Acta Neurol Scand 2001: 104: 266–270
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ACTA NEUROLOGICA
SCANDINAVICA
ISSN 0001-6314

Multiple sclerosis: modulation of apoptosis susceptibility by glatiramer acetate

Aktas O, Ari N, Rieks M, Hoffmann V, Schimrigk S, Przuntek H, Pöhlau D. Multiple sclerosis: modulation of apoptosis susceptibility by glatiramer acetate.
Acta Neurol Scand 2001: 104: 266–270. © Munksgaard 2001.

Objectives – We investigated whether therapy of multiple sclerosis (MS) with glatiramer acetate (GA) involves the modulation of programmed cell death (apoptosis) in disease-relevant T-helper lymphocytes. *Material and methods* – Blood was drawn from 15 relapsing–remitting MS patients both before (baseline) as well as 6, 12, and 18 weeks after GA therapy and from 15 healthy controls. Detection of apoptosis was performed in response to *in vitro* stimulation with GA, myelin basic protein or medium alone. *Results* – T-helper lymphocytes from untreated MS patients displayed an overall increased apoptosis susceptibility *in vitro*, compared to controls. During subsequent GA therapy, apoptosis vulnerability of these T cells in MS patients significantly declined under the initial baseline before treatment, and was finally equal in treated patients and controls. GA itself had no direct apoptosis-modulatory properties *in vitro*. *Conclusion* – Our findings indicate that therapy of multiple sclerosis with glatiramer acetate presumably involves the compensation of altered apoptosis in T-helper lymphocytes.

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Key words: multiple sclerosis; apoptosis; glatiramer acetate

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Accepted for publication May 25, 2001

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), characterized by localized myelin destruction, loss of oligodendrocytes and axonal transection. The failure of immunoregulatory mechanisms is considered to lead to the persistence of activated myelin-specific T-helper lymphocytes (T cells) which infiltrate the CNS, initiate and promote the pathological process (1). Therefore, therapeutic interventions in MS aim at the peripheral control of these cells as well as the inhibition of inflammation within the CNS. These concepts are partly based on the MS animal model experimental autoimmune encephalomyelitis (EAE), where CNS autoantigens like myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte-glycoprotein (MOG) are known to be major targets of pathogenic T-helper lymphocytes.

Apoptosis (programmed cell death) is a key regulatory control mechanism of the immune system that determines T-cell fate (2). Evidence has been provided that in autoimmune diseases such as MS the apoptosis of T-helper cells is dysregulated (3), and that the intervention in processes of apoptosis might have a therapeutic effect. Notably, the enhancement of T-cell apoptosis, especially via CD95-mediated *activation-induced cell death* (AICD) after repeated administration of encephalitogenic autoantigen, has suppressive and protective effects in EAE (4–6). Thus, the specific elimination of encephalitogenic T cells by induction of apoptosis might serve as a new therapeutic strategy in multiple sclerosis (3, 7).

Although glatiramer acetate (GA; copolymer-1; Copaxone®) has beneficial effects on the clinical course and the magnetic resonance imaging (MRI)-defined disease activity of MS patients, the mechanisms underlying this action are not fully understood (8–10). Since glatiramer acetate was originally synthesized as a functional and molecular analogue to MBP, it was found to have distinct cross-reactive properties with regard to MBP, as well as to PLP and MOG (11). Current concepts propose that the

interaction of GA with autoreactive T lymphocytes may lead either i) to a total block of recognition of encephalitogenic antigens, thus, completely inhibiting the immunological activation of these cells (12), and/or ii) to an immunomodulatory T-cell activation of the so called Th2-type, presumably resulting in the secretion of protective cytokines in the CNS (13). A further mechanism of action may include iii) the apoptotic elimination of T lymphocytes via AICD following their repeated agonistic reactivation by cross-reactive glatiramer acetate.

