Elevation of apolipoprotein E in the CSF of cattle affected by BSE

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Abstract The cerebrospinal fluid (CSF) of patients suffering from Creutzfeldt-Jakob disease (CJD) display two unique polypeptide chains by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). In the absence of a well-defined ante-mortem diagnostic test for bovine spongiform encephalopathy (BSE), spinal fluid samples of eight normal cows and eight cows known to carry BSE by post-mortem histological analysis were investigated to verify if equivalent polypeptides were present. Proteins with similar migration to human CJD polypeptides were not detected. But surprisingly, a cluster of polypeptide spots that was faint or not detected in normal bovine CSF samples was found to be elevated or massively increased in BSE CSF samples (more than 10-fold increase). These elevated polypeptide chains were identified as apolipoprotein E.

Key words: Bovine spongiform encephalopathy; Creutzfeldt-Jakob disease; Two-dimensional gel electrophoresis; Apolipoprotein E; Cerebrospinal fluid

1. Introduction

Creutzfeldt-Jakob disease (CJD) and bovine spongiform encephalopathy (BSE) are fatal degenerative neuronal disorders known as the transmissible spongiform encephalopathies (TSE). Despite the decline in the number of new cases of BSE, most likely because of preventive action, concern about BSE and its possible link to CJD remains [1]. Recently, Collinge et al. [2] suggested that the molecular weight and glycosylation pattern of the PrP^{Sc} of the new CJD variant are similar to those seen in BSE-infected cattle, but different from other CJD patterns. Their method could be used as a post-mortem diagnostic tool. It is clear that diagnostic test development is required for ante-mortem screening and/or prognostic evaluation of BSE [3]. The cerebrospinal fluid (CSF) of CJD patients displays two additional polypeptides on two-dimensional polyacrylamide gel electrophoresis [4–6]. The function of these 14-3-3 polypeptides remains unclear in TSE. To see if the same proteins are present in BSE, and thus investigate any possible link in the diseases and to search for altered expression of other proteins, we analysed CSF samples of eight normal and eight BSE-affected cows.

2. Materials and methods

Detailed materials and methods can be found on our web site at the following address: http://expasy.hcuge.ch/ch2d/technical-info.html/.

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2.1. CSF samples

Samples (a kind gift of the Ministry of Agriculture, Fisheries and Food of the UK) were collected immediately after death by cisterna magna puncture from eight BSE-affected adult British cattle and eight normal adult British cattle (not fed ruminant-derived protein). CSF samples were stored at −20°C until 2-D PAGE analysis.

2.2. Analytical two-dimensional gel electrophoresis

CSF from eight controls and eight BSE animals was analysed by high resolution 2-D PAGE. An aliquot of 500 μl of bovine CSF was mixed with 1000 μl of ice-cold acetone and centrifuged at 10 000 × *g* at 4°C for 10 min. The pellet was mixed with 10 μl of a solution containing SDS (10% w/v) and DTE (2.3% w/v). The sample was heated to 95°C for 5 min and then diluted to 60 μl with a solution containing urea (8 M), CHAPS (4% w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. The whole final diluted CSF sample (100 μg) was loaded on the first dimensional separation. A commercial sigmoidal immobilised pH gradient (IPG) from pH 3.5 to 10.0 was used for first dimensional separation [7]. After equilibration, the IPG gel strips were transferred for the second dimension onto vertical gradient slab gels (9–16% T) and run with the Laemmli-SDS discontinuous system [8]. Protein detection was achieved using a sensitive ammoniacal silver stain [7]. Silver-stained gels were scanned and the 2-D PAGE images were analysed using the MELANIE II software package. Spots were detected and quantified automatically. The optical density, the area and the volume were computed and directly related to protein concentration. The relative optical density and relative volume were also calculated in order to correct for differences in gel staining.

2.3. Preparative two-dimensional gel electrophoresis

An aliquot of 5 ml of CSF from BSE-affected cattle was mixed with 10 ml of ice-cold acetone and centrifuged at 10 000 × *g* at 4°C for 10 min. The pellet was mixed with 500 μl of a solution containing urea (8 M), CHAPS (4% w/v), DTE (65 mM), Resolytes 4-8 (2% v/v) and a trace of bromophenol blue. This whole final diluted CSF sample (1 mg) was used for in-gel sample rehydration before first dimensional separation [9]. A home-made narrow immobilised pH gradient (IPG) of pH 4–6 was used for first dimensional separation. After equilibration, the IPG gel strips were transferred onto vertical gradient slab gels (9–16% T) for the second dimension [8]. Then, electroblotting onto PVDF membranes was done using a home-made semi-dry apparatus with 10% methanol and 10 mM CAPS as buffer (pH 11) at 200 V for 2 h. After electrotransfer, the PVDF membranes were stained in a solution containing amido black (0.5% w/v), isopropanol (25% v/v) and acetic acid (10% v/v) for 1 min. Destaining was done by several soakings in deionised water. The amido black-stained polypeptides of interest were excised with a razor blade and N-terminal sequence determination was performed (20 cycles) using an ABI model 473A or 477A microsequencer from Applied Biosystems equipped with Problott cartridges.

