CLINICAL STUDY

Defective lymphocyte caspase-3 expression in type 1 diabetes mellitus

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Abstract

Objective: Activation-induced cell death (AICD) is a major mechanism in the regulation of peripheral tolerance and its impairment can determine the development of autoimmunity. In the present study, in order to evaluate the role of caspase-3 in type 1 diabetes mellitus (T1DM) AICD, caspase-3 expression was analyzed in peripheral blood lymphocytes from 37 new onset T1DM patients and from 36 normal control subjects (NC) in resting conditions and after anti-Fas-triggered apoptosis.

Methods: Caspase-3 expression was determined by semiquantitative RT-PCR and Western blot. Apoptosis was induced in activated lymphocytes by anti-Fas monoclonal antibody and quantified by flow cytometry and morphological analysis.

Results: Caspase-3 mRNA expression was reduced in resting lymphocytes in 18/37 T1DM patients and in 1/36 NC (P < 0.01). Patients studied for both Fas-mediated AICD and caspase-3 mRNA expression revealed that a reduced caspase-3 mRNA expression in resting lymphocytes occurred in all patients showing resistance to Fas-mediated apoptosis (T1DM vs NC, P < 0.02) with the exception of 3 patients who exhibited normal caspase-3 expression levels. Caspase-3 protein analysis confirmed mRNA data and showed an impaired expression of caspase-3 active form in T1DM subjects compared with NC.

Conclusions: Our data show that defective expression and function of caspase-3 in peripheral lymphocytes of T1DM patients may contribute to the development of AICD resistance in type 1 diabetes.

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Introduction

In the immune system both central and peripheral tolerance are actively involved in the regulation of responses to self antigens (1). In the thymus, the elimination of potentially autoreactive T lymphocytes occurs by positive and negative selection, the latter involving activation-induced cell death (AICD) that ensures an efficient elimination of T lymphocytes by apoptosis (2). In the periphery, AICD is still required in order to remove those cells that escape the negative selection (3). An impaired balance between Bcl-2 and its related proteins, or the engagement of tumor necrosis factor receptor (TNFR) family members such as Fas and TNFR, represent different mechanisms by which peripheral T lymphocyte deletion may occur (4). At the present time, however, the Fas system appears to be the main factor accounting for this process (5, 6). Cross-linking of Fas by its natural ligand (FasL) leads to the recruitment of the adapter molecule, FADD, and subsequently of caspase-8. The association between FADD and caspase-8 in the death-inducing signaling complex (DISC) leads to activation of a downstream protease cascade, with caspase-3 activation leading to the cleavage of vital substrates and to apoptosis (7, 8). In animal models such as the Fas-deficient mouse, an impaired AICD has been shown to contribute to the development of autoimmune phenomena (9). In the non-obese diabetic (NOD) mouse, an established model of autoimmune diabetes, different studies reported a resistance of peripheral T lymphocytes to apoptotic signals (10–12). Furthermore, a recent study described an impaired expression of Fas and FasL, and a reduced expression of caspase-8 in peripheral NOD T lymphocytes displaying AICD resistance (13). Likewise in animal models, defective AICD in peripheral T lymphocytes has been described in human type 1 diabetes mellitus (T1DM) (14, 15). Thus, peripheral T lymphocytes of HLA-DR3-positive subjects, a haplotype associated with autoimmune diseases, show resistance to Fas-mediated apoptosis (15), and, more recently, an impairment of Fas function has been
described in patients with type 1 diabetes and other autoimmune diseases (16). In the present study, in order to gain insights into the potential molecular mechanism(s) involved in the resistance to AICD in human T1DM and taking into account that caspase-3 is the central executor of AICD, caspase-3 expression and its role in the susceptibility to Fas-dependent apoptosis were evaluated in peripheral T lymphocytes from new onset T1DM patients and normal control individuals (NC). Our study shows that a reduced expression of caspase-3 is constitutively present in nearly 50% of T1DM patients’ peripheral blood lymphocytes and that such a defect is associated with a reduced expression of caspase-3 active form and with a reduced sensitivity to Fas-mediated AICD.

