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Cellular prion protein in ovine milk

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Abstract

Cellular prion protein, PrP^C, is essential for the development of prion diseases where it is considered to be a substrate for the formation of the disease-associated conformer, PrP^{Sc}. In sheep, PrP^C is abundant in neuronal tissue and is also found at lower concentrations in a range of non-neuronal tissues, including mammary gland. Here, we demonstrate the presence of soluble PrP^C in the non-cellular, non-lipid fraction of clarified ovine milk. Compared with brain-derived PrP^C, ovine milk PrP^C displays an increased electrophoretic mobility. Ovine milk PrP^C is mainly present as three species that differ in the extent of their N-linked glycosylation, with glycoform profiles varying among animals. Similar PrP^C species are also present in fresh and commercial homogenised/pasteurised bovine milk, with additional N-terminal PrP^C fragments detectable in ruminant milk and commercial milk products.

Keywords: Prion; PrP^C; Milk; Ovine

The prion protein (PrP) is a membrane-bound glycoprotein that in mammals can exist in at least two distinct structural conformations. The normal or cellular conformation (PrP^C) is an apparently benign protein whose main function, despite considerable research effort, remains enigmatic. PrP^C is capable of misfolding into a protease-resistant conformation designated PrP^{Sc} that according to the protein-only hypothesis is the infectious agent of prion diseases, a group of fatal neurodegenerative disorders also known as transmissible spongiform encephalopathies [1]. Prion diseases have been described in a number of mammalian species and include scrapie in sheep, bovine spongiform encephalopathy in cattle, and Creutzfeldt-Jakob disease in humans. PrP^C is a prerequisite for the development of prion diseases as demonstrated by $PrP^{0/0}$ transgenic mice being resistant to scrapie [2].

PrP^C, which is conserved in mammalian species, has an N-terminal basic region, a tandem repeat region near the N-terminus, a conserved hydrophobic domain, two glycosylation sites, a disulphide bond within the C-terminal domain, and a C-terminal glycophosphatidylinositol (GPI)-anchor signal sequence. PrP^C is abundant in CNS tissue but is also found at lower concentrations in non-neuronal tissues including skeletal muscle and mammary gland [3,4]. PrP^C is usually located on the extracellular surface, attached via a GPI-anchor domain to cholesterol- and sphingolipid-rich membrane microdomains called lipid rafts [5]. However, there are reports of soluble PrP^C in human cerebrospinal fluid and serum [6,7], human platelets [8], and in ovine epididymal fluid and seminal plasma [9]. The mechanism of PrP shedding from the cell surface remains to be elucidated, although in cell culture systems PrP^C can be shed via both secretase-like proteolytic cleavage of the protein and phospholipase cleavage of the GPI anchor [10]. To date, the only report of exocrine PrP^C is on the surface of ejaculated sperm where it is present as a C-terminally truncated form which is cleaved at, or N-terminal of, residue 203. Such PrP is not localised to lipid rafts but is associated with membranes via an undefined mechanism [9,11]. Here, we demonstrate for the first time the presence of soluble PrP^C and PrP^C fragments in

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fresh ovine milk as well as in commercial homogenised/pasteurised bovine milk. The presence of PrP^{C} in milk raises a number of questions: can such PrP^{C} species be converted into PrP^{Sc}, can similar secretary pathways result in the occurrence of exocrine PrP^{Sc} and therefore does milk play a role in TSE transmission?

Materials and methods

Fresh ovine milk and colostrum were obtained from individual sheep of $AA_{136}RR_{154}RR_{171}$ or $AA_{136}RR_{154}QQ_{171}$ PrP genotype (ADAS, Cambs., UK). A pool of fresh bovine milk was obtained from a Leicestershire herd of Holstein cattle. Commercially obtained milk was purchased from a local supermarket. Milk or colostrum was made up to 50 mM EDTA, 0.5% v/v Nonidet P-40, and 0.5% w/v sodium deoxycholate. Protease inhibitors were added (Roche) and samples (1 ml) centrifuged for 5 min at 16,000*g*. After cooling on ice for 5 min, clarified milk was withdrawn from under the solidified fat layer.

