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Apoptosis and genome instability in children with autoimmune diseases

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Abstract

As apoptosis and genome instability in children with autoimmune diseases (AIDs) are insufficiently investigated, we aimed to analyse them in peripheral blood lymphocytes (PBLs) of children and adolescents with Hashimoto's thyroiditis (HT), Graves' disease (GD) and type 1 diabetes mellitus (T1DM), including possible factors that could affect their occurrence. The study population included 24 patients and 19 healthy controls. Apoptotic cells were detected using an Annexin V-FITC/7-AAD kit. Genome instability was measured as micronuclei (MNs) frequency using the cytokinesis-block MN assay. In addition, comet assay was performed for determination of genome instability as genome damage index (GDI) in new subpopulation of patients with T1DM. The percentage of apoptotic PBLs in patients with AID was significantly lower than in control subjects. There was a positive correlation between thyroid-stimulating homone (TSH) concentration and the proportion of cells in late stage apoptosis in patients with autoimmune thyroid diseases (AITDs). The MN frequency in patients was significantly higher than in controls. Individuals with HT or T1DM had a significantly higher MN frequency than those with GD. Similarly, the value of GDI in patients with T1DM was significantly higher than in controls. The level of apoptosis was positively correlated with MN frequency as well as with GDI in patients with AID. In conclusion, children with AITD (HT and GD) and T1DM have a significantly lower level of apoptosis in PBLs and significantly higher MN frequency as GDI than healthy subjects. Apoptosis and the level of genome instability in these patients with AID are positively correlated.

Introduction

Apoptosis is a highly controlled form of cell death responsible for the maintenance of tissue homoeostasis. Control of apoptotic mechanisms is critical for the activity of cellular populations under physiological conditions. Besides, apoptosis participates in the elimination of pathologically altered and unnecessary cells (1). Excessive or insufficient apoptosis due to faulty pathways has an important role in the pathogenesis of many diseases (2,3). Published data indicate deregulation of apoptosis in the initiation and propagation of autoimmune diseases (AIDs) (4,5). It is assumed that apoptotic fragments produced during programmed cell death might be a potential source of the autoantigens that induce the autoimmune process (6,7).

Micronuclei (MNs) are chromosomal fragments or whole chromosomes isolated during anaphase of cell division. They appear

in the cytoplasm of daughter cells as small additional nuclei (8) and occur after exposure to many physical and chemical agents. Therefore, they are considered to be surrogate markers of genomic instability. Earlier studies on micronuclei frequency in patients with organ-specific and/or systemic AIDs have given contradictory results (reviewed in Torres-Bugarín *et al.* (9)). Analyses of MN frequency in children with autoimmune phenomena are still missing, with the exception of newborns from women with diabetes mellitus (10).

The aim of our study was to investigate the level of apoptosis in peripheral blood lymphocytes (PBLs) of young patients with three relatively frequent AIDs, two autoimmune thyroid diseases (AITDs), Hashimoto's thyroiditis (HT) and Graves' disease (GD) and type 1 diabetes mellitus (T1DM) and to correlate it with MN frequency. In addition, it was examined the correlation of the level of PBLs apoptosis and DNA damage in the particular group of patients with T1DM.

Materials and Methods

Study population

The study was approved by the ethical committee of the Clinical Center Kragujevac. All patients and control subjects gave written informed consent according to the Helsinki Declaration.

The study population included 24 children and adolescents (14 females and 10 males) with AIDs, recruited at the Pediatric Clinic of the Clinical Center Kragujevac. Eight (33.3%) patients had HT, three (12.5%) had GD and thirteen (54.2%) patients had T1DM. Those with HT and GD were diagnosed on the basis of clinical presentation, high levels of antithyroid antibodies and abnormal thyroid function. In patients with HT the concentrations of thyroglobulin antibodies and thyroid peroxidase antibodies (TPOAbs) were 1089.7 ± 1967 IU/ml and 4069 ± 4316 IU/ml, respectively. In those with GD, the concentration of thyrotropin receptor antibodies (TSHRAbs) was 1.91 ± 1.33 IU/ml. Clinical presentation, 24-h glycaemic profile, the levels of glycated haemoglobin (10.24 ± 2.03 %) and glutamic acid decarboxylase antibodies (GADAbs) (577.8 ± 644.2 U/ml) were used for diagnosis of T1DM. The mean age of the study population was 10.4 ± 3.9 years.