Materials and methods

Subjects

Peripheral blood mononuclear cells (PBMC) were obtained from 37 new onset T1DM patients (17 females, 20 males, 8-30 years old) and 36 healthy individuals (18 females, 18 males, 6-44 years old). All patients were studied within 1 month from diagnosis of diabetes and were in good metabolic control. Normal control subjects had no family history of type 1 or type 2 diabetes or of other autoimmune diseases. A first subset of subjects (25 T1DM and 18 healthy controls) was analyzed for caspase-3 expression; an additional subset (12 T1DM and 18 healthy controls) was studied for both caspase-3 expression and for sensitivity to Fas-mediated apoptosis. In addition, in order to establish whether caspase-3 mRNA expression in PBMC was stable over time and to exclude the possibility that the differences observed at disease onset could be due to the recent metabolic derangement, 5 T1DM patients and 10 control individuals were re-tested after 1 year.

Cell separation and cultures

Peripheral blood mononuclear cells were isolated from heparinized blood by Ficoll-Hystopaque density gradient centrifugation, washed twice with RPMI 1640 medium (Gibco-BRL, Grand Island, NY, USA) and either frozen in freezing media containing 10% DMSO and stored in liquid nitrogen until used for caspase-3 expression studies, or put in culture and activated to analyze susceptibility to Fas-mediated apoptosis as follows: PBMC were cultured (1 × 10^6/well) in 24-well flat-bottomed plates (Falcon Labware, Becton Dickinson, Lincoln Park, NJ, USA) with RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. PBMC contained about 90% CD3^+ T lymphocytes. PBMC were then activated with PHA (5 mg/ml) (Sigma, St Louis, MO, USA) for 72 h, followed by interleukin (IL)-2 (25 U/ml) (Biogen, Geneva, Switzerland) for an additional 48 h. Cell activation and Fas surface expression were determined by cytofluorimetric analysis (EPICS XL-MCL cytomter, Coulter Electronics, Hialeah, FL, USA) employing respectively an anti-CD25 monoclonal antibody (Dako Corporation, Santa Barbara, CA, USA) and an anti-Fas mouse monoclonal antibody (CH11, mouse IgM; Upstate Biotechnology, Lake Placid, NY, USA). At day 5 of culture, flow cytometry analysis showed that the proportion of T lymphocytes was > 95%. Cells were then incubated with or without anti-Fas monoclonal antibody (1 mg/ml) (Upstate Biotechnology) for 16 h.

Apoptosis analysis

Apoptosis was measured by both flow cytometry and morphological analysis before and after anti-Fas treatment. DNA fragmentation was studied by propidium iodide staining followed by flow cytometric analysis (17). Briefly, cells were washed twice in PBS and fixed in 70% ethanol in PBS overnight at 4°C. Following incubation, cells were washed twice with PBS, resuspended in 0.5 ml PBS, 50 ml RNase A (5 mg/ml) (Sigma) and stained with 0.5 ml propidium iodide (100 mg/ml) (Sigma) in PBS. Cells were incubated for 30 min at room temperature in the dark and kept at 4°C until analyzed. The fluorescence was measured using an EPICS XL-MCL flow cytometer (Coulter Electronics). The normal range of response to Fas, defined as the mean±2 s.d. of data obtained from 18 normal donors, was 64.1±9.8 (relative cell survival percentage). Consequently, individuals with relative cell survival values of > 83.7% after anti-Fas treatment were considered as Fas resistant. Changes in nuclear morphology after fixation in 4% paraformaldehyde were analyzed by DNA-binding fluorescent dye Hoechst 33258 (Sigma). Vital dye Hoechst 33258 was used at a final concentration of 1 mmol/l. A minimum of 200 cells were analyzed per sample employing a Leitz DMRB (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) inverted fluorescence photomicroscope (320 nm UV excitation) and photographed under fluorescent light. Cell nuclei, when characterized by marked chromatin condensation, were scored as apoptotic.

