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P-GLYCOPROTEIN EXPRESSION IS INCREASED IN THE BRAIN OF BSE AFFECTED CATTLE

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ABSTRACT

P-glycoprotein (P-gp) plays a major role as an efflux pump for endogenous and exogenous substrates at the blood-brain barrier and is localized in the brain at the apical side of capillary endothelial cells. Bovine spongiform encephalopathy (BSE) is marked primarily by the build-up of protease resistant misfolded prion protein (PrP^{res}) in the brain. In the present study, the relationship between P-gp and BSE was investigated. An increase in the expression of vascular P-gp in the obex was found inclassical BSE, more prominent in the pre-clinical cases. This up-regulation of P-gp in the early stages of the disease might be a protective regulatory mechanism to increase the clearance of the abnormal PrP^{res} protein in an attempt to protect the brain from the accumulation of PrP^{res}.

INTRODUCTION

P-glycoprotein (P-gp) was first demonstrated by Juliano and Ling (1976) and described by Thiebaut *et al*. (1987) because of its major role as an efflux pump for endogenous and exogenous substrates. Being a key-player in the blood-brain barrier, P-gp is strongly expressed in the brain at the apical side of capillary endothelial cells. Reduced expression of P-gp may diminish this barrier protection and lead to increased exposure to possible toxic compounds.There are a number of diseases of the central nervous system in humans that appear to be associated with alterations in P-gp expression. These include diseases such as Alzheimer, Creutzfeldt-Jakob and Parkinson (reviewed by Lee *et al*., 2010). Bovine Spongiform Encephalopathy (BSE) is a fatal neurodegenerative disease of cattle known as 'Mad Cow disease'. The clinical signs of BSE may include tremors, gait abnormalities particularly of thehind limbs (ataxia), aggressive behavior, apprehension, and hyper reactivity to stimuli. As in Creutzfeldt-Jakob disease a normal host cellular protein, prionprotein (PrP^{c}), is affected by conformational change and aggregation, whichleads to the accumulation of a protease resistant protein PrP^{res} usually in the nervoussystem (Bruce *et al*., 1997). BSE first appeared in the mid-80s in the UK, soon evolved to epidemic proportions in the 90s and is naturally transmissible to a number of zoo species (Sigurdson *et al*, 2003). The use of meat and bone meal (MBM), possibly contaminated with infectious mammalian pathogenic prions, in cattle feed is considered the likely cause of the BSE epidemic (Sigurdson *et al*., 2003). The characteristic histological changes in the central nervous system (CNS) are bilateral and usually symmetrical vacuolization of grey matter neuropil (spongiforme change) and/or vacuolization of neurons, astrocytosis and neuronal degeneration. In cattle withclassical BSE, these changes have predilection for certain neuroanatomical nuclei, particularly within one part of the brain stem, the obex (Novakofski *et al*., 2005). This characteristic lesion profile in cattle is the basis for routine histological screening for BSE together with the immunohistochemical detection of PrP^{res} deposits in the obex. Besides classical BSE, twowell identified atypical BSE forms have been distinguished by Western immune blot on the basis

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of the signature of the proteinase K-resistant fragment of the pathologic PrP^{res} with higher or lower molecular masses of PrP^{res} (H-type an L-type BSE respectively)(Jacobs *et al*., 2007). They differ from the classical form as they occur in older animals, have predilection forthe cerebrum, are not originating from the contamination of the MBM and might correspond to natural "sporadic" forms of BSE. Most of atypical cases have been detected during active surveillance targeting fallen stock and slaughtered animals (Ducrot *et al*., 2008).So far the pathogenesis of BSE is largely unexplained. In Creutzfeldt-Jakob disease, Vogelgesang *et al*. (2006) found a decrease of cerebrovascular P-gp expression. They suggested that decreased expression of P-gp at the level of the blood-brain barrier may facilitate the accumulation of PrP^{res} in certain areas of the brain.The purpose of this retrospective study was to evaluate P-glycoprotein expression in classical and atypical BSE cases compared to healthy animals and animalswith nervous symptoms related to other brain lesions such as inflammation and necrosis due to Listeriosis, which is the most important differential diagnosis of BSE (Roels *et al*., 2009).

MATERIAL AND METHODS

Tissue

Tissue samples of the obex of four groups of ten bovines, all between four and seven years old, were investigated. The first group consisted of ten animals with nervous symptoms due to clinical BSE, diagnosed with a rapid enzyme-linked immunosorbent assay (ELISA) (TeSeE-kit Bio-Rad, Nazareth, Belgium), and confirmed positive by histopathology and immunohistochemistry. The control group consisted of ten healthy BSE negative animals. The third group consisted of ten animals diagnosed with nervous symptoms due to Listeriosis. And the last group consisted of ten pre-clinical BSE animals, positive on ELISA, without clinical symptoms or histopathological evidence of BSE. Additionally, the obex of five animals with atypical BSE (three of the H-type and two of the L-type) were included.