The control group comprised 19 healthy subjects, 10 (52.6 %) females and 9 (47.4%) males of mean age 11.9 ± 3.7 years. They were distant relatives willing to participate, who had no clinical or laboratory signs of AID, and no family history of these disorders in first or second degree relatives.

Isolation of peripheral blood mononuclear cells

Blood samples were collected in the morning in polystyrene tubes. Heparinised peripheral blood (10 ml) was centrifuged at 400×g for 10 min to separate plasma and cells. Peripheral blood mononuclear and polymorphonuclear cells were separated by single step continuous density-gradient centrifugation with Lymphoprep (Lymphoprep 1.077; Nicomed Pharma AS, Oslo, Norway). The separated mononuclear cells were washed thrice with isotonic phosphate buffered saline (PBS) and then re-suspended in fresh PBS.

Detection of apoptosis

Apoptotic cells were detected using an Annexin V-FITC/7-AAD kit (Beckman Coulter IM3614). In the early phase of apoptosis, cell membrane integrity is maintained but the cells lose membrane phospholipid asymmetry. Phosphatidylserine (PS), a negatively charged phospholipid located in the inner leaflet of the plasma membrane, is then exposed at the cell surface. The calcium and phospholipid binding protein, Annexin V, binds preferentially to PS. In both late apoptosis and necrosis the cell membrane loses integrity and exposes DNA to viable dyes (11). It is considered that Annexin V and 7-AAD negative cells are viable, Annexin V positive and 7-Aminoactinomycin D (7-AAD) negative cells are in the early stages of apoptosis, whereas Annexin V and 7-AAD positive cells are in late stage apoptosis. Annexin V negative and 7-AAD positive cells are considered necrotic. After isolation, the cells were washed in PBS (p5493 Sigma-Aldrich) and re-suspended in ice-cold binding buffer to the final concentration of 1 million cells/ml. Samples for analysis were prepared using 100 µl of final solution incubated in the dark for 15 min with 10 µl of Annexin V-FITC and 20 µl of 7-AAD and re-suspended in 400 µl of ice-cold binding buffer. Finally, cells were analysed on an FC500 Beckman Coulter flow cytometer to the number of 20 000 events, gating lymphocytes on the FS/SS diagram. The percentages of cells in early and late stages of apoptosis were determined together with necrotic cells using CXP Cytometer software.

Cytokinesis-block MN assay

MNs were prepared using the Fenech and Morley method (12). Whole heparinised blood (0.5 ml) was added to 5 ml of PBMax Karyotyping (Invitrogen, CA, USA), the complete medium for lymphocyte culture. All cultures were incubated in duplicate at 37°C for 72 h. Cytochalasin B (Sigma, St. Louis, MO, USA) was added 44 h after initiation of incubation at a final concentration of 4 µg/ml. Cultures were harvested 28 h later. The standard cold hypotonic 0.56% KCl solution was used for preparation, followed by fixation in acetic acid–methanol fixative (1:3 v/v) three times for 15 min. The cell suspensions were dropped onto clean slides, air-dried and stained with 2% Giemsa for 12 min (Alfapanon, Novi Sad, Serbia). We used a light microscope (Nikon E50i) at ×400 magnification following the criteria for scoring MN only in binucleated (BN) cells as described by Fenech (8). The MN frequencies were determined in 1000 BN lymphocytes from each patient.

