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Distribution of the Shadoo protein in the ovine brain assessed by immunohistochemistry

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ABSTRACT

Shadow of prion protein is a gene potentially involved in the pathogenesis of prion diseases. However, the Shadoo protein encoded by this gene has not yet been studied in sheep, an important species in prion matters. Therefore, we developed a polyclonal antibody against ovine Shadoo and assessed the presence and distribution of this protein in the ovine brain by immunohistochemistry. The strongest staining level was found in the cerebellum (especially in the Purkinje cells) and in the pons, but cerebrum, hippocampus, pituitary gland, medulla oblongata, thalamus and hypothalamus were also immunopositive. Remarkably, a typical granular pattern was seen in most of the tested brain tissues, which might indicate that Shadoo is primarily expressed at synapses. The results of this study and the availability of an ovine anti-Shadoo antibody can contribute to future research on the function of Shadoo and on its potential involvement in prion diseases.

1. Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of neurodegenerative diseases including scrapie in small ruminants, bovine spongiform encephalopathy (BSE) in cattle and variant Creutzfeldt–Jakob disease (vCJD) in man (Dalsgaard, 2002). These inevitably fatal diseases are caused by prions (PrP^{Sc}), which are alternatively folded forms of the body-own protein PrP^{C} (Prusiner, 1998). PrP^{C} (encoded by the *PRNP* gene) is a cell surface protein bound to the cell membrane by a glycosylphosphatidylinositol (GPI)-anchor (Mastrangelo and Westaway, 2001). It is mainly expressed in neurons (Kretschmar et al., 1986), although substantial amounts have also been detected in a number of other tissues (for review, see Vana et al., 2007). In case of TSE infection, this PrP^{C} is converted into the PrP^{Sc} isoform through a not fully understood mechanism, which leads to PrP^{Sc} accumulation primarily in the brain (Prusiner, 1998).

In sheep, TSE susceptibility clearly depends on the *PRNP* genotype. Sheep homozygous for alanine–arginine–arginine (ARR) at positions 136, 154 and 171 of the prion protein are the most resistant to classical scrapie (Goldmann, 2008). However, this resistance is not absolute, as two cases of classical scrapie have been described in ARR/ARR sheep (Groschup et al., 2007) and as sheep with this genotype are clearly susceptible for atypical scrapie (Buschmann et al., 2004; Orge et al., 2004; Le Dur et al., 2005; Everest et al., 2006; De Bosschere et al., 2007) and experimental BSE infection (Houston and Gravenor, 2003). Therefore, it is important to evaluate the role of other genes and proteins in TSE pathogenesis and susceptibility.

A very promising candidate gene is Shadow of prion protein (*SPRN*). This gene encodes Shadoo (Sho; Premzl et al., 2003), a protein of 145 amino acids in sheep (Lampo et al., 2007). Like PrP, the Sho protein is attached to the cell membrane by a GPI-anchor and contains a hydrophobic region with a palindrome sequence of aliphatic amino acids, which is typical for PrP and PrP-like proteins (Premzl et al., 2003; Watts et al., 2007). Interestingly, the mRNA of *SPRN* has been found primarily in brain tissue, the main target organ of prion infections (Premzl et al., 2003; Uboldi et al., 2006; Lampo et al., 2007).

Several studies indicate that Sho might be involved in TSE pathogenesis. First, Watts et al. (2007) found that Sho transgenes are capable of neutralizing the neurotoxic effect of Doppel (a protein related to PrP) and $PrP\Delta 32–121$ in $prnp^{0/0}$ cerebellar granular neuron cultures, just like PrP. The same authors also reported a remarkable reduction in Sho protein levels in brain homogenates of prion-infected mice, although no effect was seen on *SPRN* mRNA levels of scrapie-infected sheep (Gossner et al., 2009). In addition, three polymorphisms in *SPRN*, discovered in a genetic screening in man, were reported to have an effect on TSE susceptibility (Beck

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et al., 2008). A single base pair insertion at codon 46 of *SPRN*, causing a frameshift, was shown to be associated with vCJD. In the same screening, two linked single nucleotide polymorphisms, one in the intron and the other in codon seven, were associated with presence of sporadic CJD. Furthermore, a positive correlation between *SPRN* and *PRNP* mRNA expression has been found in sheep cerebrum and cerebellum, which might indicate that these genes are co-regulated (Lampo et al., 2009).

In sheep, only the *SPRN* gene and its mRNA expression, but not the Sho protein have been studied to date. Therefore, we present in this article the assessment of the distribution of Sho in a number of sheep brain tissues. A newly developed polyclonal antibody against ovine Sho was used for this purpose.

2. Material and methods

2.1. Antibodies

Polyclonal antibodies against two different peptides of the presumed Sho protein, anti-RRAAGPAELGLEDAEDGA (amino acids 84–101; Genbank: DQ870545) or anti-Sho1 and anti-HRHLCPLLGGALGALRLLRP (amino acids 126–145; Genbank: DQ 870545) or anti-Sho2, were raised in rabbits by Davids Biotechnologie (Regensburg, Germany). Briefly, both peptides were synthesized, conjugated with keyhole limpet hemocyanin and purified by high pressure liquid chromatography. Next, one rabbit was immunized with each peptide. The first immunization was performed intradermally on day 0, the following peptide injections were subcutaneously given on day 14, day 21, day 28, day 35 and day 49. The sera were collected at day 70. Both sera were purified by affinity chromatography and 0.02% Natrium Azid was added for preservation.

