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Glatiramer acetate induces pro-apoptotic mechanisms involving Bcl-2, Bax and Cyt-c in peripheral lymphocytes from multiple sclerosis patients

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Abstract Apoptotic deletion of autoreactive T-cells is defective in patients with multiple sclerosis (MS). Glatiramer acetate (GA) treatment seems to restore apoptosis of detrimental T-cells. We analyzed the mitochondria membrane pro- (Bax) and anti-apoptotic (Bcl-2) and cytosolic pro-apoptotic (Cyt-c, APAF-1) proteins expression in peripheral lymphocytes from relapsing-remitting (RR) MS patients during GA treatment. Blood samples were collected from 8 healthy controls (HCs) and from 8 RR MS patients prior to and every three months during the 9 months of GA treatment. Peripheral blood mononuclear cells (PBMNCs) Bcl-2, Bax, Cyt-c and APAF-1 were quantified by western blot followed by densitometric scanning and Bax/Bcl-2, cytosolic Cyt-c/Bcl-2 and APAF-1/Bcl-2 ratios were calculated. T-cells were *in vitro* tested for oxygen consumption by a respirometric analysis. Bax/Bcl-2, cytosolic Cyt-c/Bcl-2 and APAF-1/Bcl-2 ratios in untreated MS patients were significantly ($p < 0.05$) lower than in HCs. Bax/Bcl-2 ratio increased ($p = 0.03$) and Cyt-c/Bcl-2 ratio showed a trend to increase during the 9 months of GA treatment in MS patients. A reduction of 58 % and 59 % in oxygen consumption by PBMNCs was evident after GA treatment *in vitro* or when GA treated patients' cells were compared with those from HCs, respectively. Our findings suggest that GA exerts a regulatory effect on peripheral T lymphocytes through pro-apoptosis mechanisms involving mitochondria and cytosolic proteins.

Key words apoptosis · glatiramer acetate · multiple sclerosis

Introduction

There is emerging evidence that apoptotic deletion of autoreactive lymphocytes is defective in multiple sclerosis (MS) patients and this defect permits these cells to perpetuate a continuous cycle of inflammation within the Central Nervous System (CNS) [15, 25].

Cell death occurs by necrosis or apoptosis. These two mechanisms have distinct histological and biochemical signatures. In necrosis, the stimulus of death is itself often the direct cause of cell demise. In apoptosis, by contrast, the stimulus of death activates a cascade of events that orchestrate the cell destruction. The process of apoptosis is basically mediated by receptor-induced death mechanisms, which include the surface receptor Fas (CD95) and other members of the tumor necrosis factor receptor family [14], and mitochondria-mediated mechanisms, which include the family of Bcl-2 proteins, cytocrome-c (Cyt-c) and apoptotic protease-activating factor-1 (APAF-1) [5]. The Bcl-2 family comprises the eponymous Bcl-2 protein, which has anti-apoptotic ac-

tivity in a variety of cell types including lymphocytes, and the Bax protein, which has pro-apoptotic properties [9].

Unlike necrosis, which is a pathologic process, apoptosis is part of normal development and tissue homeostasis, including that of the immune system, since it regulates the balance between susceptibility and resistance of activated T lymphocytes to cell death [7, 12]. However, the apoptotic process may result altered in a variety of diseases.

Although the cause of this lymphocyte resistance to cell death in MS is not fully understood, it does appear to involve apoptotic defects at multiple cellular levels: increased serum levels of soluble CD95 [26], known to inhibit CD95-induced apoptosis, and heightened expression of intracellular apoptosis-protective molecules such as inhibitors of the apoptosis (IAP) family, FLIP (Fas-associated domain-like interleukin-1beta-converting enzyme inhibitory protein) and Bcl-X_L [16–18].

Based on these findings, it is likely that the induction of peripheral immune cell apoptosis may represent a therapeutic target in MS.

