Activation-induced cell death and Fas-induced apoptosis in patients with systemic or pauciarticular juvenile idiopathic arthritis

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Abstract
Objective
To investigate the functionality of the Fas-induced apoptotic pathway in peripheral blood mononuclear cells (PBMC) from patients with systemic or pauciarticular juvenile idiopathic arthritis (JIA).

Methods
PBMC from 12 patients with systemic and 6 with pauciarticular JIA were activated with anti-CD3 and rhIL-2 and then incubated in the presence or absence of the anti-Fas MoAb CH11 inducing activation of the Fas apoptotic pathway. Apoptosis was evaluated by flow cytometry and fluorescence microscopy.

Results
The percentage of apoptotic cells following triggering of Fas did not differ between patients with systemic JIA (12.5 ± 9.5%) or pauciarticular JIA (18.7 ± 8.9%) and controls (16.1 ± 6.8%). Evaluation of activation-induced cell death (AICD) in the absence of exogenous triggering of Fas showed that 44% (8/18) of the patients with JIA, compared to none of the controls (0/16), had a percentage of apoptotic cells higher than the mean + 2 SD of controls. The increased AICD was neutralized by the addition of an anti-TNF- antibody.

Conclusion
Patients with systemic or pauciarticular JIA do not show a defect in the Fas-dependent apoptotic pathway of T cells. The increased AICD present in some patients with JIA appears to be at least in part related to the inflammatory cytokine TNF- .

Key words
Apoptosis, Fas, juvenile idiopathic arthritis.

Introduction

Apoptosis is an active, signal-dependent process leading to cell death with characteristic changes in the dying cells: cell shrinkage, blebbing of the plasma membrane, nuclear collapse and fragmentation of nuclear chromatin (1). In addition to representing a mechanism of negative selection employed by self-reactive thymocytes (2), apoptosis is also a key mechanism of immune homeostasis in the periphery, where activation-induced cell death (AICD) plays a crucial role in the extinction of the immune responses to foreign and self antigens (3). Although several mechanisms are involved in the induction of apoptosis, much interest has been focused on the role of the Fas/Fas ligand pathway in immune regulation. Mice with a mutation of the Fas gene (MRL/lpr/lpr) or of the Fas ligand gene (C3H/HeJ-gld/gld) present the spontaneous occurrence of autoimmunity (4). In humans mutations in the Fas, Fas ligand or caspase 10 genes (5-7), leading to impaired functionality of the Fas pathway, cause the recently identified disorder called autoimmune lymphoproliferative syndrome (ALPS) (8,9). This syndrome is characterized by a non-malignant lymphoproliferopathy with expansion of double negative (CD4+CD8-) T cells and autoimmune features such as hemolytic anemia, thrombocytopenia, neutropenia (8, 9). Therefore, defects in the mechanisms of apoptosis induction could be responsible for autoimmune and/or inflammatory chronic diseases.

While a number of studies have evaluated the mechanisms leading to T cell apoptosis (in particular Fas-induced apoptosis) in patients with systemic lupus erythematosus or rheumatoid arthritis (10), to the best of our knowledge no such studies have been carried out in patients with juvenile idiopathic arthritis (JIA). Therefore, this study set out to investigate the functionality of the Fas-induced apoptotic pathway and of activation-induced cell death in patients with systemic or pauciarticular onset JIA, the two most characteristic forms of childhood chronic arthritis. We found that the induction of apoptosis by Fas triggering was comparable between patients and controls, demonstrating that systemic JIA (s-JIA) and pauciarticular JIA (p-JIA) are not associated with functional defects of Fas-triggered apoptosis. Evaluation of AICD did not show defective AICD in JIA patients compared to controls. On the contrary, increased AICD was found in a significant percentage of the JIA patients; this increase appears to be mediated by TNF-.