Ovine brain material (medulla) from a Suffolk genotype $AA_{136}RR_{154}QR_{171}$ was obtained from the Veterinary Laboratories Agency (Addlestone, Surrey, UK). Homogenates (10% w/v) were prepared in lysis buffer (0.5% v/v Nonidet P-40 and 0.5% w/v sodium deoxycholate in PBS) by passing through needles of decreasing diameter (19G, 21G, and then 23G). Samples were centrifuged for 5 min at 2300*g* and aliquots of the supernatant stored at −20 °C.

For immunoprecipitation of PrP, antibodies were bound to protein A–Sepharose 4B beads (Sigma) and covalently coupled using dimethylpimelimidate (Sigma). Bead suspension (20 μl) was mixed with 1 ml of sample for 6 h at 4 °C. Immunoprecipitate was recovered by centrifugation for 5 min at 2300*g* and the beads washed three times in RIPA buffer (1 ml). Immunoprecipitates were eluted from the beads by boiling in 1× LDS loading buffer before analysis. Where indicated, immunoprecipitations and sample processing were carried out in the presence of 50 mM EDTA or 100 mM $CuSO_4$.

For Western blot analysis, samples were boiled in 1× LDS loading dye, 5% mercaptoethanol for 5 min and electrophoresed on 16% SDS–PAGE gels. Proteins were transferred to PVDF membrane (Roche) and blocked in 3% (w/v) powdered infant formula milk. Membranes were probed with antibodies AG4 (TSE resource centre-UK) at 1/2000, SAF32, SAF15 (provided by J. Grassi) both at 1/40,000, L42 and P4 (R-Biopharm Rhone LTD) at 1/10,000, and control 9E10 anti-C-myc antibody used at 1/2000. Bound primary antibody was detected with a 1/2000 dilution of anti-mouse antibody Fc fragment HRP conjugate (Sigma) and then visualised using POD HRP substrate (Roche) and Kodak Biomax MR film.

For de-glycosylation, samples were suspended in 20 mM phosphate buffer, pH 7.0, and 1% (w/v) SDS, heated at 100 °C for 5 min, and cooled to room temperature. NP-40 was added to 1% (v/v) along with PNGase F (Roche) to a final concentration of 50 U/ml. Samples were incubated at 37 °C for 24 h.

For protease treatments, milk was buffered by the addition of 1 M Tris to 30 mM, pH 7.0, and thermolysin or Proteinase K (Sigma) added to a final concentration of 100 μg/ml. Samples were incubated at either 70 or 37 °C, respectively, for 1 h. Digestions were stopped by the addition of EDTA to 10 mM (thermolysin digests) or PMSF to 3 mM (proteinase K digests).

Sedimentation of clarified milk or brain homogenates, prepared as described above but without detergents, followed the method described by Chen et al. [12]. Phase partitioning of milk PrP was carried out as described by Duade et al. [13].

Results

PrP^{C} was immunoprecipitated from the non-cellular, non-lipid fraction of clarified ovine milk using anti-PrP antibodies directed to the octapeptide repeat region of the protein (Fig. 1B). Western blot analysis of immunoprecipitates, with monoclonal antibody SAF32, detected three PrP species of slightly increased electrophoretic mobility compared to the three species of PrP found in brain tissue extracts (Fig. 1B). In addition, milk contains three PrP fragments between approximately 4 and 10 kDa. The three higher molecular weight PrP bands were also recognised by a range of anti-PrP antibodies with specificity for distinct epitopes: these include AG4, P4, and L42 (Fig. 1C). A previous report by Everest and co-workers highlighted the potential for certain secondary antibodies to recognise milk-derived proteins, a potential problem for the detection of milk PrP [14]. For this study, we screened several candidate secondary antibodies and selected an anti-Fc-HRP conjugate that does not detectably cross-react with any milk proteins, that could potentially be present in immunoprecipitates. Thus, the use of a primary antibody with irrelevant binding (antibody 9E10) in Western blots did not produce any detectable bands (Figs. 1C and 3). Furthermore, immunoprecipitation using beads lacking an anti-PrP antibody did not isolate any Western blot-positive bands (Fig. 3). These results demonstrate that the immunoprecipitation and Western blot detection of bands were anti-PrP-antibody specific and strongly suggest the presence of PrP^{C} in milk. The three lower molecular weight fragments of PrP isolated from milk were recognised by