2.4. Immunoblotting

In order to confirm the apolipoprotein E identification, a polyclonal sheep antibody against human ApoE was used for immunoblotting (Chemicon International Ltd). The PVDF membrane of a 2-D PAGE BSE-affected cattle CSF sample was stained with amido black and scanned. The membrane was first blocked with a blocking solution (5% (w/v) dry low fat milk, 0.5% (v/v) Tween 20 and 10 mM PBS (pH 7.4)) for 30 min, probed in the same solution containing 1:500 of the primary polyclonal antibody against apolipoprotein E for 2 h and washed for 3 × 10 min in 10 mM PBS (pH 7.4) and 0.5% (v/v) Tween

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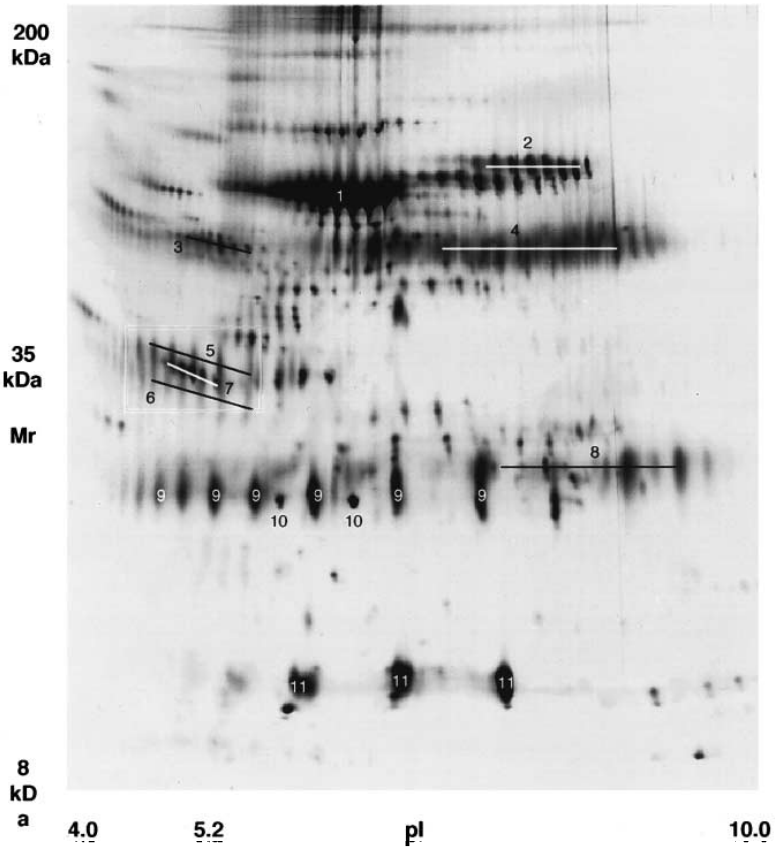

Fig. 1. Silver-stained 2-D PAGE image of BSE-affected CSF. Proteins identified by Edman degradation were: (1) albumin, (2) transferrin, (3) α-antitrypsin, (4) immunoglobulin-γ heavy chains, (5) apolipoprotein J α chain, (6) apolipoprotein J β chain, (7) apolipoprotein E, (8) immunoglobulin light chains, (9) prostaglandin-D synthase, (10) apolipoprotein A1 and (11) transthyretin. The boxed square shows the area enlarged in Fig. 2.

20. The secondary goat anti-sheep antibody (1:1000, DAKO) labelled with peroxidase was mixed with the blocking solution. The membrane was soaked in the latter solution for 1 h and then washed for 4×10 min in 10 mM PBS (pH 7.4) and 0.5% (v/v) Tween 20. Protein detection was achieved using enhanced chemiluminescence and X-ray films as described by the manufacturer (Boehringer Mannheim). The films were scanned using a laser densitometer and the 2-D PAGE image computer analysis and comparison were carried out using the MELANIE II software package.

