Veterinary Microbiology 159 (2012) 23–32

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Differential gene expression and apoptosis markers in presymptomatic scrapie affected sheep

Carlos Hedman [a,1], Jaber Lyahyai [b,c,1], Hicham Filali [a], Belén Marín [a], Carmen Serrano [b], Eva Monleón [a], Bernardino Moreno [a], Pilar Zaragoza [b], Juan José Badiola [a], Inmaculada Martín-Burriel [b], Rosa Bolea [a,*]

[a] Centro de Investigación en Encefalopatías y Enfermedades Transmisibles Emergentes (CIEETE), Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet 177, 50013 Zaragoza, Spain
[b] Laboratorio de Genética Bioquímica (LAGENBIO), Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet 177 Zaragoza, Spain
[c] Centre de Génomique Humaine, Faculté de Médecine et de Pharmacie, Université Mohammed V Souissi, Rabat, Morocco

ARTICLE INFO

Article history:
Received 19 December 2011
Received in revised form 1 March 2012
Accepted 9 March 2012

Keywords:
Apoptosis
Prion
Scrapie
Mitochondrial pathway
Presymptomatic

ABSTRACT

Neuronal loss is one of the characteristics of scrapie neuropathology. Previous analysis of brains from sheep naturally infected with scrapie that were in a terminal stage did not detect a clear induction of apoptosis, although molecular changes were evidenced. As neuronal death could be occurring early in scrapie, we developed a neuropathological and gene expression study of sheep infected with scrapie in a presymptomatic stage. The histopathology, immunolabelling of PrP^{Sc}, Bax and activated caspase-3, and the analysis of the expression of 7 genes involved in the regulation of the mitochondrial pathway of apoptosis were investigated in the following 4 central nervous system areas: medulla oblongata, diencephalon, frontal cortex and cerebellum. Moreover, TUNEL and NeuN immunolabelling was performed in the medulla oblongata. The PrP^{Sc} immunolabelling in the four areas, as well as a neuropil spongiform change, were more evident in the terminal stage than in presymptomatic animals. Cytoplasmic Bax immunostaining was observed in the presymptomatic medulla oblongata. In contrast to symptomatic animals, the immunostaining was not extended to the hypothalamus, indicating the progression of Bax induction during the course of the disease. Although neither caspase-3 immunostaining nor the TUNEL technique detected neurons with apoptosis, NeuN-immunolabelled cell counting determined that presymptomatic animals have already suffered neuronal loss in a lower or equal degree than symptomatic animals. Finally, the gene expression profiles indicated that the mitochondrial pathway of apoptosis was activated with higher intensity in presymptomatic animals than in symptomatic sheep and confirmed the implication of genes such as *BAX* or *AIF* in the disease.

1. Introduction

Scrapie is a fatal neurodegenerative disease that affects sheep and goats and belongs to the group of transmissible spongiform encephalopathies (TSEs), or prion diseases, which include bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease (CWD) in cervids. All forms of prion diseases are associated with an accumulation of abnormal isoforms (PrP^{Sc}) of the cellular prion

* Corresponding author at: Dpto. Patologia Animal, Centro de Investigación en Encefalopatías y Enfermedades Transmisibles Emergentes, Facultad de Veterinaria, Universidad de Zaragoza. 50013 Zaragoza. Spain. Tel.: +34 976762945; fax: +34976762508.
E-mail address: rbolea@unizar.es (R. Bolea).
[1] These authors contributed equally to this work.

0378-1135/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetmic.2012.03.020

protein (PrP^C) in the central nervous system (CNS). The neuropathological features of TSE also include spongiform changes, neuronal loss and gliosis (Budka et al., 1995; Kim et al., 1990). Specific lesions in the CNS may be caused by one of the following mechanisms: the toxic effect of PrP^{Sc} deposits in the tissue or the decrease of functional PrP^C being converted to PrP^{Sc}. Neuroprotective and anti-apoptotic functions of PrP^C have been proposed in previous studies (Vana et al., 2007). PrP^C prevents Bax-mediated neuronal apoptosis *in vitro* (Bounhar et al., 2001; Roucou et al., 2005) and acts as an anti-apoptotic protein through the interaction with the carboxy-terminal region of Bcl-2 (Kurschner and Morgan, 1996; Roucou et al., 2005).

Several studies of scrapie-infected sheep, mice, and hamsters have shown that apoptosis is a common cause of neuronal cell death in animal prion diseases (Siso et al., 2002; Wolf et al., 2001). Similarly, apoptosis has been considered a common form of neuronal death in CJD and fatal familial insomnia (Gray et al., 1999). Sheep naturally infected with scrapie are a good animal model for the investigation of the neuropathology of infectious prion diseases (Lyahyai et al., 2006).