Interferon Beta (IFNB) is able to enhance lymphocyte apoptosis [4, 6, 19], by upregulating the expression of the death receptor Fas on antigen-specific T lymphocytes [25] and downregulating the anti-apoptotic FLIP [19] and IAP proteins [21] in T cells. Moreover IFNB may well regulate anti-apoptotic Bcl-2 expression, though results from different reports are still discordant [4, 19, 23].

Glatiramer acetate (GA) has also been shown to modulate *in vitro* apoptosis susceptibility of T-helper lymphocytes [1] and to induce [13] a significant enhancement of the apoptosis rate in CD4+ T cells. However, the GA effects on the different mechanisms of apoptosis had never been investigated until the present work.

In this study we analyzed the mitochondria membrane pro- (Bax) and anti-apoptotic (Bcl-2) and cytosolic pro-apoptotic (Cyt-c, APAF-1) proteins expression in peripheral lymphocytes from RR MS patients during 9 months of GA treatment.

Methods

Patients and controls

Eight patients with RR MS [10] (Table 1) were enrolled into the study. Patients had to be without any immunomodulatory treatment at least 6 months prior to study entry. For each patient, blood samples were obtained at baseline (untreated) and every three months during GA (Copaxone® – Aventis) treatment (20 mg s. c./day) for a period of 9 months. At these times all patients also underwent complete neurological examinations with EDSS evaluation. None of the patients relapsed at any time during the study period. Written informed consent was obtained from each individual before the start of the study.

Blood samples were also collected from 8 sex and age matched healthy controls (HCs) (Table 1).

Table 1 Demographic and clinical characteristics of MS patients on start of treatment and HCs

	MS (no. 8)	HCs (no. 8)
Age (years)	39.4±5.5	35.12±8.3
Gender ratio (M/F)	3/5	4/4
Disease duration (years)	7.9±5.7	/
EDSS score	2.8±1.3	/

Cell preparation

Peripheral blood mononuclear cells (PBMNCs) were isolated from K3-EDTA blood by centrifugation on a Ficoll-Hypaque density gradient (density: 1.077 g/ml; Pharmacia Biotech, UK), washed twice and resuspended in culture medium (RPMI-1640 with 10% FCS; GIBCO, Karlsruhe, Germany).

Digitonin was used to obtain membranes and cytosol after incubation in ice and centrifugation at 10000 rpm for 10 minutes. These samples were then frozen at –80°C until protein dosage with Bradford method.

To concentrate proteins, samples were incubated in ice, with 12% TCA (Trychloracetic acid) for 10 minutes and then centrifuged at 13000 g. Pellets were resuspended in reducing Laemli buffer and put in boiling water. Cellular lysates equivalents of 30 µg of protein were loaded on a 12% SDS-PAGE. After run, cellular contents of Bax, Bcl-2, cytosolic Cyt-c and APAF-1 were analysed in T-cell lysates by Western-blotting.

Western blot analysis

After run, gels were equilibrated in transfer buffer (Methanol, Tris, Glycine, ph 8.5) for 30 minutes and electroblotted onto nitrocellulose membrane for 30 minutes.

After blotting, nitrocellulose membranes were transferred in TTBS (Nacl, Tris, Tween 20, ph 7.4) and incubated overnight with specific antibodies against Bax, Bcl-2, cytosolic Cyt-c and APAF-1 diluted 1:500. Following washing in TTBS, membranes were incubated with secondary antibody anti-rabbit HRP (Horse Radish Peroxidase)-conjugated diluted 1:2000 for 4 hours.

Immunoreactive bands were detected by ECL (Enhanced Chemiluminescence Reagent) and apparent molecular weights (MWs) were assigned to the specific bands using pre-stained standards MWs (Bio-Rad). Finally, bands were quantified by a computerized densitometer (BIORAD System).

We then normalized the pro-apoptotic protein activities by reporting their ratios with the anti-apoptotic Bcl-2, and then calculated Bax/Bcl-2, cytosolic Cyt-c/Bcl-2 and APAF-1/Bcl-2 ratios.