Materials and methods

Patients

Twelve patients (mean age 8.0 years, range 3-17) with s-JIA, and 6 with p-JIA who fulfilled the diagnostic criteria for JIA (11) were studied. All patients presented active disease, defined by the presence of synovitis upon examination. Table I shows the erythrocyte sedimentation rate (ESR) values, C-reactive protein concentrations, and the number of active joints. At the time of sampling, all patients were being treated with non-steroidal anti-inflammatory drugs; 9/18 patients were receiving weekly methotrexate (MTX) (7 with s-JIA, and 2 with p-JIA and a

<table>
<thead>
<tr>
<th>Table I. Erythrocyte sedimentation rate values, C-reactive protein concentrations and number of active joints in patients with systemic or pauciarticular JIA. Values are shown as mean ± standard deviation and range is indicated between parenthesis.</th>
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<tr>
<td>Erythrocyte sedimentation rate (mm/h)</td>
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<tr>
<td>(10 - 116)</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
</tr>
<tr>
<td>(3-172)</td>
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<tr>
<td>Number of active joints</td>
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340
polyarticular course). In addition, 8 patients with s-JIA were studied during treatment with prednisone (on an alternate day regimen in 5 of them). Sixteen healthy children (mean age 8.6 years, range 4-16), hospitalized for minor surgical procedures or bone marrow donation, were used as controls. Permission to draw extra blood during routine venipuncture was obtained from the parents of all children. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by standard Ficoll-Hypaque density centrifugation and resuspended in complete medium (RPMI 1640, supplemented with glutamine, gentamicine and 10% FCS).

Activation- and Fas-induced cell death, and apoptosis analysis
2.5 x 10^5 PBMC were cultured in 24-well plates in 1 ml of complete medium with 0.1 g/ml of anti-CD3 (Ortho, Raritan, NJ) and 20 U/ml of rhIL-2 (Hoffmann-La Roche, Nutley, NJ) for 5 days. To evaluate the induction of apoptosis triggered by Fas, 200 ng/ml of the activating MoAb CH11 to Fas (Upstate Biotechnology, Lake Placid, NY) were added during the last 24 hours of incubation, and then cells were harvested and analyzed for apoptosis. To evaluate activation-induced cell death (AICD), apoptosis was measured without the addition of the CH11 MoAb. In order to evaluate the relevance of the Fas or the TNF-α pathway in AICD, activation of PBMC, as described above, was performed in the presence of 2 μg/ml of a neutralizing anti-TNF-α MoAb (R&D Systems, Minneapolis, MN), or of 1 μg/ml of the Fas blocking MoAb ZB4 (Medical & Biological Laboratory Co., Nagoya, Japan).

Two different methods were used for apoptosis analysis: (i) PBMC were stained as described by Nicoletti et al. (12) with 1.5 ml of a hypotonic fluorochrome solution (PI 50 m/ml in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma, St. Louis, MO) for 18 h at 4°C before flow cytometric analysis; and (ii) PBMC were stained with 15 ng/ml of acridine orange and 50 ng/ml of ethidium bromide and analyzed for the presence of condensed and fragmented nuclei by fluorescence microscopy (13). At least 200 cells were read per sample by a blinded observer. A very high correlation in the percentage of apoptotic cells measured using the two methods was found (r = 0.81). All data shown in the figures were obtained using fluorescence microscopy.

Statistical analysis
Data were analyzed using the Mann-Whitney U test for unpaired samples, the Wilcoxon test for paired samples, and Fisher’s exact test, as appropriate. Correlations were evaluated with the Spearman rank test. A p value < 0.05 was considered statistically significant.

Results
Apoptosis induced by anti-Fas antibody in PBMC
In order to evaluate whether patients with JIA had a defect in Fas-induced apoptosis, we measured the percentage of apoptotic cells induced by triggering Fas with the anti-Fas MoAb CH11 following PBMC activation with anti-CD3 and IL-2. The percentage of apoptotic cells was comparable between JIA patients and controls (Fig. 1). When the patients were divided according to the onset type of the disease, the percentage of apoptotic cells induced by the anti-Fas antibody tended to be lower in patients with s-JIA (12.5 ± 9.5%) than in patients with p-JIA (18.7 ± 8.9%) or in controls (16.1 ± 6.8%), but the difference was not statistically significant (p > 0.1) (Fig. 1). We then evaluated whether the modest decrease in anti-Fas induced apoptosis in patients with s-JIA could be secondary to a reduced expression of the Fas molecule on the cell surface. Evaluation of the percentage of CD3+ cells expressing Fas did not show significant differences between patients with s-JIA and controls (CD3+ cells expressing Fas 33.0 ± 15.5% and 27.1 ± 7.8%, respectively). Moreover, after activation of PBMC with anti-CD3 and IL-2 for 5 days, the expression of Fas on T cells was higher than 90% both in patients with s-JIA and in controls. We did not find a significant correlation of the percentage of Fas-induced apoptosis with different treatments or with clinical and laboratory parameters of disease activity, such as the number of active joints, serum C-reactive protein concentration, and the ESR (data not shown). These data show that patients with JIA do not present a defect in the Fas-triggered pathway of apoptosis.

