Down-Regulation of Survivin Expression in T Lymphocytes After Interferon Beta-1a Treatment in Patients With Multiple Sclerosis

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Background: Treatment with interferon beta reduces clinical exacerbations in multiple sclerosis (MS) through several immunomodulatory mechanisms that involve the augmentation of programmed cell death (apoptosis) of peripheral T lymphocytes. The expression of survivin, a cell cycle–regulated antiapoptosis protein, is upregulated in mitogen-stimulated T lymphocytes from patients with MS, and this expression correlates with MS disease activity.

Objective: To evaluate the effect of interferon beta on the expression of survivin and other apoptosis regulatory molecules in peripheral T lymphocytes from patients with MS.

Patients and Methods: In a prospective, combined clinical and immunologic study, we evaluated the expression of survivin, Bcl-2 protein, and the death receptor Fas in mitogen-stimulated T lymphocytes from 26 patients with MS, before and serially after treatment with interferon beta-1a. We also investigated the long-term effects of interferon beta-1a on cellular expression of these proteins and T-lymphocyte apoptosis in a cross-sectional study of 19 patients with MS receiving long-term interferon beta-1a therapy.

Results: Treatment with interferon beta-1a reduced the expression of survivin in in vitro stimulated T lymphocytes. This reduced expression correlated with augmented T-cell susceptibility to apoptosis and with clinical response to treatment. In contrast, interferon beta-1a therapy did not significantly alter cellular expression of Bcl-2 protein or Fas. This down-regulatory effect of interferon beta-1a on cellular expression of survivin was maintained after long-term therapy.

Conclusions: Our observations suggest that interferon beta exerts a regulatory effect on peripheral T lymphocytes through an antiapoptosis mechanism that involves the down-regulation of cellular survivin expression.

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Programmed cell death (apoptosis) of lymphocytes is essential for the proper functioning and homeostasis of the immune system. Among other functions, lymphocyte apoptosis is an important antiautoimmune mechanism that deletes potentially pathogenic autoreactive lymphocytes and limits tissue damage in autoimmune diseases, including multiple sclerosis (MS). There is emerging evidence that autoreactive T lymphocytes in MS are more resistant to apoptosis than cells from healthy individuals or patients with other neurologic disorders. Such reduced susceptibility to apoptosis in MS is related to rearrangements of the apoptosis machinery at multiple cellular levels that include the death receptor Fas and Bcl-2 family of oncogenic proteins.

In patients with MS, treatment with interferon beta reduces clinical exacerbations and lessens the accumulation of disease burden as assessed by magnetic resonance (MR) imaging. The precise mechanism of action of the 2 recombinant preparations of interferon beta (1a and 1b) is not clear. These preparations exert multiple regulatory effects on peripheral lymphocytes that include augmentation of apoptosis and upregulation of the death receptor Fas on antigen-specific T lymphocytes. Ligation of Fas leads to activation of cellular caspases, a series of destructive intracellular cysteine proteases that play an essential role in mediating cell death through cleavage of structural elements of the cytoplasm and nucleus. We recently reported that interferon beta therapy regulates the activity of apical caspases, such as caspase 8. However, its effect on downstream caspases is currently unknown.

The activity of downstream caspases is partly modulated by survivin, a re-
PATIENTS AND METHODS

PATIENTS
In a prospective longitudinal study, we investigated cellular apoptosis and the expression of survivin, Bcl-2, and Fas protein in activated T lymphocytes from 26 patients with clinically active, relapsing-remitting MS before treatment with interferon beta-1a (baseline samples) and consecutively after 1, 3, 6, 9, and 12 months of this drug therapy. Their clinical features were presented earlier.10 In brief, their mean age ± SD was 32.6 ± 6.2 years, and the mean ± SD disease duration was 6.3 ± 3.6 years. The criteria for active disease were as follows: (1) history of at least 2 clearly identified clinical relapses during the 2 years preceding blood collection, and (2) the presence of 1 or more enhancing lesions on cranial MR imaging at the time when baseline blood samples were obtained. Twelve patients received Rebif (Serenos International SA, Geneva, Switzerland), 22 µg 3 times weekly; 6 patients received Rebif, 44 µg 3 times weekly; and the remaining 8 patients were treated with Avonex (Biogen, Inc, Cambridge, Mass), 30 µg once weekly.