Respirometry

Cells assayed for oxygen utilization were collected by centrifugation at 2000 rpm and pellets resuspended in 0.2 ml of the same culture medium. Oxygen utilization was measured in 650 ml of air-saturated culture medium at 37 °C with constant mixing using an oxygen-sensitive electrode and dual oxygen electrode amplifier (INSTECH Model 203). The rate of oxygen uptake driven by endogenous cellular substrates was recorded for 5 minutes. In order to test the effect of GA on oxygen consumption, non toxic concentrations of GA (50 ng/ml) were added for 24 hours to cultured PBMNCs from 5 consecutive MS patients. Moreover, GA treated patients' cells (3 MS) were compared with those from HCs (3 HCs).

Statistical analysis

Differences in Bcl-2, Bax, cytosolic Cyt-c and APAF-1 protein expressions and in Bax/Bcl-2, cytosolic Cyt-c/Bcl-2 and APAF-1/Bcl-2 ratios between MS patients and HC were evaluated using the non-parametric Mann-Whitney test. Changes in protein expression, in ratios and in EDSS score in MS patients during treatment were evaluated using the non-parametric Friedman test followed by Dunn's post-hoc test.

Results

At baseline, Bcl-2 and APAF-1 protein expressions were similar between untreated MS patients and HCs. Bax and Cyt-c protein expressions were significantly lower in untreated MS patients compared to HCs (Table 2).

Bcl-2 protein expression declined, Bax increased, cytosolic Cyt-c showed a trend to increase whereas APAF-1 expression did not change during GA treatment in MS patients (Table 2). In Fig. 1, an example of western blot analysis for each protein in a single MS patient is reported.

When examining pro-/anti-apoptotic proteins ratios, we observed that Bax/Bcl-2, cytosolic Cyt-c/Bcl-2 and APAF-1/Bcl-2 ratios in untreated MS patients were significantly lower than those in HCs (Fig. 2).

Bax/Bcl-2 ratio increased during the 9 months of GA treatment in MS patients (Fig. 3 and Table 2). But, at the same time, the longitudinal analysis showed that cytosolic Cyt-c/Bcl-2 and APAF-1/Bcl-2 ratios did not significantly change during GA time course, even though an increase after nine months for the former and after three months for the latter was evident (Table 2).

The EDSS score did not change during the time of treatment.

To validate the *in vivo* effects of GA therapy on Bcl-2, Bax, cytosolic Cyt-c and APAF-1 proteins, we analysed, *in vitro*, by a respirometric analysis, the oxygen consumption in 24 hours cultured, without and with the addition of GA, in PBMNCs from 5 consecutive MS patients (Fig. 4A). A reduction of 58% in oxygen consumption was observed in cells cultured in presence of GA. Moreover, a respirometric analysis was investigated in PBMNCs from 3 GA treated patients and 3 HCs (Fig. 4B). A similar reduction (59%) in oxygen consumption was observed in GA treated patients cells compared to those from HCs. A higher degree of oxygen consumption in untreated MS (black line in Fig. 4A) compared with that of HCs (black line in Fig. 4B) was also evident.

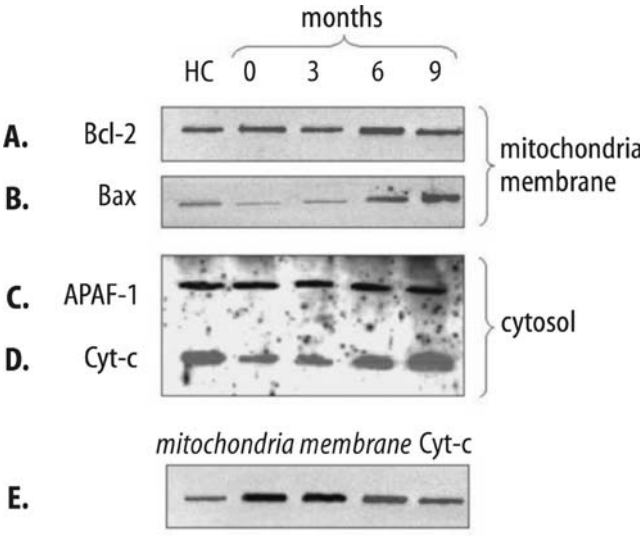

Fig. 1 Western Blot analysis of Bcl-2 (**A**), Bax (**B**), APAF-1 (**C**) and cytosolic Cyt-c (**D**) in one representative MS patient during 9 months of GA treatment and one HC. Lane **E** shows the mitochondria membrane Cyt-c protein expression in the time course