The single-cell gel electrophoresis (comet assay)

The comet assay is widely applied to assess DNA damage. The assay was performed as described by Singh *et al.* (13), with some modifications. Peripheral venous blood was collected from the additional group of 12 patients with T1DM aged from 5 to 19 years (12.42 ± 3.75) and 8 healthy control subjects aged from 21 to 25 years (21.88 ± 1.45). Lymphocytes isolated using Histopaque-1077 and incubated for half an hour at 37°C in PBS solution. Then treated cells were suspended in 100 µl of 1% low melting point agarose, and 90 µl of suspension was layered onto microscopic slides and kept at 4°C for 5 min. The slides were put in cold lysing solution at pH 10 for 2 h (2.5 M NaCl, 100 mM EDTA, 10 mM tris(hydroxymethyl) aminomethane [TRIS], 1% triton X-100, 10% dimethyl sulfoxide [DMSO]), and next were immersed in electrophoresis buffer (10 M NaOH, 200 mM EDTA) for 30 min. The electrophoresis was carried out at 4°C, 25 V and 300 mA for 30 min. After electrophoresis the slides were submerged in neutralisation buffer (0.4 M Tris–HCl, pH 7.5) three times for 5 min and stained with 50 µl ethidium bromide for 10 min. The slides were examined using fluorescent microscope (Nikon E50i) at an increase of 200×. Cells were divided into five categories based on DNA damage: 0, no damage (< 5%); 1, low-level damage (5–20%); 2, medium-level damage (20–40%); 3, high-level damage (40–95%); 4, total damage (>95%). A total of 100 cells per donor were analysed (50 cells from each of two replicate slides). The genome damage index (GDI) calculated using the formula (14):

$$GDI = \frac{Class1 + 2x\ Class2 + 3x\ Class3 + 4x\ Class4}{Class0 + Class1 + Class2 + Class3 + Class4}.$$

Statistical analysis

All values were expressed as mean ± SD. The commercial SPSS, version 13.0 for Windows was used for statistical analysis. Differences between two analysed groups were determined by the independent samples *t*-test or the Mann–Whitney *U*-test when the distribution of variables was not normal. The observed variables were compared by the bivariate correlation test and Pearson/Spearman coefficient. Multivariant regression analysis was performed for estimating the relationships among variables. *P* values less than 0.05 were considered to be statistically significant and those less than 0.01 highly significant.

Results

We determined the percentage of apoptotic cells and MN frequency as markers of genome instability in 24 patients with AIDs (11 patients with AITD and 13 patients with T1DM) and 19 control subjects. The results are shown in Tables 1 and 2, and Figures 1 and 2.

The mean percentage of PBLs in the early phase of apoptosis in patients with AID was significantly lower than that for control subjects (3.15 ± 2.55% vs. 5.63 ± 2.29%; Mann–Whitney test, $P = 0.002$). Moreover, the mean percentage of PBLs in late phase apoptosis (0.44 ± 0.54% vs. 0.89 ± 1.03%; Mann–Whitney test, $P = 0.001$) and the total percentage of apoptotic cells (early + late; 3.59 ± 2.68% vs. 6.53 ± 2.92%; Mann–Whitney test, $P = 0.002$) were also much lower in patients with AID than in the controls. However, the difference between the percentage of necrotic PBLs in patients with AID and controls was not statistically significant (0.23 ± 0.38% vs. 0.16 ± 0.34%; $P > 0.05$) (Table 1).