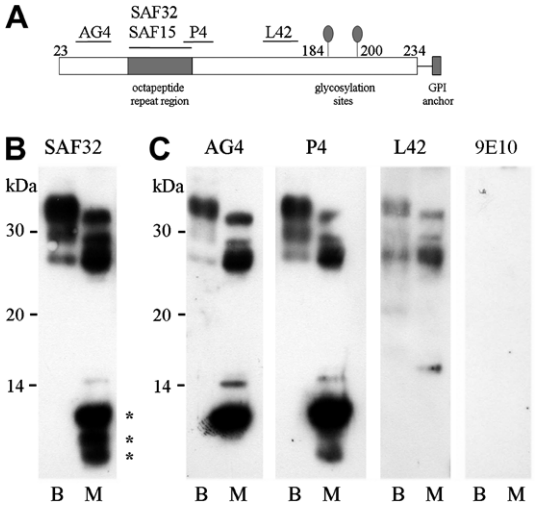

Fig. 1. Detection of milk PrP^{C} and PrP^{C} fragments with anti-PrP antibodies. (A) Epitopes of the anti-PrP antibodies used in the study are illustrated; AG4 binds between residues 31 and 51, and also has a suggested epitope between residues 147 and 163. However the antibody does not bind to the latter epitope in our hands in Western blots, as assessed using PrP with and without the N-terminal epitope domain (data not shown). SAF32 and SAF15 bind an epitope within the octapeptide repeat region, P4 binds between residues 89 and 104 and L42 binds between residues 145 and 163. (B and C) PrP^{C} from 1 ml of clarified ovine milk (M) was immunoprecipitated with antibody SAF15 and the entire eluate analysed by Western blot. Brain homogenates (B), 2.5 μl of 10% (w/v), was analysed directly. PrP was detected with anti-PrP antibodies SAF32 (B), AG4, P4, and L42 (C) as well as a control antibody with irrelevant specificity, 9E10. In (B) the three main N-terminal fragments of PrP^{C} are labeled with an asterisk.

antibodies directed to the N-terminal octapeptide repeat region including SAF32 (Fig. 1B). The largest fragment is immunostained by AG4, SAF32, and P4 indicating that it spans the entire N-terminal region of the protein; the next largest band contains only a SAF32 epitope and is assumed therefore to be truncated after the AG4 epitope and before the P4 epitope. The smallest fragment contains both SAF32 and P4 epitopes and therefore contains all or part of the octapeptide repeat region as well as further C-terminal residues.

The digestion of clarified ovine milk with either proteinase K or thermolysin abolishes the immunoprecipitation of near full length PrP^C species whereas the N-terminal fragments of milk PrP are sensitive to proteinase K treatment but resistant to thermolysin digestion (Fig. 2A). Thermolysin is predicted to cleave at the hydrophobic amino acid residues Leu, Ile, Phe, Val, Ala, and Met [15] and the N-terminal region of PrP lacks thermolysin cleavage sites, with the most N-terminally located site situated at residue Met112.

Digestion of immunoprecipitates of ovine milk with PNGase F yielded a single band from the near full length PrP triplet, as seen for similar digestion of brain homogenates (Fig. 2B), indicating that the milk-derived PrP species differ only in their extent of N-linked glycosylation, with di-, mono-, and unglycosylated PrP species being present. The de-glycosylated milk-derived PrP^C still displays increased electrophoretic mobility compared with brain-derived PrP^C. This observation suggests heterogeneity in composition of N-linked glycans cannot fully account for the differential electrophoretic mobility of PrP^C derived from milk and brain. Treatment of ovine milk and brain samples with EDTA or $CuSO_4$ did not alter the electrophoretic mobility of immunoprecipitated unglycosylated PrP^C (Fig. 2C) suggesting that differences in the Cu^{2+} occupancy of milk and brain PrP^C cannot account for their different electrophoretic mobilities.

To determine whether an operationally functional GPI-anchor domain was present on milk-derived PrP, sub-cellular fractionation and phase partitioning through Triton X-114 were performed. Following ultracentrifugation, the majority of milk-derived PrP^C and PrP fragments were soluble whilst no detectable PrP from brain extracts was present in the supernatant fraction (Fig. 2D). However, milk-derived PrP partitioned into the detergent-rich phase upon Triton X-114 phase partitioning (Fig. 2D), suggesting that although the majority of milk-derived PrP is not obviously associated with either cells or membrane fragments it nevertheless retains a hydrophobic GPI anchor.

