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Analysis of apoptosis-related genes in patients with clinically isolated syndrome and their association with conversion to multiple sclerosis

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ABSTRACT

To analyse whether the expression of apoptotic transcripts is associated with the conversion from clinically isolated syndrome (CIS) to multiple sclerosis (MS). Eleven candidate transcripts belonging to the death receptor pathway, BCL-2, the inflammasome complex and NF-KB family were studied in the nonconverting and converting CIS patients during the four-year follow-up period. Conversion to MS was associated with marked variability in the expression of proapoptotic genes that were linked to TGF-B1 gene levels. The predominant expression of proapoptotic genes in patients with CIS suggests an increased potential to undergo apoptosis with the goal of terminating immune responses and regulating immune system homeostasis.

1. Introduction

Multiple sclerosis (MS) is the most common neurological disease and is characterised by inflammation, demyelination and axonal loss leading to the development of brain atrophy and neurological disability. During the recent years, treatment options with different mechanisms of action enabling better control for MS have increased markedly (Bruck et al., 2013; Freedman, 2013). The identification of patients with clinically isolated syndrome (CIS) who are at high risk for developing MS is a prerequisite for the early initiation of such therapy. However, due to the heterogeneous genetic and immunopathogenic background of MS, therapeutic responses differ in the individual patient, and hence the selection of optimal treatment for patients is challenging. Due to these reasons, there is a high need for the identification of biomarkers with the potential to predict disease course/activity, identify different phenotypes of MS as well as monitor therapeutic responses.

MS pathogenesis is mediated by activated autoreactive CD4+ T helper 1 (Th1) and Th17 cells, as well as other immune cells, such as CD8+, natural killer cells and B cells, which contribute to neural damage (Comabella and Khoury, 2012; Chanvillard et al., 2013). Multiple cellular mechanisms have been noted in MS pathogenesis, such as the dysregulation of T-cell survival and apoptosis and the altered expression of apoptotic molecules in the mitochondria and the extrinsic pathway (Comi et al., 2012; Moreno et al., 2014).

Apoptosis plays a conflicting role with the two opposite effects in MS (Reichardt and Luhder, 2012). On one hand it mediates the death of oligodendrocytes and neurons in the central nervous system (CNS), while on the other hand it also terminates immune responses and regulates immune system homeostasis by eliminating autoreactive immune cells. In fact, the molecular mechanisms of several immunomodulatory and immunosuppressive agents are partly mediated through the apoptotic pathways (Fox and Rhoades, 2012). Factors inducing the dysregulation of T cell survival and apoptosis need to be clarified because these molecules may represent novel biomarkers as well as therapeutic targets. The predominance of the antiapoptotic BCL-2 family members and inhibitor of apoptosis proteins (IAP) has been observed in mitogen-activated T lymphocytes from patients with clinically active MS (Sharief and Semra, 2001; Semra et al., 2002a, 2002b; Sharief et al., 2002, 2003; Mandel et al., 2012). Elevated serum levels of antiapoptotic sFas have been associated with worsening disability and the accumulation of hypointense lesions seen upon magnetic resonance imaging (Hagman et al., 2011). Recently, increased gene expression levels of the proapoptotic adaptor protein FADD were observed in RRMS (Reuss et al., 2014). All of these data indicate the involvement

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of apoptotic processes in the pathogenesis of MS, but there are still no studies reporting on the use of apoptotic molecules as biomarkers for the disease. In the present study, our aim was to examine whether the apoptotic changes are already detectable after the first demyelination event and determine their potential to predict the conversion from CIS to MS.

2. Patients and methods

2.1. Study design

Gene expression analyses from peripheral blood mononuclear cells (PBMCs) were conducted with two separate cohorts. First, the expressions of 96 apoptosis-related genes were analysed from the blood samples of 12 relapsing–remitting MS (RRMS) patients and seven healthy controls (Cohort 1). Thereafter, 11 transcripts, which appeared to be upregulated in patients with RRMS, were analysed in 11 converting and nine nonconverting clinically isolated syndrome (CIS) patients over the four-year follow-up (baseline, two years and four years after the baseline visit) and 11 healthy controls at the baseline (Cohort 2).

2.2. Subjects

The study included 49 subjects: 12 untreated patients with RRMS, 19 patients with CIS and 18 healthy controls. CIS patients underwent neurological and magnetic resonance imaging (MRI) examination at baseline and two years and four years after baseline, while RRMS patients were studied only at the baseline. The diagnosis of MS was based on revised McDonald Criteria (McDonald et al., 2001; Polman et al., 2005) and all diagnoses were definite. CIS patients were defined as patients who had their first demyelinating event suggestive of MS (Miller et al., 2012). The clinical evaluation included the determination of expanded disability status scale (EDSS) score (Kurtzke, 1983) and disease activity as expressed by the number of relapses in the preceding two years. The control group consisted of 18 healthy subjects (Cohort 1 ($n = 7$): age 34.4 ± 11.4 years; 6 females, 1 male; Cohort 2 ($n = 11$): age 33.6 ± 11.0 years; 10 females, 1 male, mean ± SD). The study was approved by the Ethics Committee of Tampere University Hospital, and all subjects gave informed consent. Demographics of the RRMS and CIS groups are shown in Table 1.