Caspase-3 mRNA expression

The expression of caspase-3 mRNA, in all experimental conditions, was evaluated by RT-PCR using multiple exon-spanning primers to avoid detection of genomic DNA. Briefly, total RNA was extracted from samples by Trizol (Gibco-BRL) according to the manufacturer’s instructions. First strand cDNA synthesis was performed using 2 mg of each RNA sample primed with random hexamers with 200 U Superscript II (Gibco-BRL); 200 ng aliquots of cDNA were subsequently amplified in a 100 ml reaction volume containing 20 pmol of upstream and downstream primers, 2.5 U Taq cDNA polymerase (Gibco-BRL), 200 mmol/l of each deoxy-nucleoside triphosphate and 1.5 mmol/l MgCl2. The
primer sequences for caspase-3 were: sense 5'-ATG GAG AAC ACT GAA AAC TCA GTG-3', anti-sense 5'-TAG TGA TAA AAA TAG AGT TCT TIT G-3', and generated a 354 bp product. Expression of β-actin as an RNA control was analyzed employing the following primers generating a 354 bp product: sense 5'-ACC AAC TGG GAC GAC ATG GAG-3', anti-sense 5'-CGT GAG GAT CTT CAT GAG GTA GTC-3'. Reaction conditions were standardized in order to observe a linear amplification of PCR products. To this end, cDNA was amplified in serial cycles from 20 to 41 cycles for caspase-3 and β-actin, and linearity was demonstrated within this range. According to this result, 30 cycles were used for caspase-3 and 25 cycles for β-actin. All PCR products were electrophoresed on 1.2% 'Separede' agarose gels and the bands were visualized by ethidium bromide staining. Semiquantitative analysis was performed by densitometric gel scanning utilizing a BioRAD ‘Gel Doc 2000’ video image system (Bio-Rad Laboratories, Hercules, CA, USA) and the results have been expressed as caspase-3/β-actin density % ratio in each sample analyzed. Caspase-3 mRNA levels were considered as pathologically reduced for caspase-3/β-actin % ratio values < 1st percentile of the normal control group (ratio < 5.7%).

Caspase-3 protein expression

Cells were washed twice with Ca²⁺- and Mg²⁺-free PBS buffer. Cell extracts were prepared by lysing 10⁷ cells in 80 ml lysis buffer containing 50 mmol/l TRIS buffer pH 7.6, 150 mmol/l sodium chloride, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 100 mg/ml phenylmethylsulfonyl fluoride, and 1 mg/ml each of aprotinin, leupeptin, pepstatin A, at 4°C. Insoluble materials were removed by centrifugation for 20 min at 14 000 r.p.m. at 4°C. Protein content was determined using the Bradford method (Bio-Rad protein assay kit). Protein samples (20 mg protein) were boiled for 5 min in a sodium dodecyl sulfate (SDS) buffer and separated in 12% SDS-polyacrylamide denaturing gels (SDS-PAGE) according to the method of Laemmli (18). Proteins were electroblotted to nitrocellulose membranes (Schleicher and Schuell GmbH, Dassel, Germany). The loading and transfer of equal amounts of protein were confirmed by staining the membrane with Ponceau S. The blots were blocked by incubation in 50 mM Tris, pH 7.6, 150 mM NaCl (TBS) with 0.05% Tween 20 (TBST) and 5% nonfat dry milk at 4°C for 1 h. After a brief rinse, blots were incubated overnight at 4°C in TBST-5% milk with rabbit anti-caspase-3 polyclonal antibody (Abcam Ltd, Cambridge, Cambbs, UK) (1:1000). Mouse anti-β-actin monoclonal antibody (Sigma) (1:5000) was used as an internal control protein. Blots were washed four times with TBST, incubated with peroxidase-conjugated with anti-rabbit or anti-mouse IgG (Santa Cruz, Palo Alto, CA, USA) (1:2000) in TBST for 1 h at room temperature, washed again, and developed with an enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA).

Statistical analysis

The Mann-Whitney U test was employed to compare T1DM patients and NC in terms of (a) percentage of cell survival after anti-Fas treatment and (b) caspase-3 expression levels. Fisher’s Exact test was used to compare the frequency of subjects with pathologically reduced caspase-3 expression levels in T1DM patients and in NC.

Results

Caspase-3 expression in resting lymphocytes

Caspase-3 mRNA expression was analyzed in resting lymphocytes from 37 new onset T1DM patients and from 36 NC. Although the analysis was performed on mononuclear cells (MNC), at the time of the assay the proportion of T lymphocytes was > 90%. Caspase-3 mRNA expression, analyzed by semi-quantitative RT-PCR, was pathologically reduced (caspase-3/β-actin % ratio values < 5.7) in 18/37 T1DM patients but in only 1/36 NC (P = 0.0029 by Fisher’s Exact test) (Fig. 1). After 1 year, caspase-3 mRNA expression levels were re-tested in a subset of 5 T1DM subjects and 10 controls. The caspase-3 mRNA expression levels were unchanged in all patients and control subjects studied, including 3 T1DM individuals with significantly reduced caspase-3 levels at diabetes diagnosis, indicating that caspase-3 mRNA levels in resting lymphocytes are stable over time (data not shown). Caspase-3 mRNA expression was independent of age, sex and the presence of type 1 diabetes-associated autoantibodies such as IAA, GADA and IA-2A.