In addition to this prospective study, we investigated the long-term effect of interferon beta-1a on the expression of survivin and other apoptosis regulatory proteins in a cross-sectional evaluation of another 19 patients with relapsing-remitting MS. These patients had been receiving interferon beta-1a (Rebif, 22 µg 3 times weekly) for a mean duration of 4.8 years (range, 2.5-6.5 years). Their mean age ± SD was 35.7 ± 7.8 years, and mean disease duration was 7.3 ± 2.6 years. To control for this treatment group, we collected blood samples from 14 patients with relapsing-remitting MS who had never received treatment with interferon beta. Their mean age ± SD was 34.1 ± 8.2 years, and mean disease duration was 4.8 ± 2.9 years. These control patients had had at least 2 clinical relapses during the 2 years preceding blood collection, and in 8 patients who underwent cranial MR imaging at the time of sample collection, disease activity was confirmed by the presence of 1 or more enhancing lesions. No patient from the treatment or the control groups had received corticosteroid or immunosuppressive therapy during the 6 months before blood collection. We did not include neurologic control subjects, as we have already reported a significant difference in cellular survivin expression between patients with MS and those with other neurologic disorders.27 However, we obtained blood samples from 12 healthy individuals to determine the normal range of cellular survivin expression.

METHODS

Cell Culture and Induction of Apoptosis
Peripheral mononuclear cells were isolated from heparinized blood by centrifugation on a Ficoll-Hypaque density gradient washed twice and resuspended in culture medium. In vitro activation of T cells was established by means of nonspecific stimulation with phytohemagglutinin (1 µg per 10⁶ cells) followed by culture in continuous presence of phytohemagglutinin and interleukin 2, 100 U/mL, as previously described.31 Flow cytometry analysis of cells after 5 days of culture showed that the proportion of T cells was more than 94%. Cell viability was determined by trypan blue dye exclusion assay. Apoptosis was induced on day 5 of culture by incubation with etoposide, 10 µg/mL (Sigma-Aldrich Corp, St Louis, Mo), or culture medium as described elsewhere.31 This method was chosen to account for the fact that survivin, like other inhibitors of apoptosis proteins, may be involved in the proteolytic processing mediated by etoposide.32 Apoptosis was quantified by means of commercial cellular DNA fragmentation immunoassays (Boehringer Mannheim GmbH, Ingelheim, Germany), as presented in detail elsewhere.11,29

Quantification of Survivin and Other Proteins
All laboratory analyses were performed blind to clinical data. As we have already reported that survivin was not usually expressed in resting peripheral lymphocytes,27 survivin expression in this study was quantified in T cells that were stimulated in vitro for 5 days. Cellular contents of survivin were quantified in cell lysates by means of dot-blot immunoassay, as discussed earlier.27 In brief, cellular lysate equivalents of 5 µg of protein were probed with affinity-purified antibody raised against a peptide mapping at the carboxy terminus of human survivin (C-19; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), followed by computerized densitometry. To validate cellular expression of survivin in individual patients, we analyzed signals obtained from Western blot analysis as described earlier.31 In earlier experiments, we detected a strong correlation between cellular survivin levels as determined by the dot-blot assay and corresponding levels measured by Western blots.23 However, we used dot-blot data for statistical analyses in this study to avoid gel-to-gel variation and differences in blotting and exposure time that may occur with Western blotting. Quantification of Bcl-2 protein content in cell lysates was performed with a commercial immunoassay (Oncogene Research Products, Calbiochem, CN Biosciences, Inc, Nottingham, England). We also used commercial assay (Calbiochem, CN Biosciences, Inc), rather than flow cytometry, to measure cellular Fas protein to maintain consistency of the assays used in this study.