2.3. MRI image segmentation and volumetric analysis

All examinations were performed on a 1.5 Tesla MRI Unit (Siemens Avanto, Erlangen, Germany). The MRI protocol for this examination included a T1-weighted header followed by an axial T1-weighted magnetisation prepared rapid gradient echo (MP-RAGE), and a T2-weighted turbo spin echo (TSE), fluid attenuation inversion recovery (FLAIR), magnetisation transfer contrasts (MTC), diffusion weighted imaging (DWI), and gadolinium-enhanced T1-weighted MP-RAGE sequences. In this study, T1-weighted MP-RAGE, FLAIR and T2-weighted TSE images were used for volumetric analysis. For MP-RAGE, the imaging parameters were as follows: repetition time (TR) = 1160 ms; echo time (TE) = 4.24 ms; inversion time (TI) = 600 ms; slice thickness = 0.9 mm; and in-plane resolution = 0.45 * 0.45 mm. In FLAIR, the following parameters were used: TR = 8500 ms; TE = 100 ms; TI = 2500 ms; slice thickness = 5.0 mm; and in-plane resolution = 0.45 * 0.45 mm. In TSE, the following imaging scheme was used: TR = 750 ms; TE = 115 ms; slice thickness = 3.0 mm; and in-plane resolution = 0.90 * 0.90 mm. Volumetric segmentation of plaques in the brain was performed using semiautomatic Anatomatic™ software operating in a Windows environment, and the images were analysed blindly. RRMS patients underwent examination only at the baseline, but in the CIS group MRI examinations were performed at the baseline and at two and four years after the baseline visit followed by neurological examination on the same day.

2.4. Total RNA isolation from peripheral blood mononuclear cells

PBMCs were separated in a Vacutainer CPT cell preparation tube (Becton Dickinson and Company, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. Total RNA was isolated from stored cell lysate with a Qiagen RNeasy plus mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The total RNA was eluted with nuclease-free water, and samples were stored at −80 °C for further analyses. The concentration and purity of RNA was determined before cDNA synthesis using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA).

2.5. Quantitative real-time PCR of apoptosis-related genes in mononuclear cells

Total RNA (1 μg) was reverse transcribed to cDNA using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) with the standard protocol. Gene expression analyses were carried out with a TaqMan low-density array (LDA) (Applied Biosystems) using a 7900HT Real-time PCR system (Applied Biosystems). A human apoptosis array was used to determine the expression of 93 apoptosis-related genes and three housekeeping genes from the PBMCs of RRMS patients (Cohort 1). A custom Taqman array (format 23) was used to analyse selected apoptosis-related molecules (*BAD, BCLG, BIK, BOK, PUMA, FADD, TNFRSF25, IKBKE, NFKBID, CASP1, PYCARD*) and cytokines (*IL23A, IL12A, IL4, TGFB1, IL6, IL21, IL10, IFNG, IL17A*) from CIS patients (Cohort 2). Arrays were loaded with 4 μl undiluted cDNA, 42 μl H_2O, and 50 μl PCR Universal master mix and run according to the manufacturer's instructions. Samples were run in duplicate. The expression data were analysed with RQ manager software (Applied Biosystems) using the comparative Ct method (ΔΔCt). To normalise the results, the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used, and two healthy control samples were used as calibrators in the data analysis.

2.6. Statistical analyses

Statistical analyses were performed using SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL, USA) and R software (software environment for statistical computing and graphics, version 2.13.0, the R

Table 1
Clinical characteristics in different groups.

Characteristics	Cohort 1	Cohort 2	
	RRMS n = 12	Converting n = 10	Nonconverting n = 9
Age[a]	37 (30–45)	33 (29–36)	37 (31–43)
Gender (M/F)[b]	3/9	1/9	1/8
Age at onset[a]	26 (24–30)	29 (25–34)	33 (28–42)
Time since first symptoms (years)[a]	8.3 (2.2–14.4)	1.0 (0.7–4.1)	1.3 (0.8–1.7)
Duration of disease (years)[a]	1.0 (0–6.5)	–	–
EDSS score at baseline[a]	1.0 (0.8–2.0)	0 (0–0)	0 (0–0)
EDSS score increase over follow-up[b]	–	3	0
Number of relapses, baseline[b,c]			
0	4	4	1
1 or more	8	6	8
Number of relapses over follow-up[b]			
0	–	6	0
1	–	4	0
T1 (cm3)[a]	1.4 (0.8–2.1)	0.6 (0.2–1.3)	0.01 (0.0–0.2)
FLAIR (cm^3)[a]	4.6 (2.5–7.8)	2.0 (0.8–7.1)	0.5 (0.0–0.8)

CIS clinically isolated syndrome; RRMS relapsing–remitting MS; EDSS expanded disability status scale; FLAIR fluid-attenuated inversion recovery.
[a] Median (interquartile range).
[b] Number of patients.
[c] All relapses preceding two years.