Activation-induced cell death (AICD) in patients with JIA
Activation of T cells results in programmed cell death in the absence of any exogenous triggering of the Fas molecule. Therefore, we evaluated the percentage of apoptotic cells after the activation of PBMC with anti-CD3 and IL-2, in the absence of the anti-Fas MoAb. Results expressed as a percentage of apoptotic cells are shown in Figure 2. None of the patients with s-JIA or p-JIA showed a percentage of apoptotic cells lower than the mean - 2 SD of the controls, thus ruling out a possible defect of
Table II. Apoptosis following T cell activation (AICD) in patients with JIA divided according to the presence or absence of prednisone (PDN) or methotrexate (MTX) treatment at time of sampling. Results, expressed as percentage of apoptotic cells, are shown as mean ± standard deviation.

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<tr>
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<th>PDN</th>
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<tr>
<td>AICD (% of apoptotic cells)</td>
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<tr>
<td>Yes</td>
<td>24.0 ± 11.5 (*)</td>
<td>30.8 ± 17.0 (*)</td>
</tr>
<tr>
<td>No</td>
<td>30.7 ± 14.5</td>
<td>24.6 ± 9.0</td>
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<tr>
<td>(n = 8)</td>
<td>(n = 9)</td>
<td>(n = 7)</td>
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(*) p > 0.1 versus corresponding patients not treated with PDN or MTX.

Fig. 2. Apoptosis induced by T cell activation (AICD) in healthy controls (ctrl) and in patients with systemic-JIA (s-JIA) or pauciarticular-JIA (p-JIA). The mean value of each group is represented by the continuous lines. The upper and lower limits of normal (means ± 2 SD of controls) are shown by the dotted lines.

Fig. 3. Increased activation induced cell death (AICD) in healthy controls (ctrl) and in patients with systemic-JIA (s-JIA) or pauciarticular-JIA (p-JIA). The mean value of each group is represented by the continuous lines. The upper and lower limits of normal (means ± 2 SD of controls) are shown by the dotted lines.

AICD in patients with JIA. On the contrary, 44% (8/18) of the patients with JIA (5/12 with s-JIA and 3/6 with p-JIA), compared to none of the controls (0/16) showed a percentage of apoptotic cells higher than the mean ± 2SD of the controls (p < 0.005, by Fisher’s exact test). When patients were divided according to current treatment, we did not find any significant difference in the percentage of apoptotic cells in patients receiving MTX or corticosteroid treatment (Table II). In addition, the percentage of apoptotic cells was comparable between patients receiving daily glucocorticoids compared to those on an alternate day regimen, and no relation was found with the glucocorticoid dose (data not shown). These results rule out a direct effect of MTX or corticosteroid treatment on in vitro AICD (Table II). No correlation of the levels of AICD with clinical and laboratory parameters of disease activity, such as the number of active joints, serum C-reactive protein concentration, and the ESR was found (data not shown).

Among the various pathways capable of inducing AICD, a vast body of evidence points to the role of activation of the Fas/FasL pathway (3). Therefore, we evaluated whether in 5 patients with increased AICD, the addition of an anti-Fas neutralizing antibody inhibited AICD. As shown in Figure 3, addition of the anti-Fas antibody ZB4 did not result in any significant decrease in AICD (p > 0.1 by the Wilcoxon test for paired samples). Several studies have shown that TNF- can also cause AICD (14-16). Since increased production of TNF- is well documented in patients with JIA (17-19), we evaluated the effect of the addition of an anti-TNF- neutralizing antibody in the patients with increased AICD. As shown in Figure 3, addition of the neutralizing anti-TNF- antibody resulted in a significant decrease (p = 0.03 by Wilcoxon test for paired samples) in AICD to levels similar to those of controls.

Taken together these data show that patients with s-JIA or p-JIA do not have a defect in AICD, but rather that increased AICD occurs in some patients. This increased AICD appears to be at least in part related to the inflammatory cytokine TNF-.

