C_{18} mass spec column (220 mm × 2.1 i.d., 5 μm particle size, 300 Å pore size, Vydac, Hesperia, CA, USA) and elution was performed at a flow rate of 150 μL/min with a linear gradient from 1 to 60% acetonitrile containing 0.05% trifluoroacetic acid in 60 min. The elution of the hamster peptide 107TNMK110 (*m*/*z* of 493.2) was preliminary followed by isolation and fragmentation of the parent ions at *m*/*z* 493.2, according to Schininà et al. [17]. Moreover

ion current monitoring of the selected daughter ions (SRM mode) at m/z 216.1 (b_2 ion) and 260.4 (y_2 ion) was achieved. In heterozygous bank vole profiling, detection of the reporter peptides 107TNMK110 and 107TNIK110 was achieved on-line after isolation and fragmentation of the parent ions at m/z 493.2 and 475.0, respectively, and simultaneously monitoring ion currents of selected daughter ions (SRM mode) in the m/z range of 216.1 ± 3.0, 278.1 ± 3.0 and 260.4 ± 3.0. The spectrometer was operated under the following conditions: electrospray voltage 5.15 kV; heating capillary temperature, 260 °C. SIM and SRM chromatographic traces were automated by the Xcalibur software provided with the instrument.

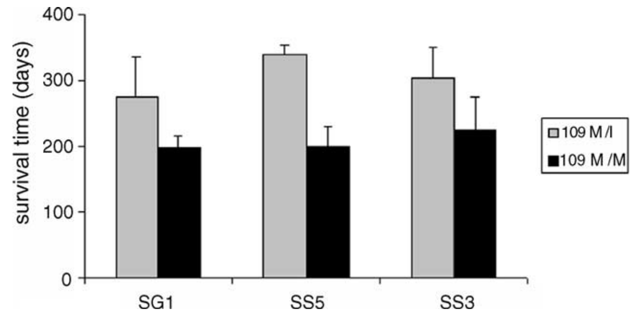

Fig. 1. Survival times in heterozygous 109Met/Ile and homozygous 109Met/Met bank voles after inoculation with two natural sheep (SS3and SS5) and one natural goat (SG1) scrapie isolates.

3. Results and discussion

Homozygous $109^{Met/Met}$ bank voles displayed similar survival times with the three different scrapie isolates and in all three experimental challenges heterozygous $109^{Met/Ile}$ voles showed longer survival times than homozygous voles (Fig. 1). Unpaired t test between the grouped $109^{Met/Met}$ and $109^{Met/Ile}$ voles showed highly significant difference ($P < 0.0001$, $t = 5.76$, d.f. = 21), with mean survival times (±standard deviation) of 202 ± 28 days for homozygous and 298 ± 51 days for heterozygous voles. These results suggest that the PrP gene polymorphism influences the incubation period of disease in voles, similarly to what occurs in mice [14].

The increased survival time observed in $109^{Met/Ile}$ voles could be due to the inability of the PrP allotype carrying Ile at residue 109 to convert into the pathological isoform. In order to evaluate this hypothesis we identified which PrP^{res} allotypes accumulate in the diseased brains of heterozygous voles. To reliably map PrP peptides in digested fractions containing highly purified PrP27-30, we recently proposed a LC–MS/MS analysis of the PrP27-30 reporter peptides [17]. This approach has been successfully applied to the analysis of amyloidogenesis in experimentally infected PrP-heterozygous mouse strains [16] and in human TSEs [19].

To optimise the mass spectrometric analysis to profiling of $109^{Met/Ile}$ voles, we performed a preliminary experiment with PrP27-30 extracted from brains of golden syrian hamster infected with 263K scrapie strain. No allelic variants are present in hamster PrP. Residues in the 107–110 region of hamster prion protein are identical to those of voles with 109^{Met} (Fig. 2). After digestion of hamster PrP27-30 with trypsin, the elution of 107TNMK110 peptide carrying the 109^{Met} residue, was selectively monitored following the ion current of its parent ion. As already reported in Schininà et al. [17], the presence of PrP27-30 in the purified fraction obtained from hamster brains infected with the 263 K strain could be inferred by detection of a chromatographic peak with a high signal to noise ratio. Moreover on-line collection of the MS/MS spectrum on this peptide ion showed a

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                   10                  20                  30                  40
                   -------------------+-------------------+-------------------+-------------------+-
Bank vole          M A N L S Y W L L A F F V T T W T D V G L C K K R P K P G G W N T G G S R Y P G
Syrian Hamster     M A N L S Y W L L A L F V A M W T D V G L C K K R P K P G G W N T G G S R Y P G

                                      50                  60                  70                  80
                   -------------------+-------------------+-------------------+-------------------+-
Bank vole          Q G S P G G N R Y P P Q G G G T W G Q P H G G G W G Q P H G G G W G Q P H G G G
Syrian Hamster     Q G S P G G N R Y P P Q G G G T W G Q P H G G G W G Q P H G G G W G Q P H G G G