3. Results

A bovine CSF 2-D PAGE protein map was first established and polypeptides were identified by amino acid microsequencing (Fig. 1). Spots equivalent to those found in CJD were not detected. However, a cluster of five or six spots was either absent or faint in normal bovine CSF and elevated or massively increased in BSE samples (Fig. 2). They covered a p*I* of 5.1–5.3 and a M_r of 34–37 kDa. N-terminal microsequencing of three of the spots gave the sequence D-M-E-G-E-L-G-P-E-E-P-L-X-X-Q-Q-P-R-X-K which identified the polypeptides as bovine apolipoprotein E (bApoE). Polyclonal antibodies against human ApoE confirmed this identification and revealed that all the polypeptides in the cluster were isoforms of bApoE. By densitometric measurement, a 10-fold difference was seen between the levels of bApoE in BSE samples. By densitometric measurement, all BSE-affected samples showed

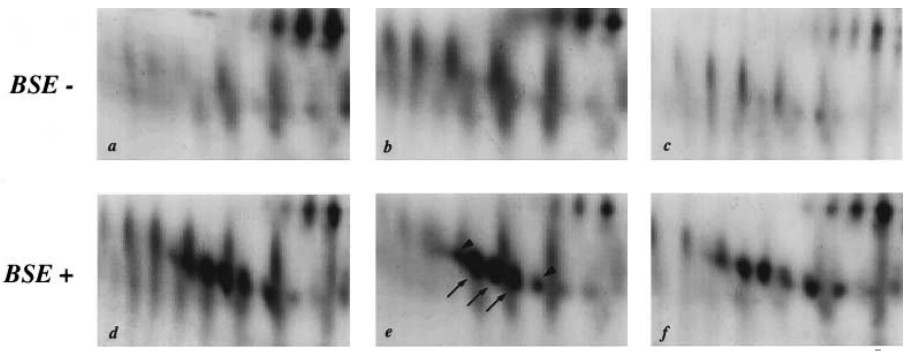

Fig. 2. Control CSF samples (a, b, c); BSE-affected CSF samples (d, e, f). The three arrows show the microsequenced spots, and the two arrowheads show additional spots identified with immunoblotting.

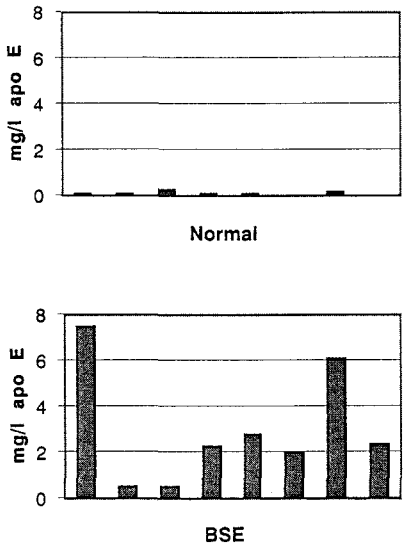

Fig. 3. Apolipoprotein E level quantitation. Total protein in each CSF sample was assayed according to Bradford [17], and percent volume of spot density was then used to calculate approximate apolipoprotein E concentration.

higher levels of ApoE than normal samples (Fig. 3). Overall, this represented a significant difference in concentration ($P < 0.001$). Interestingly, there was little variation in the quantity of ApoE detected in normal samples; however, a 15-fold difference in ApoE quantity was seen within the set of samples from BSE-affected animals. We could not establish in this study if this difference in ApoE quantity in BSE-affected animals correlated with the severity of the disease in each case.

4. Discussion

The role of the ApoE in the nervous system has been extensively studied, but its function remains incompletely understood. ApoE is known to play a role in nerve growth and nerve regeneration, and some of its phenotypes appear to affect susceptibility to Alzheimer's disease in humans [10–12]. Interestingly, the level of ApoE is known to be elevated in several situations, including following peripheral nerve injury [13] in activated astrocytes in Alzheimer's disease [14], and in the brains of mice infected with scrapie [15]. Our discovery of elevated levels of ApoE in the CSF of BSE-affected cows is thus significant and correlates with the previous results described by Jones et al. [16], as it further implicates the involvement of this protein in abnormal neuronal processes, including prion diseases. Perhaps more importantly, it presents a possible means of screening cattle for the nerve damage that accompanies BSE. The present paper described direct sequence determination of the N-terminus of three of the altered 2-D spots corresponding to bovine apolipoprotein E. We are currently undertaking a time course study in a larger group of cattle to determine when ApoE elevation takes place in the disease course, and if a threshold level of ApoE can be established for early diagnosis of BSE.

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