Low bcl-2 expression and increased spontaneous apoptosis in T-lymphocytes from newly-diagnosed IDDM patients

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Summary The bcl-2 gene product has been shown to regulate apoptotic cell death, and its dysregulation has been shown to induce several abnormalities in the immune system. No data exist regarding bcl-2 expression in autoimmune diseases, such as human insulin-dependent diabetes mellitus (IDDM). We investigated bcl-2 protein expression by testing T lymphocytes from 15 newly-diagnosed (<3 weeks) IDDM patients in comparison to 10 age-matched control subjects. The expression of bcl-2 on CD3⁺ lymphocyte subsets was investigated after membrane permeabilization by two- or three-colour immunofluorescence. When the percentage and mean fluorescence intensity (MFI) of bcl-2⁺/CD3⁺ cells from normal individuals and patients were compared, we found that bcl-2 expression within the CD3⁺ and CD4⁺ CD45R0⁺ T-cell populations was reduced significantly in IDDM patients (46.8 ± 15.4 vs 79.6 ± 11.7 ; 25.7 ± 3.8 vs 47.15 ± 5.7 , respectively;

$p < 0.001$). To establish whether low bcl-2 expression in T cells from newly-diagnosed patients reflects their susceptibility to death by an apoptotic process, we also evaluated DNA staining with propidium iodide in CD3⁺ lymphocyte suspension after a (24–72 h) culture period (spontaneous apoptosis). We found that IDDM patients have higher levels of spontaneous apoptosis (mean $\pm$ SEM: 24 h = 4.6 ± 0.8 ; 48 h = 9.9 ± 1 ; 72 h = 12.8 ± 1.1) than control subjects (24 h = 1.8 ± 0.4 ; 48 h = 4.6 ± 0.4 ; 72 h = 5.7 ± 0.3 ; $p < 0.02$ – 0.001). Our study suggests that recent onset IDDM is characterised by reduced bcl-2 expression, which in turn may be associated with the increased spontaneous apoptosis we observed. [Diabetologia (1995) 38: 953–958]

Key words Bcl-2, apoptosis, T cells, flow cytometry, cell-mediated immunity in insulin-dependent diabetes mellitus.

Apoptosis has been reported to be an important mechanism of cell death and cell self-destruction [1]. Unlike cell necrosis, apoptosis is associated with cytoplasm and nucleus condensation, nuclear frag-

mentation, formation of apoptotic bodies and reduced DNA stainability [2]. Recent studies on clonal deletion have shown that autoreactive T cells in the thymus are eliminated by an apoptotic process, and that a similar mechanism is also active in peripheral lymphoid organs to maintain self-tolerance and down-regulate an excessive immune response [1, 3–4].

Apoptosis is modulated by several inducer or suppressor molecules [3]. Among these regulatory molecules, the protein product of the proto-oncogene bcl-2 inhibits apoptosis, preventing several of its stages including blebbing, volume contraction, and endonuclease DNA cleavage [1, 5, 6]. A recent study in transgenic mice overexpressing bcl-2 in the thymus has shown that thymocytes from these animals be-

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Abbreviations: MFI, Mean fluorescence intensity; mAb, monoclonal antibody; PMA, phorbol myristate; PHA, phytoemagglutinin; Con A, concanavalin-A; PKC, protein kinase C; ICA, islet cell autoantibody; IAA, insulin autoantibody; PE, phycoerythrin; PI, propidium iodide; FSC, forward scatter; SSC, side scatter; PBMC, peripheral blood mononuclear cells.

come resistant to apoptosis induced by various stimuli such as anti-CD3 monoclonal antibody (mAb), glucocorticoids, phorbol myristate (PMA) or radiation [7]. Besides its apoptosis suppressor activity, bcl-2 also differs from other proto-oncogene products in its intracellular localization (mitochondria, endoplasmic reticula, nuclear membranes) [6].