2.2. Cloning of ovine SPRN into an expression vector

As a positive control for the antibodies, the coding sequence of ovine *SPRN* was cloned into the expression vector pEF/Myc–His A (Invitrogen, Merelbeke, Belgium), in frame with a myc epitope and a polyhistidine tag. To create the insert, PCR was conducted with the primers AAAAAGGATCCTGAGGTCCCCTCCGTCCT and AAAAAGAATTCAGCCAAGGGCCGCAGCAG (developed with the program Primer3 (Rozen and Skaletsky, 2000), based on the sequence Genbank: DQ870545). The amplicon was then digested with 10 U each of EcoRI and BamHI and purified by gel electrophoresis and subsequent elution with the Geneclean II kit (Q Biogene, Brussels, Belgium). In the meantime, 10 μg of the vector was digested with 10 U each of EcoRI and BamHI and purified by the QIAquick PCR purification kit (Qiagen, Venlo, The Netherlands). Finally, 900 ng of insert was ligated in 300 ng of vector with 8 U T4 ligase and 10× ligase buffer, and 5 μl of this mix was used for transformation in Subcloning Efficiency DH5α Chemically Competent cells (Invitrogen, Merelbeke, Belgium). DNA was isolated with the Plasmid Midi kit (Qiagen, Venlo, The Netherlands) and the position and sequence of the insert in the vector were verified by sequencing with the T7 Promoter primer (AATACGACTCACTATAGGGA) and the BGH Reverse Primer (TAGAAGGCACAGTCGAGG) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Lennik, Belgium) on an Applied Biosystems 3730× l DNA Analyser.

2.3. Western blotting

To evaluate the anti-Sho antibodies, Western blot analyses were performed. 293T (HEK) cells were transiently transfected with 500 ng plasmid encoding myc-tagged *SPRN* using FuGENE HD Transfection Reagent (Roche Applied Science, Penzberg, Germany). Non-transfected cells were included as a negative control. After 2 days, cells were washed with phosphate-buffered saline (PBS) and lysed in a reducing NP-40 buffer with protease inhibitor. Equal amounts of protein were separated on a 10% polyacrylamide gel. Membranes were blocked in a PBS solution with 5% nonfat dry milk and incubated (1 h; room temperature) with primary antibodies, diluted in 5% milk powder in PBS + 0.1% Tween. The primary antibodies used were mice anti-myc antibody (1/3000) together with anti-Sho1 or anti-Sho2 in 1/500 or 1/3000 dilution. After washing with PBS + 0.1% Tween, the fluorescently-labeled secondary antibodies rabbit-800 and mice-700 (LI-COR Biosciences, Lincoln, USA), diluted in 5% milk powder in PBS + 0.1% Tween, were added and incubated for 1 h at room temperature, followed by a final washing step with PBS + 0.1% Tween before detection of immunoreactive proteins by using Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, USA).

2.4. Immunohistochemistry

Samples of ovine cerebellum, cerebrum (frontal, temporal, parietal and occipital lobes), hippocampus, pituitary gland, pons, medulla oblongata, thalamus and hypothalamus from three different adult Texel sheep were fixed in a zinc salts-based fixative (0.1 M Tris base buffer, 0.05% Ca-acetate, 0.5% Zn-acetate, 0.5% Zn-chloride) for 48 h. Subsequently, the samples were processed automatically using successive baths of 50%, 70%, 80%, 90% and 100% alcohol, xylene and eventually embedded in paraffin wax (Tissue Processor STP 420 D, Prosan, Microm, Merelbeke, Belgium). Tissue sections of 5 μm were made (HM360, Prosan, Merelbeke, Belgium), mounted on aminopropylepoxysilane (APES) coated slides and dried overnight at 37 °C. For the immunohistochemical detection of Sho, the slides were first dewaxed and rehydrated by conventional methods, followed by 3 min rinsing in Tris-buffered saline (TBS). The non-specific binding sites were then blocked by incubating the slides for 30 min with 25% goat serum in TBS at room temperature. Primary antibody anti-Sho1, 1/200 diluted in TBS supplemented with 2% bovine serum albumin (BSA), was added and incubated for 1 h at room temperature. After 5 min washing in TBS, endogenous peroxidases were blocked with 3% H_2O_2 in TBS for 5 min at room temperature, followed by 5 min of washing with TBS. Next, biotinylated goat anti-rabbit antibody (1/500 in TBS with 2% BSA) was incubated for 1 h at room temperature followed by two times 5 min washing with TBS. Subsequently, the sections were incubated for 45 min at room temperature with horseradish peroxidase-conjugated streptavidin diluted 1/3000 in TBS with 2% BSA. Finally, immunoreactivity was detected with diaminobenzidine (DAB Dako, Glostrup, Denmark) as chromogen. Counterstaining was performed by Mayer's haematoxylin (Merck, Darmstadt, Germany) and after dehydration the slides were mounted using DPX mountant (VWR International Ltd., Lutterworth, United Kingdom). Controls were carried out either by omission of the primary antibody or the primary and secondary antibodies. A negative control using pre-immunization serum instead of anti-Sho1 antibody was also included.

2.5. Peptide blocking assay

In order to test the specificity of anti-Sho1, the antibody was incubated overnight at 4 °C with a 16-fold mass excess of peptide RRAAGPAELGLEDAEDGA. Immunohistochemistry was then performed as described above, but the blocked anti-Sho1 was used as primary antibody.