DATA ANALYSIS

All statistical analyses were performed with the SPSS/PC+ software program (SPSS Inc, Chicago, Ill). Values were compared, as appropriate, by paired Student t test, Wilcoxon rank test, analysis of variance, Kruskal-Wallis test followed by multiple comparisons, and Pearson correlation test.

cently described cell cycle-regulated antiapoptosis protein. Overexpression of survivin delays apoptosis induced by various stimuli, whereas antisense-mediated reductions in survivin sensitizes cells to apoptotic stimuli.20 While fetal tissues and normal thymus contain abundant survivin messenger RNA and protein, no such expression is detected in normal adult tissues.21,22 In contrast, the vast majority of tumors express survivin at high levels, suggesting that reactivation of survivin gene expression commonly occurs during oncogenesis.23 However, survivin is not cancer specific, as it also plays an important role in normal hematopoietic cell survival and
proliferation.\textsuperscript{24,25} Indeed, evidence is emerging that survivin expression contributes to cell survival early in T-cell activation and also in memory immune responses.\textsuperscript{26}

Overexpression of survivin has been reported in activated T lymphocytes from patients with relapsing-remitting MS.\textsuperscript{27} Moreover, this heightened expression in patients with MS seems to correlate with T-cell resistance to apoptosis,\textsuperscript{27} and with clinical features of disease activity.\textsuperscript{28} On the basis of these observations, we sought to correlate the expression of survivin in peripheral T lymphocytes from patients with MS after interferon beta therapy with clinical response to treatment, and with cellular susceptibility to apoptosis. Addressing these issues would help define the role of survivin in mediating clinical responses to interferon beta therapy.

## RESULTS

### PROSPECTIVE CLINICAL EVALUATION

After 12-month follow-up of the prospective group, 18 of the 26 patients experienced complete cessation or more than 60% reduction in clinical relapse rate compared with pretreatment levels. Thus, these 18 patients were considered interferon beta responders, according to published criteria.\textsuperscript{34,35} This favorable clinical response was verified by the lack of MR imaging activity in 8 patients who underwent follow-up MR imaging assessment. The remaining 8 of the 26 patients who were included in the prospective study experienced no or less than 20% reduction in exacerbation rate, and were therefore considered nonresponders.\textsuperscript{34}

### BASELINE CELLULAR SURVIVIN EXPRESSION

The pretreatment (baseline) expression of survivin in mitogen-stimulated T cells from patients with MS was significantly higher than corresponding expression in healthy individuals (Figure 1). More specifically, baseline survivin expression was abnormally high (ie, higher than the cutoff limit in healthy control subjects) in 16 of the 26 patients with MS who were involved in the prospective study (Figure 1). There was no correlation between survivin expression and the extent of lymphocyte activation, as measured by proliferation rate. In contrast to high survivin contents, cellular expression of Bcl-2 and Fas in mitogen-stimulated T lymphocytes from patients with clinically active multiple sclerosis compared with healthy control subjects. Closed symbols represent nonresponders to interferon beta-1a therapy. Horizontal bars depict mean values, and shaded area represents the cutoff limit (mean±2 SDs) of cellular survivin expression in healthy control subjects. Asterisk indicates \( P<.01 \) compared with corresponding levels in healthy control subjects.