                                      90                 100                 110                 120
                   -------------------+-------------------+-------------------+-------------------+-
Bank vole          W G Q P H G G G W G Q G G G T H N Q W N K P S K P K T N M K H V A G A A A A G A
Syrian Hamster     W G Q P H G G G W G Q G G G T H N Q W N K P S K P K T N M K H M A G A A A A G A

                                     130                 140                 150                 160
                   -------------------+-------------------+-------------------+-------------------+-
Bank vole          V V G G L G G Y M L G S A M S R P M I H F G N D W E D R Y Y R E N M N R Y P N Q
Syrian Hamster     V V G G L G G Y M L G S A M S R P M M H F G N D W E D R Y Y R E N M N R Y P N Q

                                     170                 180                 190                 200
                   -------------------+-------------------+-------------------+-------------------+-
Bank vole          V Y Y R P V D Q Y N N Q N N F V H D C V N I T I K Q H T V T T T T K G E N F T E
Syrian Hamster     V Y Y R P V D Q Y N N Q N N F V H D C V N I T I K Q H T V T T T T K G E N F T E

                                     210                 220                 230                 240
                   -------------------+-------------------+-------------------+-------------------+-
Bank vole          T D V K M M E R V V E Q M C V T Q Y Q K E S Q A Y Y E G R S S R A V L L F S S P
Syrian Hamster     T D I K I M E R V V E Q M C T T Q Y Q K E S Q A Y Y D G R R S S A V L F S S - P

                                     250
                   -------------------+-------------
Bank vole          P V I L L I S F
Syrian Hamster     P V I L L I S F L I F L M V G .
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Fig. 2. Alignment of Syrian hamster (*Mesocricetus auratus*) and bank vole (*Clethrionomys glareolus*) PrP amino acid sequences. Tryptic peptides used as reporter ions are bolded. Insertion in the sequence (−) is introduced to maximise homology. The bank vole polymorphic residue (109^{Met} or 109^{Ile}) is underlined.

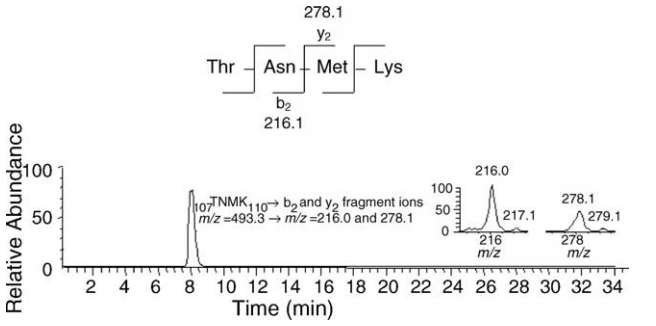

Fig. 3. Mass spectrometric detection of the hamster PrP27-30. In the upper part of the figure is reported the site of hyper fragmentation of the hamster PrP reporter peptide 107–110 resulting in b_2 and y_2 ions. In the lower part of the figure is reported the MS/MS spectra of the reporter peptide (107TNMK110); simultaneously monitoring of ion currents of selected daughter b_2 ions (*m/z* 216.1) and y_2 ions (*m/z* 278.1) were sequentially collected on-line after selective isolation and fragmentation of the parent ion (*m/z* 493.2) (SRM mode).

high fragmentation yield at the level of the peptide bound between residues 108^{Asn}–109^{Met} [17]. On the basis of this fragmentation pattern, a more accurate analysis has been approached and successfully achieved here (Fig. 3), selectively monitoring the transition of the parent ion to the b_2 and y_2 fragments.

The same HPLC–MS/MS method was used in purified fractions of PrP27-30 extracted from heterozygous bank voles. We have simultaneously monitored two different transitions of the reporter peptides 107TNMK110 and 107TNIK110: the b_2 fragments, that are identical in both reporter peptides (*m/z* of 216.1 ± 3), and the y_2 fragments, that show discriminant *m/z* values between the two allotypes (*m/z* 278.1 for the 109^{Met} and *m/z* 260.4 for the 109^{Ile} allotypes, respectively). Both 109^{Met} and 109^{Ile} PrP allotypes are unequivocally detected in PrP27-30 fractions extracted from bank voles carrying the $109^{Met/Ile}$ heterozygous PrP genotype (Fig. 4), demonstrating that they can both undergo to pathological conversion.

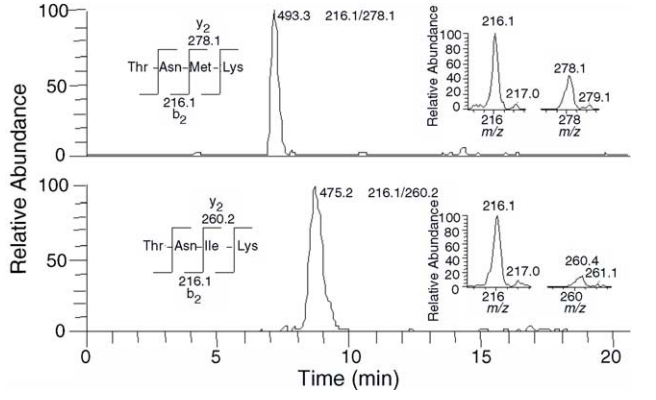

Fig. 4. Mass spectrometric detection of PrP27-30 allotypes from scrapie infected heterozygous voles. Simultaneously monitoring of ion currents of selected daughter b_2 ions and y_2 ions were sequentially collected on-line after selective isolation and fragmentation of the parent ions (SRM mode) at *m/z* 493.2 (upper panel) and at *m/z* 475.2 (lower panel); structural data on the reporter peptides (insets on the left) and mass spectra recorded on the selected fragment ions (insets on the right) are also reported.