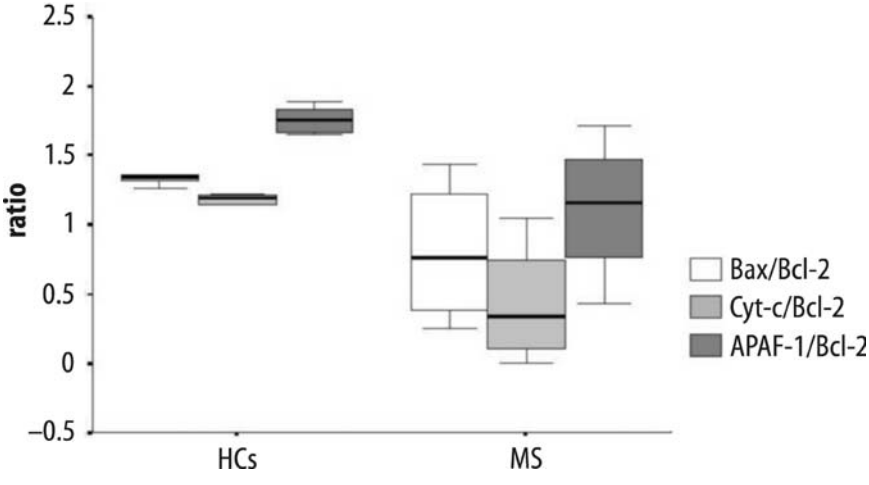

Fig. 2 Bax/Bcl-2, cytosolic Cyt-c/Bcl-2 and APAF-1/Bcl-2 ratios (*median, quartiles and extremes*) in HCs and MS patients at baseline

Table 2 Median and range of Bcl-2, Bax, APAF-1, cytosolic Cyt-c proteins and Bax/Bcl-2, cytosolic Cyt-c/Bcl-2 and APAF-1/Bcl-2 ratios in HCs and MS patients at baseline and during 9 months of GA treatment

protein/ratio	*HCs	Baseline	3rd month	6th month	9th month	Friedman test p
Bcl-2						
median	58.9	48	14	26	23.5	0.006**
range	55.2–61.5	21–102	6–32	6–39	8–66	
Bax						
median	80.15	19	12	39	48	0.02**
range	74.1–97.5	11–92	5–80	11–115	29–122	
Cyt-c						
median	69.1	17	11	23.5	53	0.07
range	62.1–71	0–22	6–31	15–25	46–76	
APAF-1						
median	102.6	43	40	46	40	0.6
range	99.8–110.1	28–118	20–43	27–110	22–44	
Bax/Bcl-2						
median	1.36	0.63	2.45	1.64	2.21	0.03**
range	1.26–1.63	0.22–1.44	0.84–8.90	1.03–6.40	1.47–8.13	
Cyt-c/Bcl-2						
median	1.19	0.11	1.06	1.1	4.45	0.1
range	1.01–1.22	0–0.22	1–1.11	0.92–1.28	3.83–5.07	
APAF-1/Bcl-2						
median	1.75	1.09	4.2	1.53	1.83	0.1
range	1.66–1.9	0.44–1.23	2.22–6.33	1.50–1.77	1.22–2.93	

* Mann-Whitney test, HCs vs. MS at baseline for:
Bcl-2 p = 0.3; *Bax* p = 0.01; *Cyt-c* p = 0.01; *APAF-1* p = 0.1; *Bax/Bcl-2* p = 0.04; *Cyt-c/Bcl-2* p = 0.02; *APAF-1/Bcl-2* p = 0.02
** Dunn's post-hoc test for:
Bcl-2 baseline vs. 3rd month $p < 0.01$; *Bax* 3rd vs. 9th month, $p < 0.05$; *Bax/Bcl-2* n.s.

