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Short communication

Increased levels of cell-free mitochondrial DNA in the cerebrospinal fluid of patients with multiple sclerosis

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ABSTRACT

Mitochondrial DNA (mtDNA) can act as damage-associated molecular pattern molecule (DAMP) and initiate an inflammatory response. We hypothesized that the concentration of mtDNA might reflect inflammatory activity in multiple sclerosis and investigated therefore levels of cell-free mitochondrial DNA in cerebrospinal fluid of patients with relapsing-remitting multiple sclerosis. Significantly higher levels of mtDNA were found in patients compared to controls and there was an inverse correlation between disease duration and mtDNA concentration. Our study suggests that mitochondria can be involved early in multiple sclerosis, but whether this is as an initiator of the inflammatory response or part of its maintenance is unclear. Further, our study suggests that changes in mtDNA may provide a novel marker for early disease activity.

1. Introduction

Multiple sclerosis is an immune-mediated, central nervous system (CNS) inflammatory disease of unknown etiology. Studies suggest that while inflammation appears to be the important component in relapsing-remitting multiple sclerosis, neurodegeneration is present at an early stage (Trapp et al., 1998) characterizing a combined inflammatory and neurodegenerative pathogenesis of the disease.

In view of the unpredictable and heterogeneous disease course of multiple sclerosis, ways of measuring disease activity and treatment response are highly relevant (Stuve and Racke, 2016). Neurofilaments (NF) are axonal cytoskeletal proteins that are released following axonal damage and can, therefore, be used as a marker for neuronal injury. Levels of NF-light (NF-L) are increased in the cerebrospinal fluid (CSF) and serum of patients with relapsing-remitting multiple sclerosis (Kuhle et al., 2016) and correlate with CSF lactate levels suggesting a role of mitochondrial dysfunction in the pathogenesis of multiple sclerosis (Albanese et al., 2016). Elevated levels of NF-L have also been found to decrease following natalizumab treatment (Gunnarsson et al., 2011). Despite these findings, there are no well-established blood or CSF markers to define disease activity, treatment response in multiple sclerosis or transition from a relapsing-remitting to a secondary progressive disease course.

Low levels of free mitochondrial DNA (mtDNA) are found in the CSF where it is assumed they reflect normal turnover of mtDNA in the brain. Recently, decreased levels of cell-free mtDNA were demonstrated in the CSF of patients with Alzheimer's and Parkinson's disease, suggesting mtDNA could be a marker for neurodegeneration (Podlesniy et al., 2013; Pyle et al., 2015). In contrast, elevated levels of mtDNA were found in the CSF of children with traumatic brain injury, indicating mtDNA could be a mediator for sterile inflammatory responses (Walko et al., 2014).

In the present study, we asked the question is cell-free mtDNA in the CSF of patients with relapsing-remitting multiple sclerosis elevated and if so, does this reflect disease activity.

2. Material and methods

2.1. Subjects

This retrospective study investigated 21 newly diagnosed patients with relapsing-remitting multiple sclerosis according to the revised McDonald criteria (Polman et al., 2011). Lumbar puncture (LP) was performed as part of their diagnostic assessment. None of the patients had received immunomodulatory therapy prior to LP. While all 21 patients

Abbreviations: DAMPs, damage-associated molecular pattern molecules; mtDNA, mitochondrial DNA; NF, neurofilaments; PAMPs, pathogen-associated molecular pattern molecules.

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had typical magnetic resonance imaging (MRI) findings, only 4 had gadolinium enhanced scans at the time of LP. The control group comprised 23 patients without inflammatory or neurodegenerative disease, and with normal CSF white cell count and normal MRI. Controls underwent LP for a variety of reasons including unspecific symptoms as headache, paresthesia, vertigo and fatigue. The groups were age-and gender-matched, but otherwise selected randomly. Table 1 summarizes the demographics and CSF findings.

The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics (No: 2014/1371), and written consent was obtained from all patients.

2.2. CSF collection and preparation

CSF was collected, centrifuged at 1900 rpm at 4 °C for 10 min, aliquoted and stored in polypropylene tubes at −80 °C in the Norwegian Multiple Sclerosis Registry and Biobank (Myhr et al., 2015). Each tube contained 500 μl CSF.

Using native, untreated CSF gave no amplification irrespective of the amount used, suggesting the presence of PCR-inhibitors. Filtering CSF using Amicon Ultra 0.5 ml, 30 kDa, columns, resulted in profound loss of mtDNA to levels below the confident detection range of our qPCR assay. To maximize DNA yield and at the same time minimize the presence of inhibitors, we therefore employed a DNA extraction protocol from 200 μl CSF using the QIAmp® DNA Mini Kit (Qiagen GmbH).