Analysis of the proportions of apoptotic and necrotic cells in the subgroups of patients with AID showed that the mean percentage of cells in the early stage apoptosis and the overall percentage of apoptotic cells in patients with AITD were each significantly lower than in patients with T1DM (1.88 ± 1.83% vs. 4.22 ± 2.64% and 2.12 ± 1.83% vs. 4.83 ± 2.72%, respectively). However, there were no significant differences between the two groups of patients with AID for percentage of PBLs in late stage apoptosis (0.24 ± 0.15% vs. 0.61 ± 0.68%; $P = 0.361$) and necrosis (0.38 ± 0.5% vs. 0.10 ± 0.13%; $P = 0.167$) (Table 2). The mean percentages of PBLs in early and late stage apoptosis in patients with GD were 1.24 ± 0.32% and 0.22 ± 0.09%, respectively. The equivalent values for patients with HT were 1.37 ± 0.84% and 0.24 ± 0.17%, respectively. The differences in the values for both early and late stage of apoptosis in PBLs between patients with GD and HT patients were not statistically significant (Independent samples *t*-test, $P = 0.798$ for early stage apoptosis and $P = 0.827$ for late stage apoptosis).

The relationships of thyroid status (TSH and FT4 concentrations) to apoptosis of PBLs in patients with AITD and that of glucose concentration to apoptosis of PBLs in patients with T1DM were also studied. We found a statistically significant positive correlation between TSH concentration and the percentage of PBLs in the late stage of apoptosis in patients with AITD (bivariate correlation test, Pearson $r = 0.627$, $P = 0.039$), whereas free T4 concentration showed no significant association with the level of apoptosis ($P > 0.05$). In patients with T1DM glucose concentration was negatively correlated with the percentage of PBLs in the early stage of apoptosis (bivariate correlation test, Pearson $r = -0.687$, $P = 0.019$) and the total percentage of apoptotic cells (bivariate correlation test, Pearson $r = -0.723$, $P = 0.012$).

The mean MN frequency in patients with AID was 6.87 ± 2.95/1000 BN cells, which was significantly higher than in PBLs of healthy controls (2.10 ± 1.99/1000 BN cells; Mann–Whitney test, $P < 0.001$). There were no significant differences in MN frequency between patients with AITD and T1DM (5.82 ± 2.71/1000 BN cells vs. 7.77 ± 2.95/1000 BN cells; $P = 0.108$). Among the patients with AID, the subjects with GD had the lowest MN frequency (Figure 1), significantly less than in patients with HT (3.67 ± 1.53/1000 BN cells vs. 7.14 ± 2.41/1000 BN cells, $P = 0.033$) or T1DM (3.67 ± 1.53/1000 BN cells vs. 7.77 ± 2.95/1000 BN cells, $P = 0.038$).

Analysis of the relationships between apoptosis and MN frequency, revealed positive correlations between MN frequency and early stage apoptosis (bivariate correlation test, Spearman $r = 0.580$, $P = 0.003$) and between MN frequency and total percentage of apoptotic cells (bivariate correlation test, Spearman $r = 0.527$, $P = 0.008$) in patients with AID (Figure 2). In the group of control subjects, the correlations between MN frequency and early stage of apoptosis (bivariate correlation test, Spearman $r = -0.664$, $P = 0.002$), and between MN frequency and total apoptosis (bivariate correlation test, Spearman $r = -0.681$, $P = 0.001$) were also statistically significant but negative.

We also analysed possible associations of specific autoantibody concentration and MN frequency in the patients with AID. The bivariate test indicated a significant positive correlation between the concentration of TPOAbs and MN frequency in patients with HT (Spearman $r = 0.795$, $P = 0.018$), but no significant correlations either between MN frequency and the concentration of TSHRAbs ($r_{GD} = 0.500$, $P_{GD} = 0.667$) in patients with GD or between GADAbs and MN frequency ($r_{T1DM} = 0.214$, $P_{T1DM} = 0.645$) in patients with T1DM.

As we showed the existence of a difference in the degree of chromosomal damage in patients and control subjects, we decided to analyse the relationship of DNA damage (expressed as GDI) in the additional group of patients with T1DM and control subjects, and their correlation with the degree of apoptosis.