Insulin-dependent diabetes mellitus (IDDM) is a T-cell-mediated autoimmune disease [8, 9]. Our *in vitro* studies in peripheral T cells isolated from patients with recent-onset IDDM and pre-diabetic subjects have suggested that the disease may also be characterised by a defect in T-cell activation. We have recently reported deficient T-cell blastogenesis after phytoemagglutinin (PHA) or concanavalin-A (Con A) stimulation [10], decreased interleukin-2 production [11], and a reduction in anti-CD3-induced proliferation with marked impairment of protein kinase C (PKC) translocation [12].

Despite the extreme dilution of islet-specific autoreactive T cells in the peripheral blood, several recent studies have shown sustained T-cell reactivity against pancreatic islet cell antigens such as glutamic acid decarboxylase, both in man [13] and nonobese diabetic mice [14, 15]. Other reports have shown reactivity against an as yet unidentified granule protein [16] and a 65 kDa heat shock protein [17]. More recently, islet-specific T cells have been isolated from the pancreases of two children who had died at diabetes onset because of ketoacidotic complications [18].

These findings are in apparent contrast with the T-cell hyporesponsiveness we have observed in our studies. Besides the dilution and the consequent poor representation of islet-specific T cells in the periphery, other factors may be responsible for this apparent paradox. One potential explanation is that islet-specific (antigen-primed) peripheral T cells, being chronically-activated cells, cause increased deletion through spontaneous apoptosis. To test this hypothesis, we directly evaluated spontaneous apoptosis and investigated its regulation by measuring bcl-2 expression in CD3-positive cells and in memory CD4-positive lymphocytes (CD45R0⁺) in patients who had recently developed IDDM in order to show an association with activation of T cells in the periphery.

Subjects and methods

Subjects. Through our 14-year family study at the University of Palermo we identified 350 patients and 18 first-degree relatives at increased risk of IDDM because of their positivity for islet cell (ICA) and/or insulin (IAA) autoantibodies.

For this study we randomly selected: a) 15 patients (6 males and 9 females) with IDDM with duration of symptoms related to the disease of less than 4 weeks (mean age 18 ± 4.3 years); b) 5 patients (2 males and 3 females) with long-standing IDDM (48–60 months), mean age 22.9 ± 2.4 years; c) 15 non-

Table 1. Baseline characteristics of subjects before entering the study

	Newly-diagnosed IDDM patients	Control subjects
<i>n</i>	15	10
Male/Female	6/9	5/5
Age (years)	18 ± 4.3	22 ± 3.6
Time since diagnosis	5.4 ± 4.5 (weeks)	–
HbA _{1c} (%)	9.2 ± 2.8	4.4 ± 2.3
Daily glycaemia (mmol/l)	7.8 ± 1.03	–
ICA ⁺ (%)	87	0

diabetic subjects with no family history of diabetes (6 males and 9 females), (mean age 22 ± 3.6 years) were studied as control subjects.

Patients were classified according to the criteria of the National Diabetes Data Group [19–20]. All patients were treated after diagnosis with human insulin, usually a combination of short- and intermediate-acting, 3 or 4 times daily. Glycaemic values, fasting and postprandial, were determined by strips and were read by reflectance method during the 24 h before the study. Glycated haemoglobin (HbA_{1c}) was determined with an autoanalyzer (Daichi, Kagaku, Kyoto, Japan) on the day of blood sampling (normal range in our cohort of normal subjects 5.5 ± 1.2 %) (Table 1).

Cells. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation from heparinized blood of IDDM patients and age- and sex-matched healthy blood donors (Table 1). Cells were washed 3 times in RPMI-1640 (Flow Laboratories, Irvine, UK) and resuspended in RPMI-1640 supplemented with 10 % fetal calf serum (Gibco, Life Technologies, Paisley, UK), antibiotics and L-glutamine. This will be referred to as complete medium. For T-cell purification, we used a two-step procedure. The majority of monocytes were removed from PBMC by plastic adherence. Non-adherent cells were then harvested and further enriched in T- or B-cells by negative immunomagnetic selection using CD15 plus CD3 and CD15 plus CD19 specific monoclonal antibodies (mAb), respectively, and goat-anti-mouse-labelled magnetic microspheres (Dyna, Oslo, Norway), as previously described [12]. This procedure resulted in high purification of CD3 positive (> 98 %) and CD3 negative (> 94 %), as measured by flow cytometry analysis.