### DYNAMICS OF SURVIVIN EXPRESSION AFTER INTERFERON BETA-1A THERAPY

Baseline survivin expression in the 18 patients with MS who later responded to interferon beta-1a was comparable to baseline expression in the 8 nonresponders. However, prospective evaluation showed a progressive decline of survivin expression after interferon beta-1a therapy in 10 of the 18 treatment responders. Serial monitoring of treatment responders showed that cellular survivin expression declined after 6 months of interferon beta-1a therapy and was significantly lower than baseline levels after 12 months of treatment (Figure 2A). In contrast, cellular survivin in the 8 nonresponders remained relatively unchanged throughout the study (Figure 2B). Similarly, cellular Bcl-2 expression in patients with MS was not significantly influenced by interferon beta-1a therapy and was comparable between interferon beta-1a responders and nonresponders. When a more stringent definition of interferon beta-1a responders was applied (ie, complete cessation of clinical and radiographic activity), all responders showed suppression of survivin expression when compared with nonresponders, and their mean±SD survivin expression decreased from a baseline of 90.6±68.7 to 26.3±20.5 densitometric units after 12 months of therapy (\( P<.001 \)). There were no differences in the dynamics of survivin expression between patients receiving the 2 doses of Rebif, or between the Rebif- and Avonex-treated groups. Cellular survivin remained undetectable after treatment in the 5 patients whose data are given in Figure 1 who showed no baseline expression. There was a tendency for increased Fas expression after interferon beta-1a therapy (Figure 2), but this was not statistically significant.

### SURVIVIN EXPRESSION AND LONG-TERM INTERFERON BETA-1A THERAPY

The down-regulatory effect of interferon beta-1a on cellular survivin expression in treatment responders

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**Figure 1.** Cellular expression of survivin (densitometric units) and Fas and Bcl-2 (units per milligram of protein) in mitogen-stimulated T lymphocytes from patients with clinically active multiple sclerosis compared with healthy control subjects. Closed symbols represent nonresponders to interferon beta-1a therapy. Horizontal bars depict mean values, and shaded area represents the cutoff limit (mean±2 SDs) of cellular survivin expression in healthy control subjects. Asterisk indicates \( P<.01 \) compared with corresponding levels in healthy control subjects.
prompted us to investigate whether such effect was main-
tained during long-term treatment. Sixteen patients from
the long-term treatment group were classified as inter-
feron beta-1a responders (complete cessation or more than
60% reduction in clinical relapse rate), whereas the re-
maining 3 patients were considered nonresponders. The
duration of MS was comparable between the 2 groups.
Cellular survivin expression in the 16 treatment respond-
ers was significantly lower than corresponding expres-
sion in the 3 nonresponders or in the 14 untreated pa-
tients with MS (Figure 3). In contrast, cellular expression
of Bcl-2 and Fas proteins in the long-term treatment group
was similar to corresponding expression in untreated pa-
tients or healthy individuals (Figure 3).

IN VITRO EFFECT OF INTERFERON BETA-1A
ON CELLULAR SURVIVIN

Having established that interferon beta therapy reduces
cellular survivin expression, we sought to validate this
down-regulatory effect in vitro. For this purpose, we ana-
yzed cellular expression of survivin and other apop-
tosis regulatory proteins in T cells after mitogen stimula-
tion in the presence or absence of interferon beta-1a.
Results confirmed that adding interferon beta-1a to the
culture medium significantly reduced survivin expres-
sion but did not alter the expression of Bcl-2 protein and
up-regulated cellular expression of Fas protein (Table).

EFFECT OF INTERFERON BETA-1A
ON LYMPHOCYTE APOPTOSIS

We then investigated the relationship between survivin
expression and T-lymphocyte apoptosis to confirm the
biological relevance of survivin down-regulation after
administration with interferon beta. After prospective treat-
ment with this drug, T-cell susceptibility to etoposide-
induced cell death was enhanced in patients who showed
progressive decline in cellular survivin expression
(Figure 4). This susceptibility to apoptosis was inde-
pendent of cellular Fas expression, and the correlation
between survivin down-regulation and augmented apop-
tosis was confirmed by T-cell cultures in the presence of
interferon beta-1a (data not shown). We observed a simi-
lar relationship between cellular survivin expression and
enhanced apoptosis in the 16 long-term treatment re-

Figure 2. Cellular expression of survivin (densitometric units) and Fas and
Bcl-2 (units per milligram of protein) in mitogen-stimulated T cells before
treatment with interferon beta-1a (month 0) and serially during 12 months of
therapy. Results are means±SE. A, Data from all 18 patients who responded
to treatment; B, data from the 8 nonresponders. Blood samples at month 12
were obtained from 8 responders and 4 nonresponders. Asterisk indicates
P<.05 compared with pretreatment levels.