2.3. Determination of mtDNA copy number

The concentration of mtDNA was determined by quantitative polymerase chain reaction (qPCR). We amplified MTND1 to quantify mtDNA and the nuclear encoded gene amyloid precursor protein (APP) to detect any nuclear DNA contamination. To ensure that we only measured cell-free mtDNA, samples showing detectable amplification of APP (CT-value <37) were discarded (n = 7). The amount of mtDNA was calculated using a standard curve derived from serial dilutions of a gel-purified MTND1 amplicon. The serial dilutions contained 10^1–10^8 copies/μl. The following primers, probes and conditions were used. *MTND1:* forward primer: L3485–3504: 5′-CCCTAAAACCCGCCACATCT-3′, reverse primer: H3553–3532: 5′-GAGCGATGGTGAGAGCTAAGGT-3′, TaqMan® MGB probe: L3506–3529: 5′-FAM-CCATCACCCTCTACATCACCGCCC-3′. *APP*: forward primer: 5′-TGTGTGCTCTCCCAGGTCTA-3′, reverse primer: 5′-CAGTTCTGGATGGTCACTGG-3′, TaqMan® MGB probe: VIC-CCCTGAA CTGCAGATCACCAATGTGGTAG. 10 μl of CSF were used per cPCR reaction. All standard curves had amplification efficiency over 90%. PCR was performed in triplicate using an ABI 7500 Fast Real-time PCR system (v2.0.6 Life Technologies Corporation) using TaqMan ® Fast Advanced Master Mix (Thermofisher). Thermal cycling consisted of following profile: one cycle at 95 °C for 20 s, 45 cycles at 95 °C for 3 s and 60 °C for 30 s. Each run contained a negative control for both MTND1 and APP. To ensure reproducibility, 12 of the samples were replicated (27%), with results within a 5% coefficient of variation range. To avoid batch effect a random mix of controls and patient samples were always run together.

Table 1
Demographic and CSF data of subjects groups.

Variables	MS n = 21	Controls n = 23
Gender, n (%)		
Men	5 (24)	7 (30)
Women	16 (76)	16 (70)
Mean age at lumbar puncture, y (±SD)	41.9 (14.1)	42.1 (16.7)
CSF oligoclonal bands, n (%)		
Negative	0 (0)	17 (74)
1–2	1 (5)	5 (22)
3–9	3 (14)	1 (4)
>10	17 (81)	0 (0)
CSF white cell counts, n (%)		
Negative (0–3)	7 (33)	23 (100)
4–10	7 (33)	0 (0)
11–15	3 (14)	0 (0)
16–20	2 (10)	0 (0)
>20	2 (10)	0 (0)

MS = multiple sclerosis, CSF = cerebrospinal fluid, SD = standard deviation.

2.4. Determination of NF-light chain

Due to limited volume of CSF, determination of NF-L was performed on half of the samples (n = 21; 10 patients and 11 controls). The concentration of NF-L in CSF was measured using enzyme-linked immunosorbent assay (ELISA), according to the manufacture's protocol (UmanDiagnostics AB, Sweden).

2.5. Statistical analysis

Statistical analyses were performed in SPSS software (v.23.0.0.2) and Prism (v6; GraphPad). We used non-parametric tests since the data did not fit a normal distribution. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Levels of mtDNA in CSF

The amount of cell-free mtDNA in the CSF of patients was significantly higher than controls ($p = 0.003$, Mann-Whitney *U* test) (Fig. 1A): mean mtDNA concentration in patients was 188.8 copies/10 μl (median 123.9, SD 205.9, range 32.7–943.2) and 75.2 copies/10 μl (median 56, SD 54.1, range 10.2–217.7) in controls. There was no correlation between mtDNA concentration and age, sex, number of oligoclonal bands or CSF leukocyte count.

3.2. Levels of NF-L in CSF

Multiple sclerosis patient samples contained a significantly higher amount of NF-L in CSF compared to controls ($p = 0.005$, Mann-Whitney *U* test) (Fig. 1B). The mean concentration in patients was 1097.9 pg/ml (SD 652.6, range 351.1–2465.1), and 525.9 pg/ml (SD 562.3, range 127.1–2116) in controls. There was no correlation between mtDNA concentration and NF-L concentration.

3.3. Correlation with duration of symptoms

There was a statistically significant inverse correlation between mtDNA concentration and time interval since disease onset ($p = 0.007$, $r = -0.57$ Spearman's correlation) (Fig. 1C).

Too few of the patients had gadolinium-enhanced MRI examinations at the time of LP to permit correlation analysis.

4. Discussion

We found a significantly higher level of cell-free CSF mtDNA in patients with relapsing-remitting multiple sclerosis compared with controls. In addition, we found an inverse correlation between length of time symptoms had been present and levels of mtDNA. Our results suggest that mtDNA concentration may reflect early, active inflammatory activity, and that this could, potentially, provide an early marker for disease activity. Whether these findings are MS-specific, or reflect neuro-inflammation in general, will need to be investigated further.

Due to the low mtDNA copy number in CSF, qPCR reproducibility was a challenge. This was particularly true when using crude CSF, but occurred also after filtration. DNA extraction provided the only consistent results and while this raises the question of mtDNA released by