Fixation protocol. For the immunodetection of the intracellular bcl-2 protein we used the paraformaldehyde/triton protocol, resuspending the cell pellet in 2 ml of 2 % paraformaldehyde (freshly prepared). After 10 min on ice, 100 µl of 1 % Triton X100 (Sigma, St Louis, Mo., USA) (final concentration 0.05 %) was added, and 10 min later the cells were washed twice in cold phosphate buffered saline [21].

Immunofluorescence staining and flow cytometry analysis. The T-cell specific monoclonal antibodies (mAb) Leu 4 (IgG1 isotype; CD3) and Leu 12 (IgG1 isotype; CD19) (Becton Dickinson, San José, CA, USA) were used to identify CD3 positive and CD19 positive cells. The anti-bcl-2 mAb (IgG₁ isotype Dako, Glostrup, Denmark) was used to evaluate the intracellular content for bcl-2 and other surface antigens by double-colour indirect immunofluorescence staining and FACS analysis. As a negative control for immunofluorescence, isotype-mat-

ched mAb (IgG₁) was employed. The fixed cells were incubated with saturating concentrations of the anti-bcl-2 mAb. After 30 min on ice, the cells were washed 3 times in complete medium and incubated again (30 min on ice) with isotype-specific, Phycoerythrin (PE) goat anti-mouse antibody (2 µg/ml) (Dako). After two more washes, the cells were incubated for 10 min with 2 µg/ml mouse IgG (Fluka, Ulm, Germany) to saturate binding sites. Finally Leu 12 or Leu 4 (FITC) was added for another 30 min on ice. After two washes in complete medium, cells were analysed by flow cytometry using a FACScan Becton Dickinson. For the analysis of bcl-2 expression in CD45R0⁺ memory CD4⁺ T-lymphocytes, three-colour immunofluorescence staining was used. The antibody combinations were fluorescein isothiocyanate anti-bcl-2 (Dako), phycoerythrin (PE) anti-CD45R0 (Dako), and Tri-colour anti-CD4 (Caltag Laboratories, San Francisco, CA, USA).

Spontaneous apoptosis. The method employed was essentially that described by Nicoletti et al. [22]. At 24, 48 and 72 h, the CD3⁺ cells were centrifuged at 200 × g for 10 min, washed, and processed. The centrifuged cell pellet was gently resuspended in 1 ml hypotonic fluorochrome solution (Propidium iodide (PI) 50 µg/ml in 0.1 % sodium citrate plus 0.1 % Triton X-100, Sigma), in 12 × 75 polypropylene tubes (Becton Dickinson). The tubes were placed in the dark at 4°C overnight before the flow cytometry analysis. This procedure gave very low coefficients of variation of DNA peaks and good discrimination of subdiploid and diploid cells and was used in the whole study.

The PI fluorescence of individual nuclei was measured using a FACScan flow cytometer. The forward scatter (FSC) and side scatter (SSC) of particles were simultaneously measured. Cell debris were excluded from analysis by appropriately raising the SSC threshold. The correct threshold value was selected according to Nicoletti's method [22]. The latter made it possible to distinguish apoptotic cell nuclei from debris by the high SSC value due to condensation of nuclear chromatin, which emitted fluorescence in channels 10–250. All measurements were made with the same instrument settings and at least 10⁴ cells were analysed.

Statistical analysis

The Student's *t*-test and the Student-Neuman-Keuls test (after variance analysis) were used for statistical calculations.