Table 3 shows the results of comet assay. The comparison of the mean GDI values of patients (1.19 ± 0.20, ranged from 0.86 to

Table 1. Apoptosis and necrosis of PBLs in patients with AIDs and control subjects

	AID patients ($n = 24$)		Controls ($n = 19$)		Significance (*P* value)
	X ± SD	min–max	X ± SD	min–max	
Early apoptosis (%)	3.15 ± 2.55	0.62–8.78	5.63 ± 2.29	1.37–9.79	0.002
Late apoptosis (%)	0.44 ± 0.54	0.01–2.05	0.89 ± 1.03	0.28–4.90	0.001
Total apoptosis (%)	3.59 ± 2.68	0.78–8.98	6.53 ± 2.92	1.65–14.69	0.002
Necrosis (%)	0.23 ± 0.38	0.0–1.65	0.16 ± 0.34	0.0–1.20	0.062

Table 2. Apoptosis and necrosis of PBLs in patients with AITDs and T1DM

	AITD patients (n = 11)		T1DM patients (n = 13)		Significance (P value)
	X ± SD	min–max	X ± SD	min–max	
Early apoptosis (%)	1.88 ± 1.83	0.62–6.97	4.22 ± 2.64	0.83–8.78	0.026
Late apoptosis (%)	0.24 ± 0.15	0.01–0.56	0.61 ± 0.68	0.05–2.05	0.361
Total apoptosis (%)	2.12 ± 1.83	0.78–7.20	4.83 ± 2.72	0.89–8.98	0.022
Necrosis (%)	0.38 ± 0.51	0.02–1.65	0.10 ± 0.13	0.0–0.43	0.167

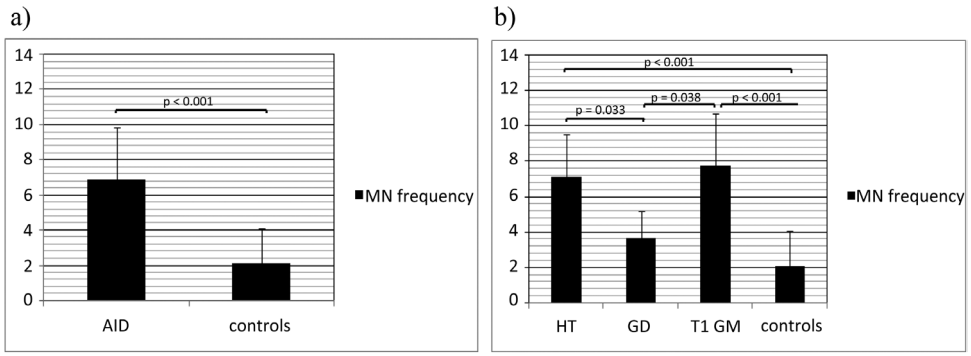

Fig. 1. Micronuclei frequency in patients with AID and control subjects (a) and micronuclei frequency in patients with HT, GD, T1DM and controls (b)

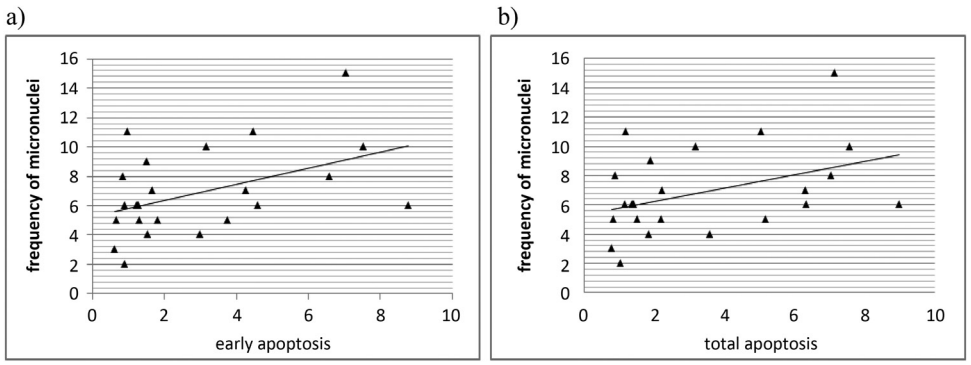

Fig. 2. Relationships between early apoptosis and micronuclei frequency (a) and total apoptosis and micronuclei frequency (b) in 24 patients with autoimmune disease

