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Aberrant expression of the apoptosis-related proteins BAK and MCL1 in T cells in multiple sclerosis

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ABSTRACT

Pathogenic T cells of multiple sclerosis (MS) patients have been suggested to be endowed with an increased resistance to apoptosis, contributing to their increased survival. We report herein increased levels of the anti-apoptotic MCL1 protein and its half-life in activated lymphocytes of MS patients, which were not associated with differences in MCL1 RNA levels or with alterations in the expression levels of the known E3 ligases of MCL1-β-TrCP and HUWE1. Concomitantly, the expression levels of the pro-apoptotic protein BAK were decreased in MS patients at relapse. These findings suggest the dysregulation of the apoptosis-related proteins MCL1 and BAK in MS.

1. Introduction

Multiple sclerosis (MS) is an immune-mediated neurological disorder in which an inflammatory response is directed against myelin components of the central nervous system (CNS). CNS myelin-specific activated T cells are considered major players in the autoimmune activity that precipitates the formation of brain lesions in MS, in concert with contribution of activated B cells and macrophages (Hemmer et al., 2006). However, autoreactive immune cells are present also in healthy individuals, though these appear to carry a different activity profile, range of antigen specificity and avidity, and migratory capacity (Danke et al., 2004). Therefore, the mechanisms that maintain self-tolerance and regulate the extent of the autoreactivity of T and B cell repertoire seem to be impaired in autoimmune diseases such as MS (von Boehmer and Melchers, 2010).

The selection and differentiation of T and B cells, which proceeds through tightly regulated steps prior to release of the mature immune cell to the periphery, involves clonal expansion and deletion via mechanisms of apoptosis, including the removal of pathogenic autoreactive immune cells and maintenance of self-tolerance. Pro- and anti-apoptotic proteins from the BCL-2 family of proteins have been reported to be involved in this process in an intricate network of inhibition and activation interactions. This protein family participates in the intrinsic (mitochondrial) apoptotic pathways, and its members share one or more BCL-2 homology domains (BH) (von Boehmer and Melchers, 2010). BAK and BAX can activate the mitochondrial pathway in a direct manner by inducing the release of proteins such as cytochrome *c* from the mitochondria. The pro-apoptotic BH3-only proteins such as BIM, can trigger apoptosis by either directly activating BAX and BAK, or indirectly, by inactivating pro-survival members of the BCL-2 family (Opferman and Korsmeyer, 2003). Bak and Bax appear to be essential for both thymic selection of lymphocytes and B cell maturation, and their ablation in mice can lead to the development of autoimmune disease (Opferman and Korsmeyer, 2003; Takeuchi et al., 2005). Studies using mice genetic models have demonstrated the critical role of the anti-apoptotic proteins Bcl-2, Bcl-X_L and Mcl1 in T cell development (Dzhagalov et al., 2008). Mcl1 specifically appears to have a major role in the survival of activated T cells and the establishment of germinal centers and memory B cells, which are likely to be mediated through its interaction with Bak (Dunkle and He, 2011; Vikstrom et al., 2010). These findings suggest that MCL1 and its interacting partners may have an important role in conditions of immune dysregulation and heightened immune activity.

Accumulating evidence suggests that apoptosis is dysregulated in immune cells of MS patients. Peripheral immune cells of MS patients appear to have increased resistance to apoptosis (Sharief, 2000; Zang et al., 1999, 2011) and an altered expression of a variety of pro- and anti-apoptotic molecules associated with both the death receptor pathway (Fas/CD95) (Comi et al., 2000; Sharief et al., 2001; Zipp et al., 2000) and the mitochondrial pathway. Higher expression levels

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of pro-survival proteins, such as the Inhibitor of Apoptosis (IAP) proteins (Hebb et al., 2008a; Semra et al., 2002; Sharief and Semra, 2001) and BCL-X_L (Waiczies et al., 2002) have been observed in resting as well as mitogen-activated lymphocytes from MS patients, and including a shift in the ratio of pro-apoptotic to anti-apoptotic protein expression (Sharief et al., 2002a, 2003). Altered expression of apoptosis related molecules was also reported specifically in T cells and B cells of MS patients (Kurne et al., 2011; Seidi and Sharief, 2002; Thangarajh et al., 2005). Moreover, some of the approved therapies for MS, such as glucocorticosteroids, interferon beta, and mitoxantrone induce apoptosis of immune cells, an activity which is considered to be part of their mechanism of action (Chan et al., 2005; Dhib-Jalbut and Marks, 2010; Tischner and Reichardt, 2007). Although it is now clear that the MCL1-BAK interaction plays a pivotal role in survival of activated lymphocytes, a cell population that has been implicated in MS pathogenesis, the previous studies did not include these proteins in their assessment of pro- and anti-apoptotic proteins in MS. This study aimed to evaluate MCL1 and its interacting pro-apoptotic protein BAK as possible dysregulated proteins in MS.

2. Materials and methods

2.1. Study population

Patients with confirmed relapsing remitting (RR) MS, according to Poser and McDonald criteria (McDonald et al., 2001; Poser et al., 1983) and healthy control volunteers were recruited at the MS clinic, Carmel Medical Center, Haifa. Subjects were excluded if symptoms of acute infection were present at time of enrollment. The study was approved by the Helsinki committee of Carmel Medical Center, and a signed informed consent was obtained from all study participants.