PrP was immunoprecipitated from milk and colostrum from 10 individual sheep. Resulting immunoblot analysis demonstrated the presence of the higher molecular weight triplet as well as N-terminal fragments, in all samples (Fig. 2F). Interestingly, these samples showed inter-animal variation in glycoform ratios; for example, mature milk from animal 1 had an apparent co-dominance of diglycosylated and unglycosylated forms of PrP whilst the glycoform

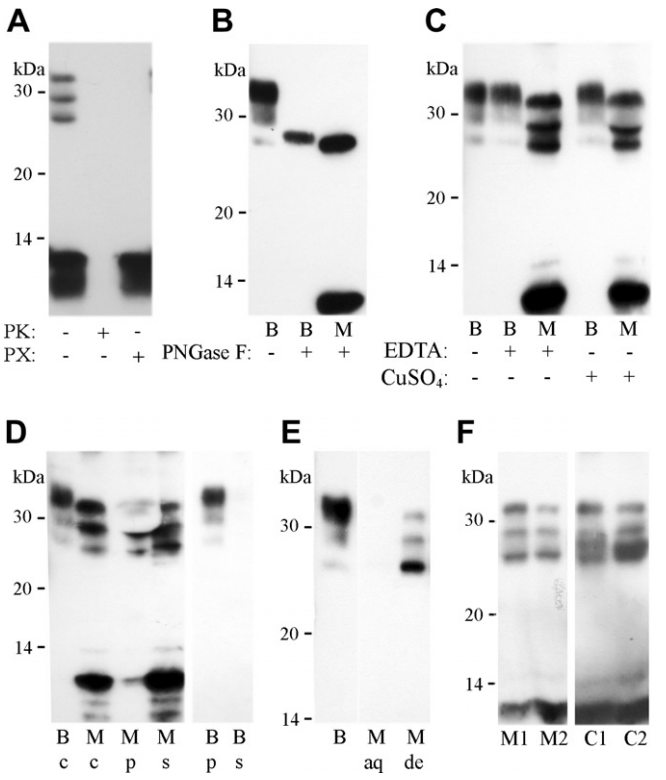

Fig. 2. Characterization of ovine milk PrP^C. Immunoprecipitations of PrP^C used antibody SAF15 and the entire eluate was analysed. All panels show the Western blot detection of PrP with SAF32. (A) Milk (1 ml) was undigested, digested with PK or thermolysin (PX) before immunoprecipitation as indicated. (B) PrP^C immunoprecipitated from ovine milk (M, 1 ml) was analysed after deglycosylated with PNGase F. Brain homogenate (B), 2.5 μl of 10% (w/v), was analysed directly or after treatment with PNGase F (as indicated). (C) PrP^C was immunoprecipitated from brain homogenate (B, 5 μl of 10% w/v within 1 ml PBS buffer) or milk (M, 1 ml) in the presence of either 50 mM EDTA or 100 mM $CuSo_4$ as indicated. (D) Milk (M) or brain homogenate (B) was prepared in 0.32 M sucrose without detergents and centrifuged at 100,000*g* for 1 h as described previously [12]. Pellets were resuspended and PrP^C within the supernatants immunoprecipitated. Both supernatants (s) and pellets (p) derived from 1 ml of milk or 10 μl of 10% (w/v) brain homogenate were analysed along with brain (2.5 μl of 10% w/v) and milk (immunoprecipitate from 1 ml) controls (c). The pellet fraction isolated from milk was distorted during electrophoresis most probably due to the precipitation of large amounts of milk proteins. (E) Brain homogenate (B), 2.5 μl of 10% (w/v), was analysed directly, 1 ml of ovine milk (M) was separated into detergent (de) and aqueous (aq) phases using Triton X-114 phase partitioning. PrP was immunoprecipitated from each phase using SAF15. (F) PrP^C from 1 ml of ovine milk (M) or colostrum (C) was immunoprecipitated. M1 and C1 represent PrP^C immunoprecipitated from milk and colostrums, respectively, from an individual animal, similarly M2 and C2 are from a second animal.

profile of animal 2 displayed dominance of the unglycosylated form only. Previous reports have highlighted PrP^C glycoform ratio variation between tissues in individual animals and even between brain regions [16,17]. Colostrum-derived PrP contained stronger immunostaining for PrP species co-migrating with the proposed unglycosylated form (Fig. 2F).

