CONCISE REPORT

Apoptosis of peripheral blood lymphocytes in patients with juvenile idiopathic arthritis

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Background: Recent data suggested that abnormalities in mechanisms regulating apoptosis may have a role in the development of the rheumatoid process.
Objective: To evaluate different aspects of apoptosis in children with juvenile idiopathic arthritis (JIA).
Methods: The frequency of TUNEL positive peripheral blood (PB) lymphocytes (apoptotic index (AI)), as well as serum CD95 (APO1/Fas) antigen expression and serum levels of sFas and interleukin 15 (IL15), were examined in 44 cases of JIA. Results were correlated with type of onset, activity of JIA, and acute phase indicators.
Results: The AI of lymphocytes was significantly higher in patients with JIA than in controls (p=0.020). The mean AI of lymphocytes was increased in JIA with systemic type of onset and high activity (p=0.001). Moreover, IL15 levels in systemic disease were higher than in controls (p=0.012). An increased AI correlated with raised IL15 (p=0.046), erythrocyte sedimentation rate (p=0.005) and C reactive protein (CRP; p=0.017). Additionally, correlation was found between IL15 and CRP levels (p=0.039). CD95 and sFas levels were unchanged compared with controls.
Conclusion: PB lymphocytes of children with JIA have an increased tendency to undergo apoptosis. The degree of apoptosis depends on the type of onset and activity of JIA and correlates with serum levels of IL15. Further studies are needed to explain whether this is an epiphenomenon of the disease activity or is related to the pathogenesis of JIA.

Recent studies suggest that abnormalities in regulation of apoptosis may have a role in the pathogenesis of autoimmune disorders, including rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA). Inhibition of apoptosis, with higher expression of anti-apoptotic Bcl-2 protein in synoviocytes, was found in patients with RA and JIA.[1 2] Additionally, raised serum levels of the soluble form of Fas, anti-apoptotic sFas, were detected in RA.[3] Proinflammatory cytokines such as tumour necrosis factor α, interleukin (IL)1, IL6, IL12, or IL15 are also believed to have a role in the development of the rheumatoid process.[4 5]

The clinical course of JIA differs from that of RA, with more frequent systemic manifestations in children. A differentiation defect of T lymphocytes and their local activation in synovial tissue is considered to be the key event in both types of rheumatoid process.[6 7] Less is known about homoeostasis maintained by equilibrium between cell proliferation and death among circulating immune cells. This study aimed at evaluating the incidence of apoptosis of lymphocytes in peripheral blood (PB) of children with JIA and correlating it with CD95 (APO1/Fas) antigen expression or serum levels of sFas and IL15, in different types of onset and activity of the disease.

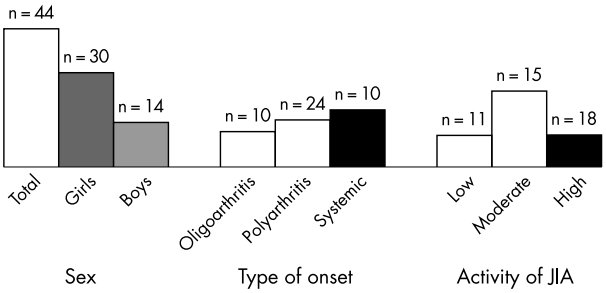

Figure 1 Clinical characteristics of children with JIA. Three subgroups with different onset of disease were established: polyarthritis, oligoarthritis and systemic disease. Three stages of JIA activity were also distinguished, based on clinical and laboratory criteria: low activity (joint movement limited, without pain or swelling, no extra-articular symptoms, ESR <20 mm/1st h, CRP <10 mg/l); moderate activity (moderate intensity of arthritis, and/or slight temperature, ESR 20–60 mm/1st h, CRP 10–30 mg/l); and high activity (morning stiffness, pain and/or swelling of joints, and/or hepatosplenomegaly, fever, rash, and raised values for laboratory tests ESR >60 mm/1st h, CRP >30 mg/l).

PATIENTS AND METHODS

Patients

Forty four children with JIA, aged 3–17 years (mean (SD) 11.6 (4.2)) were examined. A diagnosis of JIA was established according to Durban's criteria.[7] None of the children was given second line treatment before the study. Only non-steroidal anti-inflammatory drugs were used as a basic treatment, and these were stopped at least three days before collection of blood samples. The mean (SD) time interval from the first symptoms of JIA and enrolment in the study was 1.2 (1.8) years, reflecting a delayed diagnosis. Based on the type of onset, three subgroups were established: polyarthritis, oligoarthritis, and systemic disease (fig 1). Furthermore, based on clinical and laboratory criteria three stages of JIA activity were stated: low, moderate, and high activity of the disease (fig 1). The mean (SD) values of routine laboratory tests in the children with JIA were: leucocytes 9.4 (3.8)×10^9/l, lymphocytes 2.7 (2.8)×10^9/l, haemoglobin 125 (1.6) g/l, platelets 445.2 (158.6)×10^9/l, C reactive protein (CRP) 56 (16.3) mg/l, erythrocyte sedimentation rate (ESR) 46.7 (35.4) mm/1st h.