1.52) and healthy subjects (0.20 ± 0.03, ranged from 0.16 to 0.25) demonstrated that there is a significant difference. Like the MN test (8.3 ± 2.54/1000 BN cells in patients vs. 0.87 ± 0.83/1000 BN cells in controls), comet assay showed that DNA damage in patients were higher than in healthy subjects (P < 0.001), despite significantly difference of average mean age between these two groups. Multivariate regression analysis confirmed that the presence of disease significantly affects the genomic instability (P < 0.001) in contrast to the sex (P = 0.361) and age (P = 0.066) of the subjects involved in comet assay. There was significant positive correlation between MN frequency and GDI in the patients with T1DM (bivariate correlation test, Pearson r =0.716, P = 0.009) as well in the group of control subjects (bivariate correlation test, Pearson r =0.818, P = 0.013).

Analysis of the relationships between apoptosis and GDI values, revealed positive correlations between GDI and early stage apoptosis (bivariate correlation test, Pearson r =0.829, P = 0.001) and between GDI and total percentage of apoptotic cells (bivariate correlation test, Pearson r = 0.596, P = 0.041) in patients with T1DM.

Discussion

The aim of this study was to analyse the level of apoptosis and genome instability (MN frequency and genome damage index) in PBLs of paediatric patients with organ-specific AIDs: AITD (HT or GD) and T1DM. According to our best knowledge, this is the first study on possible relationships between apoptosis and MNs frequency as well as between apoptosis and GDI in patients with an AID and specifically in children.

We measured the percentage of PBLs in both the early (loss of membrane phospholipid asymmetry) and late stages of apoptosis (loss of cell membrane integrity). Our results showed that our patients with AID had a significantly lower percentage of apoptotic PBLs than control subjects. Moreover, mean values for the early stage of apoptosis and total apoptosis of PBLs in the patients with T1DM were significantly higher than those with AITD. Within the subgroup of patients with AITD, subjects with GD had a slightly lower percentage of apoptotic PBLs than those with HT, but the difference was not significant.

Table 3. Degree of genetic damage in peripheral blood lymphocytes of patients (a) and control subjects (b) obtained by comet assay

a) Patients no	Number of analysed cells	Comet classification					GDI (%)
		0 (<5%)	1 (5–20%)	2 (20–40%)	3 (40–95%)	4 (>95%)	
1	100	54	14	9	13	19	1.11
2	100	49	11	12	9	19	1.38
3	100	36	14	24	14	12	1.52
4	100	50	18	15	8	9	1.08
5	100	63	15	5	7	10	0.86
6	100	50	24	11	9	6	0.97
7	100	45	21	24	5	5	1.04
8	100	44	23	14	6	13	1.21
9	100	42	28	17	6	7	1.08
10	100	41	23	17	9	10	1.24
11	100	32	24	29	15	/	1.27
12	100	37	12	24	19	8	1.49
b) Controls no	Number of analysed cells	Comet classification					GDI (%)
		0 (<5%)	1 (5–20%)	2 (20–40%)	3 (40–95%)	4 (>95%)	
1	100	83	15	2	/	/	0.19
2	100	86	9	2	3	/	0.22
3	100	82	12	5	1	/	0.25
4	100	86	9	4	1	/	0.20
5	100	88	8	2	2	/	0.18
6	100	86	12	2	/	/	0.16
7	100	88	5	6	1	/	0.20
8	100	84	13	3	/	/	0.19