Figure 3. Cellular expression of apoptosis regulatory proteins in
mitogen-stimulated T cells from patients with multiple sclerosis receiving
long-term interferon beta-1a therapy compared with untreated patients with
multiple sclerosis. Closed symbols represent treatment nonresponders.
Horizontal bars depict mean values, and shaded area represents the cutoff
limit (mean±2 SDs) of cellular survivin expression in healthy control
subjects. Survivin expression is given in densitometric units, and Fas and
Bcl-2 in units per milligram of protein. Asterisk indicates P<.01 compared
with corresponding levels in healthy control subjects.
The efficacy of interferon beta in the treatment of MS has been established by several international multicenter trials and involves multiple immunomodulatory actions. In this study, we investigated the effects of interferon beta-1a therapy on survivin expression and subsequent apoptosis of mitogen-stimulated T lymphocytes from both interferon beta-1a responders and nonresponders. Our results demonstrate that interferon beta therapy preferentially down-regulates the expression of survivin in mitogen-stimulated T lymphocytes from treatment responders. Since interferon beta is known to exert antiproliferative actions that include potent proapoptotic activities,15,16,36-39 our findings are of therapeutic relevance in view of the apoptosis-regulatory effects of survivin40,41 and the suggestion that clinical remissions in MS could be induced by apoptotic elimination of activated T lymphocytes.3

Impairments of activation-induced apoptosis of lymphocytes may play a role in the perpetuation of the immune response in MS.42-43 Indeed, there is emerging evidence that antigen-specific T lymphocytes from patients with MS are less susceptible to apoptosis than are healthy cells, and may therefore maintain a continuous cycle of inflammation and tissue destruction within the central nervous system.7,9,12 Interferon beta is known to inhibit the proliferation of antigen-specific T lymphocytes,44 probably through apoptosis-regulatory mechanisms.8 It also augments apoptosis of activated polyclonal T lymphocytes from patients with MS,13,45 although this has not yet been confirmed in myelin-specific T-cell lines.17 This interferon beta–mediated increase in T-cell apoptosis is due to several mechanisms that include up-regulation of Fas or its ligand,39 induction of genes that down-regulate protein synthesis,46,47 overexpression of cellular apoptosis-inducing ligands,37,48 and down-regulation of apical caspase inhibitors.10 Here, we expand on these mechanisms by reporting that interferon beta down-regulates T-cell expression of survivin, a potent bifunctional protein that suppresses apoptosis and regulates cell division.22

Human survivin, a member of the inhibitor-of-apoptosis family, plays a pivotal role in the regulation of cell death.12,49 Overexpression of survivin inhibits apoptosis induced by various stimuli and has the potential to suppress terminal caspases that mediate cell death.19 Survivin is expressed in a cell cycle–regulated manner, with high levels in the G2/M phase but rapid down-regulation after cell cycle arrest. Although high expression is seen in most cancers, survivin is also overexpressed in nonmalignant cells, such as the proliferative basal layer of normal skin,50 endothelial cells during angiogenesis,33 and activated and memory T cells.39 Accordingly, the antiapoptotic properties of survivin may be necessary to counteract increased apoptosis sensitivity of proliferating cells during development or homeostasis,21 or to escape their normal proliferative constraints.52 Moreover, evidence is accumulating that derangements of survivin expression are involved in inflammatory diseases,39 including MS.27 Indeed, survivin overexpression in T lymphocytes seems to correlate with MS disease activity.26 These observations help explain our findings that down-regulation of cellular survivin corresponds with a favorable clinical response to interferon beta therapy. In this study, we adopted strict criteria for the definition of interferon beta responsiveness that did not require MR imaging confirmation.34,35 None-}