High molecular weight PrP^C species were also present in full fat, semi skimmed and skimmed bovine milk obtained

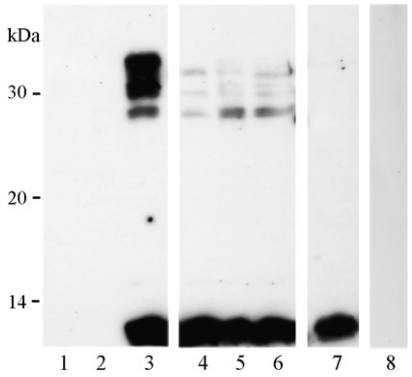

Fig. 3. Presence of PrP^C in commercial milk products. PrP^C from 1 ml of clarified bovine milk (unprocessed, lanes 2, 3, and 8) or commercial full fat pasteurised milk (lane 4), semi-skimmed pasteurised milk (lane 5), skimmed pasteurised milk (lane 6), and 1 ml 10 % (w/v) skimmed milk powder (lane 7) was immunoprecipitated with antibody SAF32 and the entire eluate analysed. Lane 1 shows the direct analysis of an equivalent amount of SAF-32 coated beads and lane 2 shows the immunoprecipitation of bovine milk with beads that lack an anti-PrP antibody. PrP was detected with SAF32 in lanes 1–7 or an antibody with irrelevant binding, 9E10, in lane 8. All samples were analysed on a single Western blot and represent identical exposure times.

from a supermarket albeit at reduced levels compared with unprocessed milk (Fig. 3). This indicates that PrP species survive the homogenisation and pasteurisation processes. However, near full length PrP^C species were not detected in powdered bovine milk including baby formula. Milk processing had little or no effect on the levels of N-terminal PrP fragments found in milk with the exception of baby formula, where they were present at considerably lower levels and were often below the limits of detection.

Discussion

Near full length PrP^C as well as N-terminal fragments of PrP were isolated from ovine milk by immunoprecipitation with anti-PrP antibodies. These PrP species were recognised by a range of anti-PrP antibodies with distinct epitopes and displayed protease susceptibilities and interspecies molecular weight variance consistent with PrP^C. Furthermore, the near full length species of milk PrP consists of di-, mono-, and unglycosylated forms of the protein, consistent with neuronal PrP^C.

The near full length, soluble PrP^C derived from ovine milk has a slightly increased electrophoretic mobility compared to neuronal PrP^C. This molecular weight difference is not due to differences in sample pH or Cu^{2+} occupancy of PrP and is consistent with previous reports that describe differences between neuronal and non-neuronal PrP^C [18].

Milk-derived PrP is immunostained with the AG4 antibody, which binds to at an epitope near to the extreme N-terminus of PrP, indicating that the peptide backbone is not significantly truncated in this region. Exocrine PrP^C has been reported in mature human and bovine sperm, where the protein is cleaved towards the C-terminus near residue 203 and lacks the GPI anchor [11]. A similar study on ovine samples also reported truncated PrP^C associated with sperm as well as a soluble PrP^C within epididymal fluid and seminal plasma [9]. Milk-derived PrP^C is hydrophobic, partitioning to the detergent phase in Triton X-114, suggesting the presence of the C-terminal GPI moiety. However, the majority of milk-derived PrP^C could not be pelleted by high speed centrifugation suggesting that it is not obviously cell- or membrane-associated, perhaps indicating an absence of GPI-anchor function. Loss of the GPI anchor would not account for the increased electrophoretic mobility of the unglycosylated form of milk PrP as several studies have shown that its removal results in a decrease in apparent electrophoretic mobility, due to the extremely hydrophobic nature of the lost anchor [19]. It seems possible that an alteration of the composition of the GPI-anchor domain and/or slight truncation of the protein, N-terminal of the AG4 epitope, could be responsible for the altered mobility of milk-derived PrP^C.

Near full length PrP^C species were found in fresh and commercially processed bovine milk sources, although they were absent or below the limits of detection in powdered milk. N-terminal fragments were present in all milk analysed but were present at much reduced levels, and often below the limits of detection, in baby formula. It is of interest that powdered skimmed milk is commonly used in research and analytical laboratories as a blocking agent in immunological assays. In the light of the results presented here, the use of skimmed milk powder is inappropriate in prion diagnostics as the presence of N-terminal prion fragments in the blocking solutions could potentially compete for binding of antibodies directed to the N-terminal region of PrP.