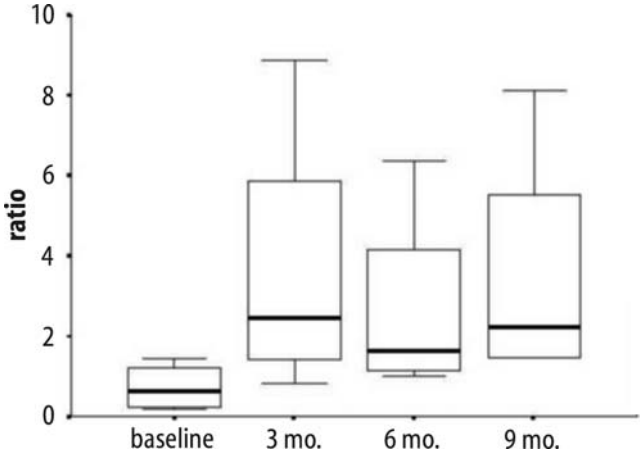

Fig. 3 Bax/Bcl-2 ratios (*median, quartiles and extremes*) in MS patients during the 9 months of GA treatment

Discussion

Autoreactive T lymphocytes are believed to mediate the disease process in MS. These cells are eliminated by apoptosis either in the periphery or in the CNS [25]. An impaired regulation of apoptotic pathways may lead to an insufficient immunological control of these autoreactive T-cells [11, 27].

We find lower Bax and cytosolic Cyt-c protein expressions and lower Bax/Bcl-2, cytosolic Cyt-c/Bcl-2 and APAF-1/Bcl-2 ratios in untreated MS patients than in HCs. This is consistent with previous reports showing lower Bax/Bcl-2 ratio in MS compared to HCs [20] and in active MS compared to stable MS and HCs [22].

This study is the first to investigate the effect of GA treatment on the expression of proteins such as Bcl-2, Bax, Cyt-c and APAF-1 which are involved in the alternative pathway of apoptosis, in MS patients. The analysis of cytosol and mitochondria membranes from MS PBMNCs shows a significant increase in Bax/Bcl-2 ratio and a trend to increase of cytosolic Cyt-c protein (but not of Cyt-c/Bcl-2 ratio) whereas APAF-1/Bcl-2 ratio does not significantly change during GA treatment. It is stated that the formation of Bax dimers at the mitochondria membrane level is needed for the exit of Cyt-c from mitochondria into the cytosol in order to induce and to bind with APAF-1 in the apoptosome complex. Although we are now still evaluating the mitochondria membrane Cyt-c expression, Fig. 1 (lane E) shows a decrease during the treatment time of one patient. If this tendency is confirmed in the other patients, it may sug-