Results

Newly-diagnosed IDDM T cells express low levels of bcl-2. Quantification of bcl-2 on lymphocyte subsets was achieved by two-colour immunofluorescence staining for bcl-2 (PE) and either the B- or T-cell markers (FITC). The results (Table 2) showed that 55.2 ± 5.6 % of blood B⁺ cells and 66.6 ± 4.2 of CD3⁺ cells were bcl-2⁺. Whereas cases analysed in the two groups expressed similar percentages of bcl-2, differences in the intensity of fluorescence were noticed. In particular, CD3⁺ cells isolated from 15 normal individuals and analysed for bcl-2 expression revealed that the majority (mean 79.6 ± 11.7) expressed bcl-2. The staining intensity was slightly high-

Table 2. Anti-bcl-2 reactivity with lymphocytes from control subjects and newly-diagnosed IDDM patients

Cell	Subjects	Bcl-2	
		%	MFI
CD19 ⁺	Control subjects	55.2 ± 5.6	82 ± 9.8
	Newly-diagnosed IDDM patients	56.6 ± 4.2	79.1 ± 10.5
CD3 ⁺	Control subjects	66.6 ± 4.0	79.6 ± 11.7
	Newly-diagnosed IDDM patients	75.2 ± 7.9	46.8 ± 15.4

Fig. 1. Mean fluorescence intensity (MFI) of bcl-2 expression in CD3⁺ cells in the newly-diagnosed IDDM patients (■) with respect to the mean values ± 2 SD in the normal population. A significant difference was found between the normal subjects and the newly-diagnosed IDDM patients (*p* < 0.001)

er in B than T cells (MFI difference 12 ± 4.2 channels).

When we analysed the group of newly-diagnosed IDDM patients, their T cells expressed lower levels of bcl-2 (46.8 ± 15.4) than those of control subjects (*p* < 0.001) (Fig. 1). Patients with IDDM of long duration showed an intermediate level of bcl-2 (63.4 ± 6.4) with respect to control subjects and newly-diagnosed patients (data not shown).

Figure 2 shows the different levels of MFI in one control subject and in one patient with IDDM. The dramatic difference is apparent and this is particularly evident when an overlay histogram examined on the same day with an identical setting of the flow-cytometry analysis is considered. No difference in MFI of B cells was detected between control subjects and IDDM patients (82 ± 9.8 and 79.1 ± 10.5, respectively; *p* = NS) and the other groups studied (data not shown).

Figure 3 shows the percentage levels of bcl-2 expression in CD45R0⁺ memory CD4⁺ T-lymphocytes. Bcl-2 levels were significantly lower in newly-diagnosed IDDM patients with respect to normal subjects (*p* < 0.001).

High levels of spontaneous apoptosis are found in CD3⁺ cells from IDDM patients during the 24–72-h

Fig. 2. Expression of bcl-2 in human CD3⁺ from one normal subject (C) and one newly-diagnosed IDDM patient (P). Histograms show negative control staining on the left, while MFI of CD3⁺ cells is indicated on the right. Numbers indicate MFI values. Identical setting of the flow-cytometry analysis was considered

Fig. 3. Percentage levels of bcl-2 in CD4⁺ CD45RO⁺ lymphocytes in newly-diagnosed IDDM patients (□) and control subjects (■). Bars represent mean values $\pm$ SD. $p < 0.001$ for differences between the two groups

culture period. Flow cytometry analysis of PI-stained nuclei was performed on parallel cell cultures from control subjects and newly-diagnosed IDDM patients. For the whole duration of culture, higher levels of spontaneous apoptotic cells were found in the diabetic cohort than in control subjects ($p < 0.02$ – 0.001) (Fig. 4).

PI-stained nuclei from parallel cultures of T cells in control subjects revealed a typical diploid DNA peak and a minor hypodiploid peak (Fig. 5, left panels); T cells from IDDM patients showed an impressive enhancement of the hypodiploid DNA peak (Fig. 5, right panels) during the 48–72 h period of observation.