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**Table 1**

<table>
<thead>
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<th>Protein</th>
<th>Interferon Beta-1a</th>
<th>Culture Medium</th>
</tr>
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<tbody>
<tr>
<td>Survivin, densitometric units</td>
<td>43.2 ± 17.7†</td>
<td>77.4 ± 25.6</td>
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<tr>
<td>Bcl-2 proteins, U/mg of protein</td>
<td>140.6 ± 17.2</td>
<td>137.8 ± 22.3</td>
</tr>
<tr>
<td>Fas protein, U/mg of protein</td>
<td>9.7 ± 1.8†</td>
<td>7.5 ± 2.8</td>
</tr>
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*Data are mean ± SD of experiments on T cells from 5 interferon-naive patients.
†P<.05 when compared with corresponding expression in cells incubated with culture medium.

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**Figure 4**

Effect of cellular survivin expression on etoposide-induced apoptosis of mitogen-stimulated T cells from 14 interferon beta-1a responders who were studied prospectively. The declining survivin group represents the 10 patients in whom survivin expression declined during treatment, whereas the persistent survivin group refers to the 4 patients in whom cellular survivin was detectable but did not decline after treatment. Blood samples from the declining survivin group at month 12 were obtained from 5 patients only. Values represent mean ± SE. Asterisk indicates P<.01 compared with baseline DNA fragmentation.
reflected interactions among populations. Although it would be of interest to determine whether interferon beta therapy differentially modulates survivin expression in T-cell subgroups or antigen-specific lymphocytes, recent evidence indicates that the drug does not alter survival of myelin-specific T-cell lines.17

Our findings indicate that reduced cellular survivin expression and the corresponding increased susceptibility to apoptosis are maintained during long-term treatment with interferon beta. Since the down-regulatory effect of interferon beta on cellular survivin expression is confirmed by in vitro experiments, it is likely that the cellular changes reported herein are functionally related to interferon beta therapy. These changes also appear to correlate with clinical response to treatment. However, it is not clear from our data whether pretreatment levels of cellular survivin would predict clinical response to interferon beta therapy. Further evaluation is necessary to examine the clinical relevance of our findings and the molecular mechanisms through which interferon beta regulates cellular expression of survivin. It is of note, however, that interferon beta therapy in this study did not modify the expression of the antiapoptotic protein Bcl-2. This is in agreement with previous reports that interferon beta activates apoptosis without modulating the expression of Bcl-2 family proteins.39 We specifically sought to evaluate Bcl-2 expression because of its unique role among oncogenic proteins, as it enhances lymphoid cell survival by interfering with apoptosis rather than promoting cell proliferation.39 Thus, our results implicate survivin, rather than Bcl-2, as the modulator of T-cell susceptibility to apoptosis during interferon beta therapy. Yet, our findings of augmented T-cell apoptosis after interferon beta therapy may be related to the observed increase in cellular Fas expression, a finding consistent with earlier studies,39,45,50 or to down-regulation of the apical caspase inhibitor FLIP (Fas-associated death domain–like interleukin-1beta-converting enzyme inhibitory protein).16 Taken together, these observations and the current results indicate that interferon beta regulates T-cell death at multiple cellular levels that involve modulation of both apical and downstream caspase inhibitors.

In conclusion, apoptotic elimination of potentially pathogenic T cells in MS might accelerate termination of the inflammatory response. Our results indicate that interferon beta therapy augments apoptosis of T lymphocytes by down-regulating cellular expression of survivin. Since interferon beta is known to induce multiple immunomodulatory pathways and apoptosis-regulatory genes,17 the observed changes in cellular survivin expression might not represent a primary therapeutic mechanism. Nonetheless, our findings improve understanding of the therapeutic effects of interferon beta and suggest that survivin expression is an additional immunologic variable in MS. The identification of cellular pathways that modulate T-lymphocyte apoptosis may open avenues to more targeted therapies for MS.

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