All the MS patients recruited to this study were on interferon beta disease modifying drug therapy, and included patients in remission and patients in relapse. Patients in remission were included only if they had not received any steroid treatment for at least 1 month prior to recruitment. Relapses were confirmed and defined as previously described by us (Galboiz et al., 2001), and blood samples were withdrawn prior to initiation of steroid treatment. Demographic and clinical data were recorded from all participants, including expanded disability status scale (EDSS) scores (Kurtzke, 1983) and Multiple Sclerosis severity scores (MSSS) (Roxburgh et al., 2005) (Table 1). The healthy control group was selected to match for age and gender to the MS patient groups.

2.2. Lymphocyte preparation

Activated lymphocytes for assessment of apoptosis-related molecules were created as previously described (Sharief and Semra, 2001). Briefly, PBMCs isolated from heparinized blood on a Ficoll-Hypaque gradient (Novamed) were cultured in the presence of 1 μg/ml phytohemagglutinin (PHA-P Sigma) for the first 2 days following isolation and maintained in continuous presence of PHA (0.1 μg/ml) and 100 U/ml interleukin-2 (IL-2, PeproTech) for 5 more days in RPMI-1640 medium containing 10% FCS, penicillin–streptomycin (100 U/ml) and L-glutamine (2 mM) (all from Biological Industries Bet HaEmek). Flow cytometry analysis showed that at this time the mean proportion of $CD3^+$ T cells in this lymphocyte population was 94%, 62% of which also expressed the activation marker CD25. A portion of these cells was lysed for preparation of RNA and protein extracts, and the rest were frozen and stored in aliquots in liquid nitrogen for cell-based assays.

2.3. Western blot analyses

Whole cell protein extracts were prepared by lysis in HCMF buffer that contained 137 mM NaCl, 5.7 mM KCl, 0.34 mM Na_2PO_4, 5.6 mM glucose (all from Sigma), 10 mM HEPES (Biological Industries Bet HaEmek) and protease inhibitors ('complete' protease inhibitor tablets — Roche).

Equal protein amounts from cell lysates (30 μg per lane, determined by Bradford protein quantification — BioRad Laboratories) were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes for immunodetection. The membranes were subsequently incubated with the appropriate primary antibodies (rabbit anti-MCL1 1:200 — Santa Cruz biotechnology, rabbit anti-BAK 1:500, rabbit anti-BIM 1:1000 — Millipore, rabbit anti-HUWE1 1:500 — Bethyl Laboratories, mouse anti-β-TrCP 1:500 — Invitrogen, mouse anti-β-actin 1:5000 — Sigma-Aldrich) and the peroxidase conjugated secondary antibodies goat anti-rabbit or anti-mouse 1:2500 (Jackson Immunoresearch), followed by ECL detection, according to standard protocols (Biological Industries Bet HaEmek). Quantification of relative expression levels was performed by densitometric analysis using TINA 2.09 software (Raytest Isotopenmeßgeräte GmbH).

2.4. Determination of MCL1 protein half life

Activated lymphocytes that were frozen and then thawed were used for these experiments after preliminary experiments we conducted established that the half-life of MCL1 in fresh and thawed cells from the same donors remained similar. The cells were thawed into RPMI-1640 medium containing 10% FCS, penicillin–streptomycin (100 U/ml) and L-glutamine (2 mM) (all from Biological Industries Bet HaEmek), and allowed to recover for 24 h before initiation of the experiment. Protein half-life was assessed by the addition of 100 μg/ml cycloheximide (Sigma) to lymphocytes from MS patients or healthy controls, followed by harvesting at 30 min intervals for preparation of whole cell lysates for MCL1 western blot analysis.

2.5. RNA preparation and cDNA synthesis

TRI Reagent (MRC) was used for RNA preparation from freshly activated lymphocytes according to the manufacturer's protocol,

Table 1
Demographic and clinical characteristics of the study participants at the time of enrollment.

Participant group	Gender female/males (% females)	Age years mean ± SD	Disease duration years mean ± SD	Relapses in past 2 years number mean ± SD	EDSS[a] median [range]	MSSS[b] median [range]
MS patients at relapse	11/10 (52%)	34.9 ± 10.3	6.9 ± 4.5	2 ± 1.4[c]	3 [0–5.5][d]	5.4 [0.24–8.70][d]
MS patients in remission	7/9 (44%)	36.7 ± 10.1	8.4 ± 5.4	1.2 ± 1.1	1 [0–4]	1.13 [0.04–5.64]
Healthy control subjects	9/7 (56%)	34.3 ± 7.2				

[a] EDSS — Expanded disability status scale.
[b] MSSS — Multiple Sclerosis Severity Score.
[c] Not including the relapse at the time of enrollment.
[d] EDSS and MSSS scores at the last remission period before the relapse. EDSS and MSSS remission scores were significantly different between the relapse and remission MS patient groups (EDSS: P = 0.003; MSSS: P = 0.001).