Fig. 4. Mean values of percent DNA spontaneous fragmentation in newly-diagnosed IDDM patients (□) and in control subjects (■) during the time course experiments. The mean values $\pm$ SD examined are indicated

Discussion

Apoptosis is an important feature during embryonic or postnatal development. In the thymus, antigen receptor stimulation induces apoptosis and this is the mechanism believed to be responsible for clonal deletion of autoreactive T cells [23]. Bcl-2 has a well-established role in the regulation of cell survival associated with the selection and/or deletion of autoreactive cells in lymphoid organs. The loss of bcl-2 is linked to a short life span whereas the upregulation of this protein results in the relatively longer survival of immune cell types. Bcl-2 has been shown to block apoptosis [6].

To test the hypothesis that the hyporesponsiveness of peripheral T cells we have reported in IDDM patients is at least partially due to increased spontaneous apoptosis of T cells, we directly evaluated spontaneous apoptosis and investigated its regulation by measuring bcl-2 expression in CD3-positive peripheral T cells from patients who had recently developed IDDM. Our findings show that bcl-2 expression is drastically reduced on the patients' T cells whereas normal expression is found on their B lymphocytes. Thus, this is a T-cell-specific deficit. Furthermore, we have demonstrated that T cells from newly-diagnosed IDDM patients are characterised by high levels of spontaneous apoptosis. As one would predict from the available knowledge on bcl-2 function, the reduced bcl-2 expression we observed is associated with an increased level of spontaneous apoptosis in T cells from IDDM patients *in vitro*. These findings are consistent with the hypothesis that reduced bcl-2 levels may lead to increased apoptosis of antigen-primed T cells in the peripheral blood. As a hypothetical consequence, islet-specific activated T-cells could be deleted from the periphery, causing the *in vitro* deficit of T-cell activation [10–12, 24]. However, the fact that diabetic patients transplanted with iso-

Fig. 5. DNA fluorescence flow cytometric profiles of PI-stained lymphocytes after 24-, 48- and 72-h incubation in medium alone. DNA histograms (log scale) after direct staining with hypotonic PI-fluorochrome solution. Left panels show values exhibited by one representative control subject; right panels indicate values found in one representative newly-diagnosed IDDM patient. Numbers indicate their relative apoptotic levels

grafts still reject them would indicate that islet reactive cells have not been fully deleted [25]. Whether the deficient expression of bcl-2 is an acquired defect or an inherited trait is at present not known. However, recent studies have shown that bcl-2 down-regulation is associated with the development of CD45RO⁺ cells after activation, and that bcl-2-CD45RO⁺ include a subset of both CD4 and CD8 lymphoblasts found in infections with Epstein-Barr and VZV viruses [26]. Elevated percentage levels of CD45RO⁺ lymphocytes were found in diabetes-protected twins, whereas their percentages were significantly reduced in pre-diabetic twins [27]. The expansion of CD45RA⁺RO⁺ cells in pre-diabetic twins in association with a decrease in CD45RO⁺ cells has suggested that pre-diabetic twins have either a defect in T-cell maturation or an increased clearance of CD45RO⁺ memory cells. In this light, CD4⁺CD45RO⁺T cells expressing low bcl-2, as found in the present study, might be those cells targeted by apoptosis. These findings would suggest that bcl-2 down-regulation and the consequent increase in apoptotic activity we found in IDDM may be due to environmental factors. This hypothesis is also supported

by a recent study which provided clear evidence for the involvement of a superantigen as the priming antigen of islet-specific autoreactive T cells isolated from the pancreases of children who died at the time of IDDM onset [18].

In conclusion, IDDM is characterised by decreased bcl-2 expression and increased apoptosis of peripheral T cells. These abnormalities may represent the consequence of a viral infection, and increased apoptotic activity would result as a mechanism to eliminate T cells exposed to the viral agent. Further investigation is necessary to elucidate how these mechanisms relate to the pathogenesis of IDDM.

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C. Giordano et al.: Bcl-2 and spontaneous apoptosis in IDDM