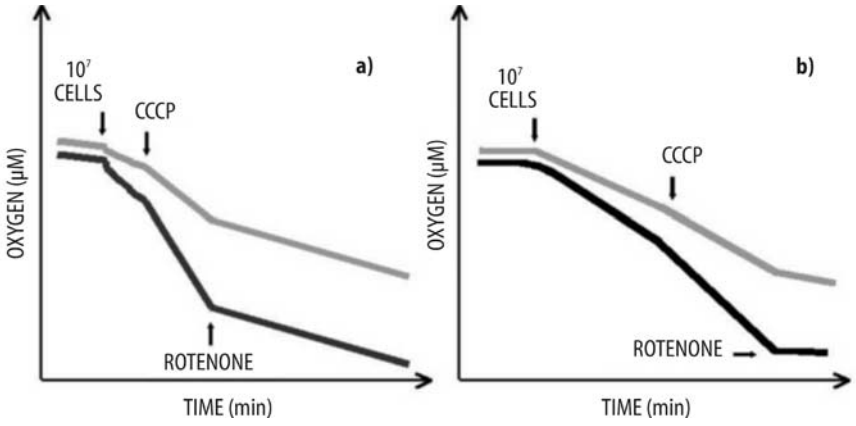

Fig. 4 Cellular oxygen consumption measurement. 10^7 cells were resuspended in 620 µl of respiration medium. CCCP (carbonyl-cyanide-m-chloro-phenyl-hydrazione) was added to the cell cultures as a decoupling agent phosphorylation and respiration. After 5 minutes of registration, Rotenone was added in order to block respiration. Differences were observed (**A**) between cells treated with GA (50 ng/ml; grey line) and those untreated (black line) and (**B**) between cells from GA treated patients (grey line) and those from HCs (black line)

gest that GA induces upregulation of Bax and favours the exit of Cyt-c.

In two reports [1, 13] evidence of a modulation in peripheral T-cell apoptosis during treatment with GA has been also provided. Aktas and colleagues [1] showed that T-cells apoptosis, after *in vitro* stimulation with GA, declined under the baseline and was equal in treated patients and controls. Hence the authors demonstrate a general involvement of GA in the compensation of altered apoptosis in T helper cells. More recently, Rieks et al. [13] showed a GA-induced apoptosis of CD4+ T cells together with an increase in activated and IL-4-producing T cells. It is widely accepted that GA induces a rather protective Th2-biased cytokine phenotype and a depletion of Th1 proinflammatory cells in MS patients. Considering apoptosis as a tightly regulated biochemical process that depends on the ability of cells to self-destruct by activation of an intrinsic suicide program and as a mechanism for the homeostasis of the immune system [8, 24], GA therapy may foster the elimination of detrimental T-cells since it restores a correct balance in the process of apoptosis.

The more evident oxygen consumption in untreated MS than in HCs seems to confirm the Bax/Bcl-2 ratio data between the two groups. Moreover, the *in vivo* evidence of a GA-linked improvement in the pro-apoptotic Bax/Bcl-2 ratio in MS PBMNCs seems to be also confirmed *in vitro* since, in presence of GA or in GA treated patients, the oxygen consumption by PBMNCs clearly decreases as a consequence of the increased rate of cell death.

We report that the increased expression of pro-apoptotic Bax/Bcl-2 ratio in MS PBMNCs occurs early on (after 3 months of treatment) and remains for the rest of the GA studied time course. This seems to be discordant with a previous report [13] showing a certain delay (24 to 30 weeks) in the CD4+ apoptosis induction after the beginning of GA treatment but also with previously published studies showing that the beneficial effect of GA as detected by clinical and MRI requires several months to develop [2, 3]. This apparent discrepancy may be explained because we investigated the effects of the treatment on the single proteins, effects that may occur early on, rather than the whole apoptotic phenomenon and the clinical and MRI manifestations.

We have examined Bcl-2, Bax, cytosolic Cyt-c and APAF-1 proteins expression in unfractioned lymphocytes. Examination of isolated T-cell subsets and B-cells, however, may provide information on cell death as resultant of interactions amongst different cell populations. It could be of interest to investigate whether GA therapy differentially modulates anti- and pro-apoptotic protein expression in different T-cell subsets and B-cells separately. Indeed, as far as T-cells are concerned, apoptosis in pro-inflammatory Th-1, including those specific reactive to myelin antigens, may be induced differently from regulatory T cells.

It is likely that the observed changes in the proteins involved in the alternative pathway of apoptosis do not represent a primary therapeutic mechanism of GA. Nevertheless, these results provide new insights into the therapeutic effects of GA and since these proteins certainly represent additional immunological variables in MS, they could become possible targets of intervention to modify MS pathology and the corresponding clinical course. But, even if of interest, the present findings need to be confirmed in further experiments and in a larger number of patients.

Finally, we do not find any correlation between biological and clinical findings since the EDSS did not change, nor were clinical relapses observed during the follow-up study. It is clear that the clinical score is not sensitive enough to be influenced by such biological changes. Therefore, the correlations between these biological findings and conventional and non-conventional MRI parameters of disease activity and progression may be worth evaluating in future studies.

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