The controls comprised 30 healthy children, 22 girls and 8 boys, aged 4–17 years (mean (SD) 12.2 (2.9)).

Abbreviations: AI, apoptotic index; CRP, C reactive protein; ESR, erythrocyte sedimentation rate; IL, interleukin; JIA, juvenile idiopathic arthritis; PB, peripheral blood; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; RA, rheumatoid arthritis; TUNEL, TdT mediated dUTP-biotin nick end labelling

Table 1 The rate of apoptosis (TUNEL positive) of lymphocytes, expression of CD95, and serum levels of sFAS and IL15 in children with JIA according to the type of onset and activity of the disease. Correlation between examined parameters and acute phase indicators

Examined groups	No	TUNEL (% cells) Mean (SD)	CD95 (% cells) Mean (SD)	sFAS (pg/ml) Mean (SD)	IL15 (pg/ml) Mean (SD)
Controls	30	2.3 (2.9)	9.8 (5.5)	5024.5 (2184.5)	1.7 (0.6)
JIA - whole group	44	6.5 (7.4)	12.1 (7.3)	5761.3 (2856.1)	1.9 (1.3)
Type of onset					
Oligoarthritis	10	3.7 (3.9)	11.2 (9.4)	6308.0 (3512.1)	1.0 (0.4)
Polyarthritis	24	4.8 (7.1)	13.1 (7.2)	5661.0 (2427.2)	1.1 (0.6)
Systemic	10	13.5 (7.1)	9.7 (3.8)	5502.9 (3557.6)	3.6 (2.1)
Activity of the disease					
Low	11	2.7 (2.5)	13.2 (13.2)	6894.0 (2736.1)	1.6 (0.1)
Moderate	15	4.9 (8.1)	10.9 (6.9)	5492.5 (2345.1)	1.8 (1.4)
High	18	9.2 (8.0)	12.6 (5.5)	5455.0 (3196.0)	2.3 (1.6)
Statistics (p values)					
Control *v* JIA		0.020*	0.331	0.424	0.825
Control *v* oligoarthritis		0.381	0.859	0.351	0.046*
Control *v* polyarthritis		0.123	0.206	0.411	0.950
Control *v* systemic disease		0.001*	0.780	0.867	0.012*
Control *v* low activity		0.437	0.938	0.071	0.612
Control *v* moderate activity		0.517	0.596	0.496	0.517
Control *v* high activity		0.001*	0.195	0.878	0.203
Acute phase indicators		*v* TdT	*v* CD95	*v* sFAS	*v* IL15
Lymphocyte count		0.098, r_s=0.313	0.368, r_s=–0.196	0.038*, r_s=–0.313	0.867, r_s=–0.035
Platelet count		0.495, r_s=–0.137	0.068, r_s=0.404	0.438, r_s=–0.122	0.543, r_s=–0.133
CRP		0.017*, r_s=0.460	0.469, r_s=–0.166	0.172, r_s=–0.225	0.039*, r_s=0.442
ESR		0.005*, r_s=0.502	0.867, r_s=0.036	0.114, r_s=–0.241	0.089, r_s=0.347

*Significant.
CRP, C reactive protein; ESR, erythrocyte sedimentation rate; r_s, Spearman rank coefficient.

Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood by centrifugation on Histopaque 1077 (Sigma, St Louis, MO, USA). Cells were then washed twice in phosphate buffered saline (PBS) buffer (Sigma) and resuspended in 0.5 ml of mixture of PBS/human serum albumin (1:1). Fifty microlitres of cell suspension, at a concentration of 5×10^5 cells/ml, was cytocentrifuged. Slides were fixed in 1% methanol-free formaldehyde (15 min/0°C), then in 70% ethanol (30 min/0°C). Simultaneously, serum was collected and stored at –80°C.

Apoptosis evaluation

Apoptosis was assessed by an in situ TdT mediated dUTP-biotin nick end labelling (TUNEL) of DNA strand breaks assay. Slides with fixed PBMC were washed in PBS subjected to the TUNEL assay using the APO-DIRECT kit provided by Phoenix Flow Systems, Inc (San Diego, CA, USA) and counterstained with propidium iodide in the presence of RNase. The intensity of cell fluorescence was measured by a laser scanning cytometer (CompuCyte, Cambridge, MA, USA); about 10 000 cells were measured for each sample. Taking advantage of the morphometric capabilities of laser scanning cytometry,[8] we identified lymphocytes from other PBMC, based on the differences in area/red integral and red maximal pixel of DNA fluorescence. The percentage of TUNEL positive cells thus represents the apoptotic index (AI) of lymphocytes. The relocation feature of laser scanning cytometry allowed us to confirm the apoptotic mode of cell death by morphological criteria, as well as by the "sub-G1" DNA content based on cell staining with propidium iodide.[8]

CD95 antigen

Expression of CD95 on cytospin preparations was examined immunohistochemically in an ABC system using anti-CD95 antibody (DAKO, Denmark), and assessed by light microscope (×40 and ×100 magnification) as the frequency of CD95 positive lymphocytes.

