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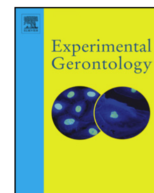

Proteomic analysis of age-related changes in ovine cerebrospinal fluid

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ABSTRACT

Cerebrospinal fluid (CSF) circulates through the brain and has a unique composition reflecting the biological processes of the brain. Identifying ageing CSF biomarkers can aid in understanding the ageing process and interpreting CSF protein changes in neurodegenerative diseases. In this study, ovine CSF proteins from young (1–2 year old), middle aged (3–6 year old) and old (7–10 year old) sheep were systemically studied. CSF proteins were labelled with iTRAQ tagging reagents and fractionated by 2-dimensional high performance, liquid chromatography. Tryptic peptides were identified using MS/MS fragmentation ions for sequencing and quantified from iTRAQ reporter ion intensities at *m/z* 114, 115, 116 and 117. Two hundred thirty one peptides were detected, from which 143 proteins were identified. There were 52 proteins with > 25% increase in concentrations in the old sheep compared to the young. 33 of them increased > 25% but < 50%, 13 increased > 50% but < 1 fold, 6 increased > 1 fold [i.e. haptoglobin (Hp), haemoglobin, neuroendocrine protein 7B2, IgM, fibrous sheath interacting protein 1, vimentin]. There were 18 proteins with > 25% decrease in concentrations in the old sheep compared to the young. 17 of them decreased > 25% but < 50%, and histone deacetylase 7 (HDAC7) was gradually decreased for over 80%. Glutathione S-transferase was decreased in middle aged CSF compared to both young and old CSF. The differential expressions of 3 proteins (Hp, neuroendocrine protein 7B2, IgM) were confirmed by immunoassays. These data expand our current knowledge regarding ovine CSF proteins, supply the necessary information to understand the ageing process in the brain and provide a basis for diagnosis of neurodegenerative diseases.

1. Introduction

Cerebrospinal fluid (CSF) is a clear, colourless bodily fluid that circulates through the brain and communicates freely with the brain extracellular fluid. In adult humans, about two-thirds of CSF is secreted by the choroid plexus (CP) at the rate of about 0.35–0.4 ml/min or 500–600 ml/day, while the remainder is from the extrachoroidal sources (Ghersi-Egea et al., 2018). CSF has a unique composition that is different from the plasma, due to the presence of blood-brain barrier (BBB) and blood-CSF barrier (BCSFB), the active secretion at the CP and specific transport systems for moving substances between blood and CSF. In comparison to plasma ultrafiltrate, CSF contains higher concentrations of sodium, chloride, and magnesium and lower concentrations of glucose, proteins, amino acids, uric acid, potassium, bicarbonate, calcium and phosphate (Shen et al., 2004). The CSF is in direct contact with the brain interstitial fluid, and the composition of the CSF therefore reflects biological processes occurring in the brain (Stoop et al., 2010). This understanding has led to an interest in the potential for discovering biomarkers within the CSF, which can be used to monitor brain function and aid in diagnosis of neurological diseases.

During ageing, several structural, chemical, and functional changes occur in the brain. The weight and volume of both cerebral cortex and hippocampus reduce, and the ventricular system expands to fill the space vacated by the brain parenchyma (Wyss-Coray, 2016). In contrast, the CP in the lateral ventricles doubles in weight, leading to significant increase of the ratio of the CP and the brain in weight (Spector and Johanson, 1989; Chen and Preston, 2012). The CSF secretion by the CP is decreased, leading to a decreased overall turnover

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of CSF which can affect its protein composition.

There are few studies on the CSF biology during ageing in absence of any neurological diseases, and the drastically incomplete knowledge hinders us from understanding the ageing process in the brain. In this study, we systemically characterized age-related protein changes in ovine CSF using a gel free proteomic mass spectrometry (MS) approach with isobaric labelled samples (iTRAQ) techniques. The advantage of using sheep in this study is that adequate CSF samples of all age groups can be obtained, with facile control over gender selection and environmental factors. Furthermore, we have previous characterized the age-related changes in the CP structure and function in the sheep, and found that the secretion rate of the CP is decreased thus slowed overall CSF turnover, which contributed to the increased protein concentrations within the CSF with increasing age (Chen and Preston, 2012; Chen et al., 2009; R.L. Chen et al., 2010; C.P. Chen et al., 2010; Chen et al., 2012).

2. Methods

2.1. Sample collection

Clun Forest strain adult female sheep aged between 1 and 10 year old were divided into 3 groups: young (aged 1–2 years); middle-aged (3–6 years) and old (7–10 years). Each group comprises 7 sheep. Sheep were anaesthetized with *i.v.* thiopentone sodium ($20\ mg\cdot kg^{-1}$) and heparinised ($20{,}000$ IU heparin kg^{-1}). CSF samples were collected from the cisterna magna by needle puncture (Chen et al., 2006). Samples were spun at 10,000*g* for 10 min at 4 °C. CSF samples with any blood contamination determined by the presence of erythrocytes in the solution or precipitation were discarded. Supernatants were immediately stored at −80 °C until being analysed. All procedures were within the Home Office Scientific procedures Act, 1986 (HMSO, London, UK), and were approved by King's College London research ethics committee.