Thus, we investigated whether immunomodulation with glatiramer acetate involves the alteration of apoptosis of T-helper lymphocytes. The susceptibility of these cells to undergo programmed cell death was studied in functional assays, where mononuclear cells from MS patients were stimulated with GA, MBP or pure medium. Further, in order to study the *in vivo* effect of glatiramer acetate, samples from these initially untreated patients were obtained both before and at different time points after onset of GA treatment. To find out whether the observed findings in MS patients were related to the presence of disease, the same assays were undertaken with samples from corresponding healthy individuals. Our findings indicate that glatiramer acetate therapy is associated with the modulation of apoptosis pathways in disease-relevant T-helper lymphocytes.

Material and methods

Donors

Blood samples were acquired with informed consent from 15 initially untreated patients (10 female, 5 male) with clinically definite RR-MS (14) and 15 healthy age- and sex-matched individuals (controls) without concomitant disease, acute infections or allergic disposition. After the first sampling (baseline), patients were started on Copaxone (20 mg s.c. per day; Teva, Petach Tiqva, Israel), and subsequently blood was drawn after 6, 12, and 18 weeks of treatment. Patients assessed at having an expanded disability status score (EDSS) ≤ 6.0 (15) were enrolled in the study, and the resulting median EDSS was 2.0 (ranging between 1.0–5.5). All patients were in a stable phase of disease (i.e. no relapses, infections or other complaints within the last 6 weeks prior to inclusion) and they had not received any immunomodulatory medication during the last 90 days prior to inclusion. During the observation period, patients did not have relapses and had no additional immunomodulatory therapy such as, e.g. corticosteroids.

Experimental studies

Heparinized blood was used to isolate peripheral blood mononuclear cells (PBMC) by the Ficoll density gradient technique under sterile conditions. PBMC were resuspended in culture medium (RPMI 1640 with 5% fetal bovine serum), and 2 ml of cell suspension were plated in sterile 24-well flat-bottom tissue culture plates (2×10^5 cells/ml). Specific stimulation was performed either by the addition of glatiramer acetate in its pharmacological preparation as Copaxone® (Teva, Petach Tiqva, Israel; 1 μg/ml), bovine MBP (Sigma-Aldrich, Deisenhofen, Germany; final concentration: 2 μg/ml), or with pure medium. Samples were incubated at 37°C in humidified atmosphere (7% CO_2) for 48 h. To detect apoptosis, fluorescein isothiocyanate (FITC)-labeled annexin V and propidiumiodide (PI) were used according to the manufacturer's instructions (Bender Medsystems, Vienna, Austria). For assessment of the CD4 status, a phycoerythrin cyanin 5-conjugated monoclonal anti-CD4 antibody was applied at the same time. The controls included matched isotype antibodies (all antibodies Immunotech, Marseille, France). The amount of apoptotic cells was determined by flow cytometric analysis on a FACScan with CellQuest software (Becton-Dickinson, Heidelberg, Germany). The control samples were used both to evaluate the physiological background fluorescence and to assess the initial gate for picking out peripheral blood lymphocytes according to size (FSC) and granularity (SSC). For each sample, 5000 events in this lymphogate were scored. To evaluate this approach, we also studied the proportion of $CD4^+$ cells in the examined population of flow-cytometrically gated lymphocytes. Statistical analysis was performed with SPSS software (SPSS Inc., Chicago, USA).

Results

The initial investigation of T-helper lymphocytes from untreated RR-MS patients (baseline) showed median apoptosis rates of 11.9%, 11.4%, and 11.8% in cells incubated with medium only, GA, and MBP, respectively. These apoptosis rates were not statistically different from each other (Table 1). In healthy individuals, an apoptosis rate between 7.0% and 8.4% was found, still without any statistically significant difference between cells incubated with GA, MBP, or pure medium (Table 1). As indicated above, T-helper lymphocytes from MS patients showed an increased apoptosis rate compared to cells from healthy people, both when treated with GA ($P = 0.02$) and MBP ($P = 0.03$), and an apoptosis rate which was also increased, although not statistically significant

Table 1. *In vitro* apoptosis frequencies of T-helper lymphocytes from MS patients and healthy controls