HT and GD are the most common organ-specific autoimmune disorders. The overall prevalence peaks in adulthood, but it does occur in children and adolescents (15). In the young population, it usually appears during early to mid-puberty (16,17). HT is characterised by an inflammatory cell infiltrate that damages the thyroid tissue and leads to gland fibrosis that is clinically manifested by hypothyroidism. In patients with HT, high levels of autoantibodies to thyroid peroxidase and/or thyroglobulin are present. On the other hand, GD is characterised by production of autoantibodies to the TSH receptor resulting in abnormal stimulation of the thyroid gland with elevated thyroid hormone secretion and is clinically manifested by hyperthyroidism. Some previously published studies indicate that apoptosis may have a role in the pathogenesis of AITDs (18,19). Thus, Bona *et al*. detected a significantly lower level of apoptosis of lymphocytes in patients with GD, as well as in patients with HT on replacement therapy, when compared to control subjects (20). There was a decrease in function of Fas molecule, associated with Fas resistance (20). On the other hand, Fountoulakis *et al*. found greater expression of the Fas molecule on peripheral blood T cells from patients with GD and HT than in control subjects, but the Fas ligand was not demonstrated, which could explain the inability of these cells for apoptosis (21). In our study, there were significantly lower percentages of apoptotic lymphocytes in both patients with GD and HT than in controls. The lowest relative numbers of cells in both early and late stages of apoptosis were detected in patients with GD. On the basis of our results, we cannot conclude whether this decrease in apoptosis of PBLs in patients with AITD is a cause or consequence of the disease. However, our findings indicate that thyroid status may in some way be associated with the level of apoptosis in PBLs of patients with autoimmune thyroid disorders. Namely, there was a positive correlation between TSH concentration and the late stage of apoptosis in PBLs from our patients with AITD. One possibility is that hypothyroidism might prolong the relatively short late stage of apoptosis (22,23), due to an inhibitory effect of TSH (24). Prolonged late phase apoptosis would lead to an increase in the percentage of cells in this stage. The effects of thyroid autoimmunity on apoptotic cell death have been studied earlier (25), and it was suggested that some autoantibodies can abrogate most of the inhibitory effect of TSH on apoptosis (12). Moreover, some published data indicate greater expression of many apoptosis-related genes in patients with HT than in patients with GD (26). On the basis of earlier findings, we can assume that thyroid status and/or thyroid autoimmunity might influence the process of apoptosis, resulting in slightly higher levels of apoptosis in PBLs of patients with HT than in patients with GD. The lack of statistical significance for the difference between the two subgroups was probably due to the small number of cases examined.

In T1DM, there is chronic immune-mediated destruction of pancreatic β-cells, leading to insulin deficiency. It becomes clinically evident when approximately 90% of the β-cells have gone. The aetiology is multifactorial, but the specific roles of genetic susceptibility, environmental factors, the immune system and β-cells in the pathogenesis of T1DM remain unclear (27). T1DM is characterised by the presence of several autoantibodies, including islet cell cytoplasmic autoantibodies, insulin autoantibodies and GAD autoantibodies. Studies using an experimental model of T1DM have indicated an increase of apoptosis in PBLs, especially in early stages of the disease (28). Enhanced spontaneous apoptosis of T lymphocytes was also found in patients with newly diagnosed T1DM (29). However, defective expression of an apoptosis-inducing CD95 (Fas/APO-1) molecule on T and B lymphocytes was observed in patients with long-standing T1DM (30). Although we found a higher percentage of apoptotic cells in PBLs of patients with T1DM than in patients with AITD, the level of apoptosis in PBLs of patients with T1DM was less than in control subjects. Even though our study included 10 newly diagnosed patients with T1DM, that does not mean that

they were in the early stage of disease. We do not know the time elapsed from initiation of the autoimmune process until diagnosis. It is interesting that in this group of patients there was a statistically significant negative correlation between glucose concentration and the total percentage of lymphocytes as well as those in the early stage of apoptosis. It seems that a high blood glucose level in patients with T1DM may reduce apoptosis of PBLs. Our results are in agreement with those of Ramakrishnan *et al.* (31), who suggested an anti-apoptotic effect of hyperglycaemia that contributes to the survival of potentially autoreactive T cells, with a possible impact on the pathogenesis of T1DM.