4. Conclusions

The described HPLC–MS/MS method is able to identify the two allotypes of PrP^{res} from heterozygous scrapie affected voles. This proves that the long incubation times observed in $109^{Met/Ile}$ voles is not imputable to the complete inability of 109^{Ile} to convert in its pathological isoform. Additional studies are underway to improve quantification of the two allotypes. This will allow to investigate if there are differences in the relative amounts of the two allotypes accumulating in the brain of scrapie infected voles and if the long incubation times observed in $109^{Met/Ile}$ is explained by a lower propensity of the 109^{Ile} allotype to undergo pathological conversion. Since the role of codon 109 on the susceptibility of heterozygous voles could also be influenced by the strain of TSE agent, similarly to the mouse model [14], further transmission studies on voles are ongoing involving the inoculation of different TSE strains. The HPLC–MS/MS analytical approach described here can be a valuable tool for investigating the molecular basis of amyloidogenesis in TSEs and to gain insight in the phenomenon of the different susceptibility to TSE diseases of heterozygous animals.

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References

[1] J. Collinge, Annu. Rev. Neurosci. 24 (2001) 519.
[2] B. Oesch, D. Westaway, M. Walchli, M.P. McKinley, S.B. Kent, R. Aebersold, R.A. Berry, P. Tempst, D.B. Teplow, L.E. Hood, S.B. Prusiner, C. Weissmann, Cell 40 (1985) 735.
[3] S.B. Prusiner, S.J. De Armond, Annu. Rev. Neurosci. 17 (1994) 311.
[4] N. Stahl, M.A. Baldwin, D.B. Teplow, L. Hood, B.W. Gibson, A.L. Burlingame, S.B. Prusiner, Biochemistry 32 (1993) 1991.
[5] R.K. Meyer, M.P. McKinley, K.A. Bowman, M.B. Braunfeld, R.A. Barry, S.B. Prusiner, Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 2310.
[6] K.M. Pan, M. Baldwin, J. Nguyen, M. Gasset, A. Serban, D. Groth, I. Mehlhorn, Z. Huang, R.J. Fletterick, F.E. Cohen, S.B. Prusiner, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 10962.
[7] R. Riek, S. Hornemann, G. Wider, M. Billeter, R. Glockshuber, K. Wüthrich, Nature 382 (1996) 180.
[8] M.S. Palmer, A.J. Dryden, J.T. Hughes, J. Collinge, Nature 352 (1991) 340.

[9] J. Collinge, M.S. Palmer, A.J. Dryden, Lancet 337 (1991) 1441.
[10] L. Monari, S.G. Chen, P. Brown, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 2839.
[11] N. Hunter, Trends Microbiol. 5 (1997) 331.
[12] W. Goldmann, N. Hunter, G. Smith, J. Foster, J. Hope, J. Gen. Virol. 75 (1994) 989.
[13] D. Westaway, P.A. Goodman, C.A. Mirenda, M.P. McKinley, G.A. Carlson, S.B. Prusiner, Cell 51 (1987) 651.
[14] M.E. Bruce, I. McConnell, H. Fraser, A.G. Dickinson, J. Gen. Virol. 72 (1991) 595.
[15] M. Pocchiari, M. Puopolo, E.A. Croes, H. Budka, E. Gelpi, S. Collins, V. Lewis, T. Sutcliffe, A. Guilivi, N. Delasnerie-Laupretre, J.P. Brandel, A. Alperovitch, I. Zerr, S. Poser, H.A. Kretzschmar, A. Ladogana, I. Rietvald, E. Mitrova, P. Martinez-Martin, J. de Pedro-Cuesta, M. Glatzel, A. Aguzzi, S. Cooper, J. Mackenzie, C.M. van Duijn, R.G. Will, Brain 127 (Pt. 10) (2004) 2348.
[16] B. Maras, D. Barra, M.E. Schinina', F. Cardone, M. Pocchiari, Eur. J. Mass Spectrom. 10 (2004) 371.
[17] M.E. Schinina', B. Maras, F. Cardone, C. Mancone, S. Principe, M.A. Di Bari, P. Parchi, M. Pocchiari, Pure Appl. Chem. 75 (2003) 317.
[18] H. Diringer, M. Beekes, M. Ozel, D. Simon, I. Queck, F. Cardone, M. Pocchiari, J.M. Ironside, Intervirology 70 (1997) 238.
[19] S. Capellari, F. Cardone, S. Notari, M.E. Schininà, B. Maras, D. Sità, A. Baruzzi, M. Pocchiari, P. Parchi, Neurology 64 (2005) 905.