The source of the near full length species of PrP^C found in milk is of interest. There are several routes through which proteins are secreted into milk: the exocytotic secretion of proteins produced within mammary gland epithelial cells, the secretion of proteins associated with milk fat globules, the vesicular transcytosis of serum- and stromal cell-derived proteins as well as through a paracellular transport system that is responsible for the secretion of plasma components and leukocytes. A recent study by Didier and co-workers demonstrated that within the bovine mammary gland, PrP^C is selectively localized in mammary gland epithelial cells and expressed largely at the basolateral surface of cells that show active secretion [20]. Therefore, is seems likely that PrP^C found in milk is actively produced within, and secreted from, the epithelial cells.

The presence of PrP^C species in milk raises the question of whether the disease isoform of PrP can also be secreted into milk and whether milk can harbor TSE infectivity. A few studies have investigated BSE infectivity in milk by challenge of transgenic mice with concentrates of milk from BSE-affected animals or investigating vertical transmission of BSE to calves [21–23]. No evidence of BSE transmission from milk was found. However, bovine BSE-associated

PrP^{Sc} and infectivity is known to be detected almost exclusively within the CNS and not within peripheral lymphoreticular tissues [24,25]. In contrast, scrapie PrP^{Sc} and infectivity is far more widely disseminated [26]. To our knowledge, the study of milk from scrapie affected ewes for possible TSE infectivity has not been published. However, one study has demonstrated the presence of PrP^{Sc} within mammary glands of mastitic scrapie infected sheep and speculates that the coexistence of prion infection and inflammation in secretary organs may lead to the prion contamination of secretes [27]. The present study provides experimental evidence of PrP^{C} in milk, it remains to be ascertained whether similar secretary pathways can result in the occurrence of exocrine PrP^{Sc} or whether milk PrP^{C} species can be converted into PrP^{Sc}.