Data in the literature relating to MNs frequency in patients with AID are quite contradictory (reviewed in Torres-Bugarín *et al.* (9)). Some authors showed significantly increased MN frequency in patients with AID compared to healthy subjects, whereas others found no difference between patients and control subjects. Here, we have shown that PBLs from young patients with AID have a significantly higher MN frequency than control individuals. Our results confirm those of Scarpato *et al.* (33), who documented markedly increased MN frequency in hypothyroid patients with AITD in comparison to controls. The patients with HT included in our study had significantly higher MN frequency than those with GD, as well as a higher MN frequency than control subjects.

Zúñiga-González *et al.* observed an increase in genome instability and MN formation in patients with T1DM (34), whereas Cinkilic *et al.* found no differences in chromosomal aberrations or MN frequency between patients with T1DM and controls (35). Because chronic hyperglycaemia is associated with excessive production of free radicals and oxidative damage of cellular molecules, including DNA (36), that could be the reason for the increase in MN frequency detected here, as well as in the earlier study of Zúñiga-González *et al.* (34).

The relationship between apoptosis and MN frequency is very complex. The process of apoptosis is accompanied by chromatin condensation that may contribute to the formation of MN. In addition, the MN-bearing cells show severe DNA damage that can continue to apoptosis (37). However, overexpression of the anti-apoptotic protein, Bcl-2, may allow cells with a significant level of DNA damage to survive as MN-bearing cells (38). In this study, there was a positive correlation between MN frequency and apoptosis of PBLs in children with AID. This finding is in agreement with our earlier published results obtained in patients with differentiated thyroid carcinoma (39). Although PBLs of the patients with AID included in this study had significantly lower levels of apoptosis and significantly higher MN frequency than control subjects, the level of apoptosis and MN frequency were positively correlated, i.e. patients with more apoptotic PBLs had a higher frequency of MN in these cells. On the other hand, the lymphocytes with genetic damage were removed by apoptosis to a lesser extent. Thus, we can assume that some factors might induce both apoptosis and MN formation (40), but their levels are under the influence of some additional factors. In addition, there was a positive correlation between the concentration of specific autoantibodies and MN frequency in all three groups of patients with AID but this was statistically significant only in our patients with HT. Namely, the correlation of the concentration of TPOAbs and MN frequency in patients with HT was positive and significant. The association of the autoantibody concentration with MN frequency does not necessarily mean that antibodies were directly included in the process of apoptosis and/or MN formation. The pathogenesis the AITD, especially HT, as well as T1DM, includes many inflammatory cells and molecules, some of which might be involved in cell damage and/or death (41,42).

The comet assay, known as single-cell gel electrophoresis, is a sensitive method for detecting DNA strand breaks and alkali-labile sites in individual cells (43), that may be suitable for *in vivo* human biomonitoring (44). When compared to other DNA damage assays, comet assay possesses a number of advantages it is a relatively fast, simple and sensitive assay.

In this study, we detected a significantly higher level of DNA damage in PBLs of patients than in controls ($P < 0.001$). A qualitative analysis of cells showed that patient's cells exhibited a distorted appearance, with tail (comet formation), and in contrast, healthy persons had mostly compact cells and maintained the circular form, without comet formation. Obtained data are the consequence of disease existence since the influence of the subjects demographic characteristics (gender and age) on the degree of genetic damage is excluded by multivariant regression analysis. Our results are in agreement with previously published data of authors who showed a high level of DNA damage in PBLs in T1DM patients using a comet assay (45,46). T1DM is associated with oxidative stress (47,48), which is due to reduced antioxidant defence system, and can be one of the causes of an increased level of oxidative DNA damage in our patients.

In conclusion, children with AITD (HT and GD) and T1DM have a significantly lower level of apoptosis in PBLs and a significantly higher MN frequency, i.e. genome damage index than healthy subjects. There is positive correlation between the level of apoptosis and the level of genome instability in these patients with AID.

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