	Percentage of apoptotic cells (median ± SEM)								
		MS patients							
		Baseline		Under treatment with glatiramer acetate					
Stimulation	Healthy controls	0 weeks	P^a	6 weeks	P^b	12 weeks	P^b	18 weeks	P^b
GA	7.49 ± 1.31	11.91 ± 1.38	0.021	11.25 ± 0.95	NS	9.86 ± 1.41	NS	3.12 ± 1.69	0.020
MBP	7.04 ± 1.15	11.38 ± 1.86	0.028	11.76 ± 1.74	NS	9.12 ± 1.06	NS	3.86 ± 1.78	0.009
BLANK	8.43 ± 1.16	11.82 ± 1.86	0.089 (NS)	11.44 ± 0.75	NS	11.21 ± 1.16	NS	2.70 ± 1.51	0.017
P^c	NS	NS		NS		NS		NS	

P^a : Mann–Whitney *U*-test for comparison of healthy controls and untreated MS patients (at baseline).
P^b : Wilcoxon-test for comparison of baseline and different time points under treatment of MS patients with glatiramer acetate (GA).
P^c : Kruskal–Wallis *H*-Test for comparison of different stimulation conditions.
NS: not significant. MBP: myelin basic protein. BLANK: without specific stimulus.

($P = 0.089$; Table 1) when the cells were left untreated. For evaluation purposes, we studied the proportion of $CD4^+$ cells in the examined population on non-adherent mononuclear cells (mostly lymphocytes). In line with published findings (16), equal frequencies of $CD4^+$ cells were detected in MS patients and controls, regardless of the type of stimulation used (data not shown).

In view of the higher *in vitro* apoptosis rate in untreated MS patients, we extended our studies to find out whether treatment of these patients with glatiramer acetate may have a compensatory effect. Using the same stimulation assays, apoptosis of T-helper cells was initially not altered after 6 weeks of treatment, with an apoptosis frequency between 11.3% and 11.8%, thus corresponding to the baseline (Table 1). However, after 12 weeks decreasing apoptosis rates were found, with 9.9%, 9.1% and 11.2% in cells incubated with GA, MBP and medium only, respectively, although not statistically significant. This tendency towards reduced apoptosis susceptibility *in vitro* was significant after 18 weeks: T-helper cells from glatiramer acetate-treated MS patients were less sensitive to undergo apoptosis when stimulated *in vitro* with GA ($P = 0.02$), MBP ($P = 0.01$) or pure medium ($P = 0.02$; Table 1), compared to the initially observed apoptosis frequencies at baseline (Fig. 1). The decrease in apoptosis susceptibility during therapy was also obvious in comparison to healthy donors. Whereas T-helper cells from MS patients were initially more sensitive to undergo apoptosis, the apoptosis susceptibility at the end of the study was equal (or even decreased) compared to cells from healthy donors after *in vitro* stimulation with GA ($P = 0.12$; Mann–Whitney *U*-test), MBP ($P = 0.07$) or pure medium ($P = 0.02$). There were principally no differences between different *in vitro* stimulation conditions (GA, MBP, medium) at any time point during GA treatment (Table 1).

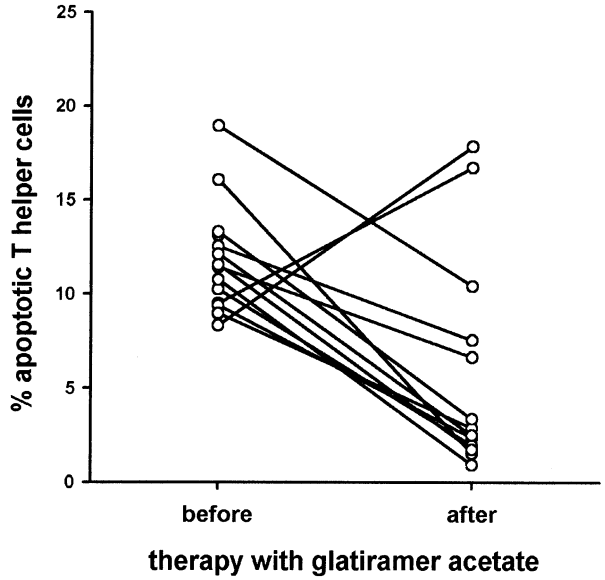

Fig. 1. Apoptosis susceptibility of T-helper lymphocytes decreases under therapy with glatiramer acetate. Mononuclear cells were left untreated after isolation from peripheral blood, and apoptosis of $CD4^+$ lymphocytes was detected after 48 h by annexin V-staining. Displayed are the proportion of apoptotic T-helper lymphocytes from individual MS patients ($n = 15$) before and 18 weeks after onset of GA treatment. The apoptosis frequency significantly declined under the initial baseline ($P = 0.017$, Wilcoxon-test). This holds also true for apoptosis in cells after *in vitro* stimulation with glatiramer acetate or myelin basic protein (see Table 1).