We have previously reported the induction of different apoptotic pathways in sheep infected with natural scrapie in a late symptomatic phase (Serrano et al., 2009, 2011). The Bax protein distribution correlates with prion protein immunolabelling in the CNS of scrapie sheep (Lyahyai et al., 2006, 2007) at terminal phases of the disease. The highest intraneuronal and neuropil Bax staining was observed in locations where the prion protein accumulates in the classic forms of scrapie, including the spinal cord, brain stem, hypothalamic region, mesencephalon and colliculus (Bolea et al., 2005).

In a recent study, we analysed the relationship between the expression profiles of genes involved in the mitochondrial and extrinsic pathways of apoptosis and the presence of neuropathological features (Serrano et al., 2009) in the terminal phase of scrapie. Both pathways seemed to be activated in natural scrapie, although the regulation was different depending on the area studied. The mitochondrial apoptosis pathway was positively related to prion deposition in the frontal cortex, an area with a very low lesion degree, suggesting that this pathway could also be activated during the early stages of prion deposition. Despite the significant increase of *BAX* (Lyahyai et al., 2007) in the scrapie-infected CNS, we reported a lack of apoptosis induction.

In addition to protective mechanisms, the lack of apoptosis in the animals analysed could reflect that apoptosis has already occurred during the early phases of scrapie. There is evidence of oxidative stress (Yun et al., 2006) and activation of the mitochondrial pathway of apoptosis (Brown et al., 2005) in the presymptomatic phase of mouse scrapie. Moreover, changes in global gene expression activities manifest prior to the PrP^{Sc} accumulation and appearance of symptomatic signs in cattle orally infected with BSE (Tang et al., 2009). These changes may therefore be responsible for the subsequent pathological and symptomatic events.

We present in this report the first neuropathological and gene expression analysis in presymptomatic scrapie sheep. Changes in scrapie-related lesions, Bax and caspase-3 immunolabelling and the apparition of cell death, measured using TUNEL and NeuN cell counting, were assessed by comparing the presymptomatic and symptomatic phases of the disease. Moreover, the activation of different factors involved in the mitochondrial pathway of apoptosis was studied by the analysis of gene expression profiles using real time quantitative polymerase chain reaction (RT-qPCR).

2. Material and methods

2.1. Animals and sample collection

Nineteen female *Rasa aragonesa* sheep were included in the present study; these included 6 animals in the presymptomatic (PS) and 4 symptomatic (S) phases of scrapie as well as 9 control animals. The use of the animals included in the study as well as the age and outbreak details is shown in Table 1. Symptomatic scrapie was diagnosed *in vivo* by means of neurological examination. When these animals were sacrificed, all exhibited symptomatic signs of scrapie in a terminal state. Presymptomatic animals were chosen by third eyelid biopsy (Vargas et al., 2006). Post-mortem diagnosis was carried out in medulla oblongata using commercial rapid tests (Idexx Herd Chek®/BioRad TeSeE® sheep/goat detection kit) and immunohistochemical techniques to detect PrP^{Sc} (Bolea et al., 2005). The animals came from a closed regulatory monitored flock and the Spanish Scrapie Surveillance Programme. Control animals of the same breed were selected from a different flock in which no scrapie cases have been reported to date. All animals analysed were adults of different ages that displayed the ARQ/ARQ genotype for *PRNP*, which is the most susceptible genotype in this breed of animals (Acin et al., 2004). Care and handling of the animals were performed according to the rules established by the National Research Council's Guide.

Table 1
Details of the analysed animals and their use for immuno-histochemistry (IHC) or Real-Time quantitative PCR (RT-qPCR). Symptomatic status of the animals: control (C), presymptomatic (PS) or symptomatic (S).

Animals	Age	Symptomatic status	IHC	RT-qPCR
C1	8	C	–	✓
C2	9	C	–	✓
C3	8	C	✓	✓
C4	8	C	✓	✓
C5	8	C	✓	✓
C6	7	C	✓	✓
C7	7	C	✓	✓
C8	6	C	–	✓
C9	4	C	–	✓
SC1	4	PS	✓	✓
SC2	4	PS	✓	✓
SC3	4	PS	✓	✓
SC4	1	PS	✓	✓
SC5	4	PS	✓	✓
SC6	2	PS	–	✓
SC7	3	S	✓	–
SC8	4	S	✓	–
SC9	6	S	✓	–
SC10	3	S	✓	–