Relationship between caspase-3 expression and AIDC

In order to evaluate whether reduced caspase-3 expression levels may account for a reduced susceptibility to Fas-mediated apoptosis in lymphocytes of T1DM subjects compared with NC, sensitivity to Fas-mediated apoptosis and caspase-3 expression were tested after activation in a subset of 12 new onset T1DM patients and in 18 healthy subjects. After activation, Fas surface expression, evaluated by indirect immunofluorescence and flow cytometry in T1DM patients and in control subjects, was similar in the two groups ranging from 40% to 60% of PBMC (data not shown). After anti-Fas treatment, T lymphocytes of T1DM patients showed resistance to Fas-mediated apoptosis, with a cell survival percentage of 98.8 ± 6.9% compared with 64.1 ± 9.8% detected in NC (P < 0.01 by Mann-Whitney U test) (Fig. 2). According to the previously mentioned criteria, 11/12 T1DM...
patients were classed as Fas resistant. Figure 3 (A-D) shows a representative flow cytometric analysis of a normal control subject (A, B) and a T1DM patient with deficient caspase-3 expression (C, D) in which the DNA of lymphocytes was stained by propidium iodide before (A, C) and after (B, D) anti-Fas stimulation. The nuclear changes that define apoptosis are represented by a typical DNA fragmentation, which is identified by the subdiploid peak in the flow cytometry histograms (cursor 1). In Fig. 3 (a-d), DNA-binding fluorescent dye Hoechst 33258 was used to analyze changes in nuclear morphology in T lymphocytes before (a, c) and after (b, d) anti-Fas stimulation. After anti-Fas treatment, T lymphocytes from the T1DM patient (Fig. 3d) showed reduced proportions of apoptotic bodies compared with T lymphocytes from the control subject (Fig. 3b). The fact that different Fas surface expression might account for these results was excluded by indirect immunofluorescence and flow cytometry analysis (data not shown). As for the relationship between Fas resistance and caspase-3 expression, 8/12 T1DM patients showed reduced caspase-3 levels. The only T1DM patient with a normal response to Fas had normal caspase-3 levels, while reduced caspase-3 expression in basal conditions was found to occur in 8/11 patients with resistance to Fas-mediated apoptosis. Of note, 3 individuals displayed normal caspase-3 levels despite Fas resistance, suggesting a possible defect in the generation of caspase-3 active form or the existence of other defects affecting the Fas-mediated pathway. With regard to caspase-3 protein expression, Western blot analysis performed in resting lymphocytes from 18 NC individuals and from 12 T1DM patients, confirmed the mRNA data (Fig. 4 shows a representative panel of 5 NC and 5 T1DM). We then analyzed the kinetics of caspase-3 activation in order to determine the relationship between caspase-3 expression in basal conditions and the generation of its active form. Caspase-3 expression in lymphocytes from 5 NC and 5 T1DM patients was studied before and after activation as described in Materials and methods. In NC, Western blot analysis revealed that lymphocyte
activation resulted in caspase-3 cleavage and generation of its active form (Fig. 5A). In contrast, as shown in Fig. 5B, in 5 representative T1DM patients lymphocyte activation resulted in an impaired cleavage of caspase-3 with absence (patient 1) or reduction (patients 2 and 3) of its active form in 3/5 subjects. Among the 12 T1DM patients studied, 3/12 showed normal caspase-3 cleavage and generation of its active form, while 9/12 showed a lack of or a greatly reduced cleavage.

**Discussion**

In the immune system, AICD is actively involved in limiting the immune responses. The process is mainly dependent on the death receptor Fas, although tumor necrosis factor and Bcl-2 family-related proteins have also been suggested as participants in this process. According to their sensitivity to Fas-induced apoptosis, T lymphocytes can be distinguished into two different types. Type I cells are characterized by direct activation of caspase-8 at the death-inducing signaling complex (DISC), while type II cells require an amplification of the caspase cascade by mitochondria. Both pathways, however, lead to caspase-3 activation (19). More recently, caspase-3 has also been reported to be a component of DISC and to be required for a complete activation of caspase-8 in Fas-mediated apoptosis (20). Therefore, caspase-3 covers a key role in the amplification of Fas-mediated signaling in T lymphocytes.