Discussion

In this study we show that patients with s-JIA or p-JIA do not have a defect in the induction of apoptosis following T cell activation (AICD). This suggests that no major defects in the mechanisms leading to T cell apoptosis are present in patients with these onset forms of JIA. Apoptosis induced in T cells after continuous TCR stimulation is in great part determined by activation of the Fas dependent cascade (3). In this study we also found that patients with s-JIA or p-JIA do not show a defect in apoptosis induced by the triggering of the Fas molecule. Consistent with these functional data, we found that the cell surface expression of Fas on T lymphocytes was comparable between patients and controls. Adding further evidence to the presence of normal Fas expression, Knipping et al. have reported normal levels of soluble Fas in patients with JIA (20).
As mentioned above, a marked increase in double negative (CD4 CD8) T cells is a characteristic feature of ALPS (8, 9). We have previously reported that patients with JIA, particularly s-JIA, also show an increase in double negative T cells, albeit at an apparently lower level than patients with ALPS (21). Incidentally, this finding was confirmed in the present study (data not shown). In ALPS the increase in double negative T cells is believed to be secondary to defective Fas-induced apoptosis, leading to the accumulation of T cells at a late stage of activation in lymphoid organs and in the periphery (8, 9). Since we excluded a defect in Fas-induced apoptosis in JIA, the same mechanism does not appear to be involved in this disease. The mechanism leading to the increased number of double negative T cells in JIA remains to be established. Recent observations showed that double negative T cells may represent a T cell population with regulatory and/or immunosuppressive functions (22, 23).

In this study we also found that in a significant percentage of patients the AICD was higher than that of controls. It is noteworthy that MTX (0.1 - 10 M) induces the apoptosis of in vitro activated T cells from human peripheral blood (24). A significant increase in the apoptosis of in vitro activated T cells from patients with RA treated with MTX was found only at 8 hours, with a subsequent decrease following MTX injection (24). However, all of our patients were studied at least 24 hours from the last MTX administration, and we did not find a correlation between increased AICD and treatment with MTX, nor with PDN, at the time of sampling. Increased AICD might be secondary to a pre-existing in vivo cellular activation state of the PBMC of patients, with the in vitro stimulation actually representing a re-stimulation, therefore leading to increased apoptosis. However, although several reports have demonstrated that synovial T cells are activated in JIA, the available evidence, albeit limited to a few and not very recent papers, does not suggest an activation state of peripheral blood T cells in JIA. For example, it has been reported that the percentage of HLA-DR-positive T cells in JIA patients was low and not different from that of controls (25). As previously mentioned, activation of the Fas-dependent cascade plays a major role in activated T cell apoptosis. However, the increased AICD in patients with JIA was not inhibited by the addition of a neutralizing anti-Fas MoAb, thus ruling out a role for the Fas-dependent cascade in this phenomenon. On the contrary, we found that the addition of a neutralizing anti-TNF-MoAb markedly decreased AICD to levels similar to those of controls. TNF- levels are increased in vivo in patients with JIA (17-19), and increased TNF- production plays a role in the pathogenesis of the disease, as demonstrated by the therapeutic efficacy of treatment with a TNF- antagonist (26). Further supporting a relationship between increased AICD and TNF-, we found a positive correlation (Rs= 0.540) between serum TNF- levels, measured in 10 of the patients studied, and AICD (data not shown).

Although our data point to the TNF pathway as being responsible for the increased AICD in patients with JIA, it remains to be established whether this finding is secondary to the increased production of TNF- in the culture system or to increased sensitivity to the pro-apoptotic effects of TNF- by T cells from patients with JIA. Indeed, the role of TNF- in mediating pro-apoptotic effects in T cells has been a matter of debate. While some authors have shown that TNF- induced apoptosis in mature T lymphocytes (14-16), other studies showed a protective effect of TNF- on T cell apoptosis, with the protective effect being mediated by the TNF-induced activation of NF- B (27). Interestingly, both salicylates and glucocorticoids have been shown to inhibit the activation of NF- B (28,29). It is therefore conceivable to speculate that both increased production of TNF- and reduced activation of NF- B, secondary to treatment, may be involved in the increased AICD in JIA patients.

In conclusion, this study shows that patients with s-JIA or p-JIA do not have a defect in AICD or in the Fas-dependent apoptotic pathway, thus ruling out an association between JIA and abnormalities in the Fas-dependent mechanisms leading to T cell apoptosis. The increased AICD observed in a significant percentage of the patients appears to be related to TNF-.

References
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