Histopathology and Immunohistochemistry

Samples of the brain stem (obex) were fixed in neutral-buffered formalin, embedded in paraffin wax, sectioned at 4µm and stained with haematoxylin and eosin (HE) according to standard protocols. For demonstration of P-glycoprotein, themonoclonal antibody C219 (SIG-38710; SA Eurogentec, Ougreé Seraing, Belgium) was applied on paraffin sections at adilution of 1:5. For visualization Envision/HRP mouse (DAB+) kit (DAKO, Glostrup, Denmark) was used for immunolabeling. This kit also blocks endogenous peroxidase. An antibody diluent (Dako, Glostrup, Denmark) with background-reducing components was used to block hydrophobic reactions.

Image analysis and Statistical analysis

Quantification of P-gp positivity was performed on five randomly chosen fields in the obex of each animal, using an Olympus BX50 F4Microscope and Digital Camera PM-C35B (Olympus NV, Aartselaar, Belgium) and a personal computer-based image analysis system (Motic image advanced 3.2, Motic group CO. LTD, Xiamen, China) measuring the area of positivity relative to the total area (%) of the fields. Data and Post Hoc analysis of the results was performed using the 'pgirmess' package (Giraudoux, 2012) in the statistical R environment(R Core Team, 2012). The area of positivity relative to the total area (%) of the fields was compared between the groups, using then on-parametric Kruskal-Wallis multiple comparison test. Significance was declared when $P<0.05$. In all samples, immunohistochemistry and image analysis was performed blinded.

RESULTS

P-gp expression was found at the level of the endothelial cells in all samples of all animals (Fig 1). Pairwise comparison of the mean ranks between groups, however, indicated that percentage of P-gp positive area was significantly higher in the group of pre-clinical BSE positive animals (Fig 2 and 3) compared to controls and animals with Listeriosis ($P<0.05$).

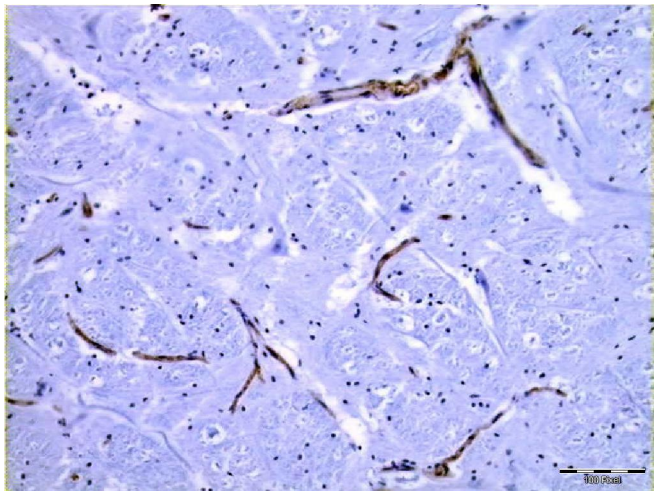

Fig. 1. P-gp expression (brown) in endothelial cells at the level of the brain stem (obex) of a classical BSE positive animal. Immunohistochemistry

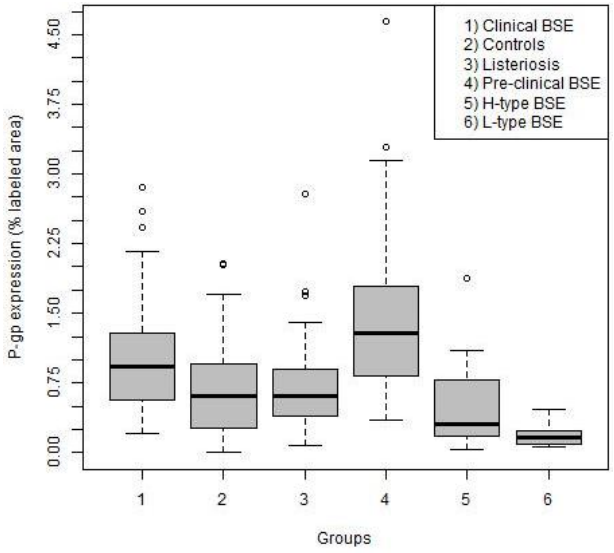

Fig. 2. Boxplots of P-gp positive area in the obex of all types of cattle investigated

Animals with clinical BSE also had a tendency towards increased P-gp expression. Listeriosis didn't affect the

percentage of P-gp positive area compared to the healthy animals. Atypical BSE cases of the L-type, however, had significantly decreased percentage of P-gp positive area in the obex. Also atypical BSE cases of the H-type tended to express P-gp in smaller percentage of the area when compared to the controls.