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References

[1] S.B. Prusiner, Prions, Proc. Natl. Acad. Sci. USA 95 (1998) 13363–13383.
[2] H. Büeler, A. Aguzzi, A. Sailer, R.-A. Greiner, P. Autenried, M. Aguet, C. Weissmann, Mice devoid of PrP are resistant to scrapie, Cell 73 (1993) 1339–1347.
[3] M. Horiuchi, N. Yamazaki, T. Ikeda, N. Ishiguro, M. Shinagawa, A cellular form of prion protein (PrP^{C}) exists in many non-neuronal tissues of sheep, J. Gen. Virol. 76 (1995) 2583–2587.
[4] M. Moudjou, Y. Frobert, J. Grassi, C.J. La Bonnardière, Cellular prion protein status in sheep: tissue-specific biochemical signatures, Gen. Virol. 82 (2001) 2017–2024.
[5] M. Vey, S. Pilkuhn, H. Wille, R. Nixon, S.J. DeArmond, E.J. Smart, R.G. Anderson, A. Taraboulos, S.B. Prusiner, Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains, Proc. Natl. Acad. Sci. USA 93 (1996) 14945–14949.
[6] F. Tagliavini, F. Prelli, M. Porro, M. Salmona, O. Bugiani, B. Frangione, A soluble form of prion protein in human cerebrospinal-fluid—implications for prion-related encephalopathies, Biochem. Biophys. Res. Commun. 184 (1992) 1398–1404.
[7] P. Parizek, C. Roeckl, J. Weber, E. Flechsig, A. Aguzzi, A.J. Raeber, Similar turnover and shedding of the cellular prion protein in primary lymphoid and neuronal cells, J. Biol. Chem. 276 (2001) 44627–44632.
[8] F. Perini, R. Vidal, B. Ghetti, F. Tagliavini, B. Frangione, F. Prelli, PrP(27-30) is a normal soluble prion protein fragment released by human platelets, Biochem. Biophys. Res. Commun. 223 (1996) 572–577.
[9] H. Ecroyd, P. Sarradin, J.-L. Dacheux, J.-L. Gatti, Compartmentalization of prion isoforms within the reproductive tract of the ram, Biol. Reprod. 71 (2004) 993–1001.
[10] E.T. Parkin, N.T. Watt, A.J. Turner, N.M. Hooper, Dual mechanisms for shedding of the cellular prion protein, J. Biol. Chem. 279 (2004) 11170–11178.
[11] Y. Shaked, H. Rosenmann, G. Talmor, R. Gabizon, A C-terminal-truncated PrP isoform is present in mature sperm, J. Biol. Chem. 274 (1999) 32153–32158.
[12] S.G. Chen, D.B. Teplow, P. Parchi, J.K. Teller, P. Gambetti, L. Autilio-Gambetti, Truncated forms of the human prion protein in normal brain and prion diseases, J. Biol. Chem. 270 (1995) 19173–19180.
[13] N. Duade, S. Lehmann, D.A. Harris, Identification of intermediate steps in the conversion of a mutant prion protein to a scrapie-like form in cultured cells, J. Biol. Chem. 272 (1997) 11604–11612.
[14] S.J. Everest, L.T. Thorne, J.A. Hawthorne, R. Jenkins, C. Hammersley, A.M. Ramsay, S.A. Hawkins, L. Venables, L. Flynn, R. Sayers, J. Kilpatrick, A. Sach, J. Hope, R. Jackman, No abnormal prion protein detected in the milk of cattle infected with the bovine spongiform encephalopathy agent, J. Gen. Virol. 87 (2006) 2433–2441.
[15] U. Arnold, K.P. Rűcknagel, A. Schierhorn, R. Ulbrich-Hofmann, Thermal unfolding and proteolytic susceptibility of ribonuclease A, Eur. J. Biochem. 237 (1996) 862–869.
[16] N. Stahl, D.R. Borchelt, K. Hsiao, S.B. Prusiner, Scrapie prion protein contains a phosphatidylinositol glycolipid, Cell 51 (1987) 229–240.
[17] R.A. Somerville, Host and transmissible spongiform encephalopathy agent strain control glycosylation of PrP, J. Gen. Virol. 80 (1999) 1865–1872.
[18] A. Jiménez-Huete, P.M.J. Lievens, V. Rubén, P. Piccardo, B. Ghetti, F. Tagliavini, B. Frangione, F. Prelli, Endogenous proteolytic cleavage of normal and disease-associated isoforms of the human prion protein in neural and non-neural tissues, Am. J. Pathol. 153 (1998) 1561–1572.
[19] P.A. Lewis, F. Propoerzi, K. Prodromidou, R.A. Clarke, J. Collinge, G.S. Jackson, Removal of the glycosylphosphatidylinositol anchor from PrP^{Sc} by cathepsin D does not reduce prion infectivity, Biochem. J. 395 (2006) 443–448.
[20] A. Didier, R. Dietrich, M. Steffl, M. Gareis, M.H. Groschup, S. Muller-Hellwig, E. Martbauer, W.M. Amselgruber, Cellular prion protein in the mammary gland is selectively expressed in active lactocytes, J. Histochem. Cytochem. 54 (2006) 1255–1261.
[21] J. Castilla, A. Brun, F. Días-San Segundo, F.J. Salguero, A. Gutiérrez-Adán, B. Pintado, M.A. Ramírez, L. del Riego, J.M. Torres, Vertical transmission of bovine spongiform encephalopathy prions evaluated in a transgenic mouse model, J. Virol. 79 (2005) 8665–8668.
[22] D.M. Taylor, C.E. Ferguson, C.J. Bostock, M. Dawson, Absence of disease in mice receiving milk from cows with bovine spongiform encephalopathy, Vet. Rec. 136 (1995) 592.
[23] J.W. Wilesmith, J.M.B. Ryan, Absence of BSE in the offspring of pedigree suckler cows affected by BSE in Great Britain, Vet. Rec. 141 (1997) 250–251.
[24] G.A.H. Wells, J.W. Wilesmith, The neuropathology and epidemiology of bovine spongiform encephalopathy, Brain Pathol. 5 (1995) 91–103.
[25] G.A.H. Wells, S.A.C. Hawkins, R.B. Green, A.R. Austin, I. Dexter, Y.I. Spencer, M.J. Chaplin, M.J. Stack, M. Dawson, Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update, Vet. Rec. 142 (1998) 103–106.
[26] L.J.M. van Keulen, B.E.C. Schreuder, R.H. Meloen, G. MooijHarkes, M.E.H. Vromans, J.P.M. Langeveld, Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie, J. Clin. Microbiol. 34 (1996) 1228–1231.
[27] C. Ligios, C.J. Sirgurdson, C. Santucciu, G. Carcassola, G. Manco, M. Basagni, C. Maestrale, M.G. Cancedda, L. Madau, A. Aguzzi, PrP^{Sc} in mammary glands of sheep affected by scrapie and mastitis, Nat. Med. 11 (2005) 1137–1138.