Discussion

Our data indicate for the first time that glatiramer acetate may influence apoptosis pathways of T-helper lymphocytes in MS patients. As shown in Table 1, the susceptibility of such cells to undergo apoptosis *in vitro* declined in the majority of patients after they were started on subcutaneous injections of GA. Finally, the apoptosis rate was significantly decreased at the end of the observation period, compared to the baseline when patients were untreated. Interestingly, as indicated by the individual courses of apoptosis in Fig. 1, we found a small number of

clinically stable patients (2 out of 15) with an increased *in vitro* apoptosis susceptibility of T cells after GA treatment. It remains to be clarified whether this finding reflects the pathological and immunological heterogeneity of multiple sclerosis, as suggested in recent publications (17). However, we observed in the majority of glatiramer acetate-treated patients a decrease of apoptosis susceptibility which did not occur immediately, but was achieved with a certain delay after the beginning of treatment (Table 1). This finding is in line with a recently published report showing that the beneficial effect of glatiramer acetate on disease activity, as measured by clinical and MRI parameters, is obtained some months after start of therapy (10). The necessity of such a delay might also explain why the here used short-term incubation assay did not show an isolated *in vitro* effect of GA on apoptosis of T-helper cells from untreated RR-MS patients, compared to cells without specific antigen challenge.

To reveal a possible link between disease presence and apoptosis susceptibility, samples from matched healthy controls were examined with the same functional assays. We found that the frequency of apoptotic T-helper cells was significantly lower in healthy people, compared to MS patients *before* the start of GA therapy (Table 1). This augmented apoptosis vulnerability in relapsing–remitting multiple sclerosis presumably reflects the dysregulation of apoptosis pathways, being in line with studies reporting elevated levels of CD95 in serum and cerebrospinal fluid from MS patients (18–20). Soluble CD95 has been repeatedly shown to inhibit CD95-mediated apoptosis, thus protecting T cells from programmed death (7, 21). Furthermore, another publication supporting our data demonstrated that an increased proportion of MBP-reactive T cells from MS patients is sensitive to CD95-mediated apoptosis and that these are not deleted *in vivo* in MS, unlike cells from healthy individuals (22). Therefore, our results are possibly due to the neutral culture conditions chosen, where, in the absence of any apoptosis-inhibiting factors, T-helper cells from *untreated* MS patients underwent apoptosis at an increased rate. In our hands, treatment with glatiramer acetate was able to compensate these differences between healthy controls and MS patients, and at the end of the study, the apoptosis susceptibility of probably disease-relevant T-helper cells was equal in MS patients, compared to controls.

In MS, the aim of increasing efficacy in future therapeutic strategies makes it essential to clarify how established pharmacological principles interfere with mechanisms of the disease. Autoaggressive T-helper lymphocytes are considered to initiate and, in cooperation with B cells, perpetuate disease pathology. The here observed altered apoptosis vulnerability of T-helper cells in untreated MS patients presumably reflects the impaired regulation of apoptotic pathways, leading to an insufficient immunological control over autoreactive T cells (3, 7). So far published studies investigating the mechanism of action of GA mainly focused on its interaction with the cytokine network, indicating that GA shifts the population of T-helper cells from a pro-inflammatory Th1- to a rather protective Th2-phenotype (13, 23). It remains to be elucidated how glatiramer acetate exactly modulates apoptosis pathways in MS, and whether this immunoregulatory effect is linked to the reported Th2-shift.

Acknowledgements

This work was supported by a project grant from Teva Pharmaceutical Industries, Petach Tiqva, Israel. The authors express appreciation to Mrs Iris Schweppe-Salewski for her assistance with the recruitment of patients with multiple sclerosis.

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