Multiple Comparison test after Kruskal Wallis:			
p.value: 0.05			
Groups compared	obs.dif.	crit.dif.	obs.>crit.
1-2	37.14000	38.21400	FALSE
1-3	35.77000	38.21400	FALSE
1-4	28.87000	38.21400	FALSE
1-5*	62.55667	56.24948	TRUE*
1-6*	106.49000	66.18859	TRUE*
2-3	1.37000	38.21400	FALSE
2-4*	66.01000	38.21400	TRUE*
2-5	25.41667	56.24948	FALSE
2-6*	69.35000	66.18859	TRUE*
3-4*	64.64000	38.21400	TRUE*
3-5	26.78667	56.24948	FALSE
3-6*	70.72000	66.18859	TRUE*
4-5*	91.42667	56.24948	TRUE*
4-6*	135.36000	66.18859	TRUE*
5-6	43.93333	78.00400	FALSE

DISCUSSION

The four groups were age matched, as age related decrease of P-gp is described in older humans (Vogelgesang *et al*., 2004) and dogs (Pekcec *et al*., 2011). Our results show that P-gp expression within endothelial cells of the capillaries in the obex is significantly higher in cases of pre-clinical BSE than in control cases. This increase in P-gp expression is in contrast to the decrease in P-gp described in Creutzfeldt-Jakob disease in humans (Vogelgesang *et al*., 2006). It is known that P-gp can be induced by a wide range of endogenous and exogenous substrates such as cytokines, toxic components (Epel, 1998; Borst and Elferink 2002), drugs (Thuerauf and Fromm, 2006), food (Zhang *et al*., 2009) and in certain diseases in humans and animals such as epilepsy (Pekcec *et al*., 2009). This up-regulation of P-gp is also described in dogs with diffuse Abeta (major component of amyloid) plaques (Pekcec *et al*. 2011) and in the early stages of Alzheimers' disease in humans (Vogelgesang *et al*., 2004). As suggested by these authors, the P-gp up-regulation might be induced by the diffuse Abeta plaques in the brain and might act as a compensatory mechanism to increase Abeta clearance from the brain. In later stages of this disease P-gp is diminishing and finally strongly reduced or completely lost.

A possible explanation for our results is an up-regulation of P-gp induced by the protease resistant protein PrP^{res}, acting as a defense mechanism of the body in an attempt to eliminate this PrP^{res} in BSE positive animals.In the present study P-gp increase is more evident in the pre-clinical stage than in the clinical stage of BSE. As the animals are killed immediately when showing clinical symptoms we might miss the down-regulation in further stages of this neurological disorder as described in Alzheimers' disease (Vogelgesang *et al*., 2004) and Creutzfeldt-Jakob disease (Vogelgesang *et al*; 2006). As mentioned by Vogelgesang *et al*. (2006 and 2011) there is no clear evidence that PrP itself is substrate for P-gp and the possibility of P-gp indirectly interacting with protein-degrading enzymes is suggested. It seems that P-gp is involved in the protection of the brain against accumulation of misfolded proteins in several neurodegenerative diseases such as Alzheimer, Parkinson, Amyotrophic Lateral Sclerosis and probably Huntington disease too (Bartels, 2011). Further studies are needed to clarify the relationship between PrP and P-gp in BSE and Creutzfeldt - Jakob disease. In contrast to classical BSE, atypical L-type BSE cases showed a significant reduction of P-gp. H-type also tended to show reduced expression. Few explanations for the low P-gp levels in the atypical cases can be proposed. On one hand, it is possible that the brain stem is not the best part of the brain to examine atypical BSE as it is not the predilection site for PrP^{res} in L- and H-type BSE. On the other hand, it is more likely that the findingsare more in line with the decrease of P-gp in idiopathic Creutzfeldt-Jakob disease in humans as atypical BSE is also an idiopathic sporadic variant in older animals.In this way we didn't miss the down-regulation in further stages of this neurological disorder as described in Creutzfeldt-Jakob disease (Vogelgesang *et al*; 2006). Because of the low occurrence of the atypical form worldwide(only 80 cases, according to the latest updates by personal communication (Polak)) and thus the low sample size in this study, the results have to be interpreted carefully.

Conclusions

This study demonstrated for the first time a significant up-regulation of P-gp in the brain stem in pre-clinicalclassical BSE, which might be the result of a protective regulatory mechanism in an attempt to increase the clearance of the abnormal PrP^{res} protein.

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