2.2. Sample preparation for MS analysis

Equal volumes of CSF samples from seven individuals within each group were pooled to give a final volume of 80 μl per age group. An internal standard (IS) was prepared by pooling equal amount of each sample from the analysis set. The pooled samples were then digested with sequencing grade porcine trypsin (Promega, Fitchburg, Wisconsin, USA) overnight at 37 °C, followed by reduction and alkylation steps performed according to the instructions outlined in the iTRAQ labelling kit (AB Sciex, Framingham, MA, USA). Following this, the digests were then dried down in a vacuum centrifuge and iTRAQ labelling carried out also according to instructions in the iTRAQ labelling kit. Each iTRAQ tag was assigned as follows: iTRAQ reagent 114 for Young, 115 for Middle-age, 116 for Old and 117 for the IS.

Both MS and MS/MS analysis was performed on the fractionated peptides using an Applied Biosystems 4800 MALDI TOF/TOF mass spectrometer. The mass spectrometer was operated under control of 4000 Series Explorer v3.5.2 software (Applied Biosystems, Waltham, Massachusetts, USA). A total of 1000 shots per MS spectrum (no stop conditions) and 2500 shots per MS/MS spectrum (no stop conditions) were acquired. The following MS/MS acquisition settings were used: 2 kV operating mode with CID on and precursor mass window resolution set to 300.00 (FWHM). Peak lists of MS and MS/MS spectra were generated using 4000 Series Explorer v3.5.2 software and the following parameters were used after selective labelling of monoisotopic mass peaks: MS peak lists: S/N threshold 10, Savitzky Golay smoothing (3 points across peak (FWHM)), no baseline correction, MS/MS peak lists: S/N threshold 14; smoothing algorithm: Savitzky Golay, smoothing (7 points across peak (FWHM)).

2.3. Quantitative MS analysis

Liquid chromatography fractionation and subsequent MS analysis was conducted as described before (Fuller et al., 2010). Briefly, labelled tryptic peptides obtained from protein digestion were pooled and dissolved in 2.4 ml of SCX buffer A (10 mM phosphate, 20% acetonitrile) and centrifuged at 15,000 × *g* for 5 min to remove any insoluble debris. The peptide mixture was loaded onto a Polysulphoethyl A column (4.6 mm × 100 mm, 5 μm, 300 Å, PolyLC Inc.) using a flow rate of 800 μl/min. The bound sample was washed using SCX buffer A for approximately 20 min, until non-binding mixture components were removed (as determined by the UV traces returning to baseline levels). Peptides were then eluted at 400 μl/min using a gradient of SCX buffer B (SCX A containing 1 M potassium chloride) of 0–30% over 30 min. Fractions were collected manually at 60 s intervals. SCX fractions containing peptides were dried to completeness and dissolved in 30 μl of RP buffer A (2% acetonitrile) before separating further using an Ultimate 3000 chromatography system connected to a Probot fraction collector (Dionex, Sunnyvale, CA, USA). Samples were randomised and loaded onto a C18 trapping column before eluting onto a 75 μm internal diameter C18 PepMap column. Peptides were washed for 15 min in RP buffer A before eluting with a 2–50% gradient of acetonitrile over 120 min, followed by further elution with 90% acetonitrile for 15 min. Eluted peptide fractions were spotted onto a MALDI-MS target plate every 10 s with 3 mg/ml α-cyano-2-hydroxy cinnamic acid at a flow rate of 1.2 μl/min. A blank injection was performed between peptide runs to minimise sample carry-over.

Peptides were analysed using an Applied Biosystems 4800 Proteomics Analyser with close external standards to calibrate the instrument. Survey spectra were collected from the range 900 to 4000 Da, with a focus mass of 2500 Da. Fifty shots were fired per sub-spectrum, with 1000 total shots per spectrum without stop conditions. All subspectra were accepted, and laser intensity was set to 3750. For product ion analysis, a maximum of 10 precursor ions per spot were selected automatically with a minimum signal-to-noise ratio of 40. Lowest-intensity precursors were acquired first.

An automated database search was run using GPS Explorer v3.6 (AB Sciex, Framingham, MA, USA). MASCOT was used as the search engine to search the NCBI non-redundant database version Oct 04, 2011, using the following search parameters: species = mammals; precursor ion mass tolerance of 150 ppm; MS/MS fragment ion mass tolerance of 0.3 Da; iTRAQ fragment ion mass tolerance of 0.2 Da. Oxidation of methionine residues were allowed as variable modifications, and N-term (iTRAQ), lysine (iTRAQ) and MMTS modification of cysteine residues were set as fixed modifications. Protein was identified on the basis of having at least one peptide with an ion score above 95% confidence. All identified CSF proteins were subjected to functional analyses using PubMed search (https://www.ncbi.nlm.nih.gov/pubmed/) and the PANTHER classification system (http://www.pantherdb.org/genes/batchIdSearch.jsp) (Mi et al., 2013). Only peptides unique for a given protein were considered for relative quantification. iTRAQ Ratios were normalised using the following formula: iTRAQ Ratio = Ratio / (median iTRAQ Ratio of all found pairs) that was applied in GPS Explorer software.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Experiments were performed using commercially available ovine protein ELISA kits: sheep haptoglobin (HP), sheep neuroendocrine protein 7B2 (MyBioscience, San Diego, CA, USA), according to the manufactory instructions. The target protein concentrations in CSF were determined according to the standard curve with known concentrations of the target proteins.

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