Serum sFas and IL15 levels

Serum concentrations of sFas and IL15 (both R&D Systems, UK) were measured by an enzyme linked immunosorbent assay (ELISA).

Other laboratory parameters

In all cases, in parallel with the evaluation of apoptosis, the PB, ESR, and CRP concentrations were estimated by standard methods.

Statistical analysis

Mean and standard deviations were calculated and compared using the Mann-Whitney U test. Correlations between variables were evaluated by the Spearman r_s test. Results were considered significant at p values <0.05.

RESULTS

Table 1 shows a detailed comparison of results.

Apoptotic index

The mean values of all parameters evaluated (AI, CD95+ lymphocyte rate, levels of sFas, and IL15) were higher in children with JIA than in healthy controls. However, significant differences were only found for AI (p=0.020). The AI correlated positively with IL15 levels (p=0.046, r_s=0.402). No correlation between AI and CD95 expression, serum sFas, or IL15 levels was apparent.

Type of onset and apoptosis

The mean AI of lymphocytes was significantly higher in systemic JIA than in controls (p=0.001).

No significant correlation was found between either the frequency of CD95+ lymphocytes *v* serum sFas levels or *v* type of onset of JIA.

The mean serum IL15 level in systemic disease was increased (p=0.012), whereas in oligoarthritis it was decreased (p=0.046) compared with controls. IL15 levels in systemic JIA were higher than those in oligoarthritis (p=0.009) and polyarthritis (p=0.006).

Activity of JIA

There were significant differences between the AI in high activity JIA and low (p=0.040) or moderate (p=0.025) activity of the disease, as well as in healthy controls (p=0.001).

Acute phase indicators

The AI of lymphocytes correlated with the CRP concentration and the ESR (p=0.017 and p=0.005, respectively). Additionally, there was significant correlation between serum levels of IL15 and CRP (p=0.039). Serum sFas correlated inversely with lymphocyte count (p=0.038).

DISCUSSION

The results of our study provide evidence for enhanced apoptosis of PB lymphocytes in children with JIA who were not treated with immunosuppressive drugs. The highest AI rate was seen in systemic and in highly active disease.

A search of published reports produced only one related to apoptosis of PB lymphocytes in JIA. In that in vitro study Pignatti *et al* evaluated anti-Fas monoclonal antibodies induced apoptosis, in CD3 and IL2 stimulated PBMC from patients with systemic and pauciarticular JIA.[9] The authors found no significant differences in the frequency of apoptosis triggered by the Fas dependent pathway between children with JIA and controls, whereas distinctly increased cell death of PBMC induced by treatment was seen in children with JIA. Our study was the first attempt to evaluate the frequency, not induced by treatment, of in vivo apoptosis of PB lymphocytes in this disease; therefore it is difficult to compare these data directly. Our findings may reflect mechanisms which play a part in the development of the rheumatoid process. In an experimental animal model of RA, enhancement of apoptosis in synovial lining cells was seen at an early stage of the arthritis progression, followed by its inhibition in the prolonged phase of inflammation.[10] A similar course of events may take place in PB lymphocytes.

For RA, the data are also fragmentary. Decreased catecholamine induced cell death of RA B lymphocytes was reported by Wahle *et al*.[11] Courtney *et al* found an increased rate of apoptotic PB lymphocytes in patients with RA compared with controls, without correlation with serum sFas levels,[3] similar to our JIA data.

Haas *et al* showed different Fas/sFas in PBMC, serum, and synovial fluid in oligoarthritis JIA, suggesting a role for Fas expression in dysregulation of apoptosis in this disease, which may take place not only in affected joints but also in circulating immune system cells.[12] In our study, expression of CD95 antigen on PB lymphocytes, as well as serum sFas levels, was not significantly higher in JIA than in healthy children.

Scola *et al* are the only authors who reported IL15 in children with JIA.[13] They found raised IL15 levels in synovial fluid, with a population of IL15 positive cells located predominantly within perivascular aggregates. In our patients we found increased serum IL15 levels in children with systemic disease, correlating with the CRP concentration, which may reflect an association between IL15 and joint tissue alterations. Harada *et al* found raised levels of IL15 mRNA in the synovium of patients with RA,[14] probably responsible for local activation of T lymphocytes. IL15 may cause the proliferation of fibroblast-like synoviocytes and inhibit their apoptosis, leading to uncontrolled hyperplasia of the synovium.[15]

In conclusion, we found an increased tendency for PB lymphocytes to undergo apoptosis in children with JIA. The intensity of this phenomenon depended on the type of onset and activity of the disease and corresponded with raised serum levels of IL15, but not with CD95 expression or serum concentration of sFas. Both the intensity of apoptosis and serum IL15 correlated with the acute phase indicator CRP levels. It is unclear, at present, whether the observed changes represent an epiphenomenon of disease activity or are related to the pathogenesis of JIA. To answer this question, further studies, including a detailed analysis of the mechanisms of apoptosis modulation during the rheumatoid process, are needed.

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