**Figure 3** Evaluation of AICD in T cells from a representative type 1 diabetic patient with deficient caspase-3 expression (T1DM) and from a control individual (NC). Activated T cells were treated with anti-Fas monoclonal antibody for 16 h, fixed with 70% ethanol in PBS and then stained with propidium iodide solution. (A-D) Representative flow cytometric analysis of one normal control subject (A, B) and one T1DM patient (C, D). DNA of T cells was stained by propidium iodide before (A, C) and after (B, D) anti-Fas stimulation. The nuclear changes that define apoptosis are represented by typical DNA fragmentation which is identified by the subdiploid peak in the flow cytometry histograms (cursor 1). Cursor 2 reveals the diploid peak, cursor 3 the hyperdiploid peak, and cursor 4 the tetraploid peak. Cell number is indicated on the y-axis and fluorescence intensity is represented on the x-axis. (a–d) Hoechst dye staining of T lymphocytes before (a, c) and after (b, d) anti-Fas stimulation. Higher proportions of apoptotic cells were detected in lymphocytes of the normal control subject (b) compared with the T1DM patient (d). Cells with fragmented nuclei were scored as apoptotic.
human autoimmune diseases such as multiple sclerosis and rheumatoid arthritis, an impaired balance between pro- and anti-apoptotic molecules accounts for the existence of Fas-resistant T cell clones (21–23). In T1DM patients, different studies reported similar data (14–16), although the role of signaling molecules involved in such resistance to Fas-mediated AICD has not been evaluated. Hence, in the present study we decided to investigate caspase-3 expression, the major executioner of Fas-mediated apoptosis in peripheral blood lymphocytes of new onset T1DM subjects, and its role in Fas-mediated AICD. Our data show that a reduced caspase-3 expression in resting lymphocytes is detectable in almost 50% of T1DM patients. As far as the relationship between reduced susceptibility to anti-Fas-induced apoptosis and caspase-3 expression levels in resting lymphocytes is concerned, data obtained from those individuals studied for both these parameters indicate that reduced caspase-3 levels are associated with resistance to Fas-mediated AICD, although we observed 3 T1DM patients with Fas resistance expressing normal caspase-3 levels. It is, therefore, of interest that in some T1DM patients, caspase-3 activation resulted in lower levels of active fragments compared with controls. This finding suggests the possibility that in a given patient with normal caspase-3 expression in resting lymphocytes and resistance to Fas-mediated apoptosis, the levels of active caspase-3 may be inadequate for the transmission of apoptotic signal (e.g. T1DM patient in lane 3, Fig. 5B), although a correct processing and activation of caspase-3 was clearly observed in 2 Fas-resistant T1DM patients with normal caspase-3 levels (e.g. T1DM patients in lanes 4 and 5, Fig. 5B). As a matter of fact, it is likely that in such patients other defects affecting the Fas-mediated pathway do exist. In the NOD mouse, for example, low caspase-8 levels in peripheral T cells are associated with resistance to AICD (13), and in multiple sclerosis, the overexpression of the cellular caspase inhibitor, FLIP, confers resistance to Fas-mediated apoptosis in patients’ peripheral T cells (21). Mice lacking Fas show impaired Fas-mediated AICD and an accumulation of activated lymphocytes with an unusual phenotype. In humans, mutations of the Fas receptor result in a similar phenotype characterized by non-malignant lymphoproliferation with lymphadenopathy and autoimmune phenomena (e.g. hemolytic anemia, thrombocytopenia, etc.) (24). It has been reported that T and B lymphocytes of T1DM patients show a reduced Fas surface expression suggesting an impairment of the Fas-mediated pathway (14). Recently, De Franco et al. (16) described a partial resistance to Fas-mediated apoptosis in T1DM patients as well as in subjects affected by autoimmune polyendocrinopathies, without Fas surface expression defects, suggesting an impairment of the pathway downstream of the Fas receptor. Our data are in agreement with and extend the results of this study, suggesting that resistance to Fas-mediated apoptosis may be due, at least in a subset of patients, to a defective caspase-3 expression, the major executioner of Fas-dependent apoptotic death. Certainly, the Fas system represents a redundant pathway that involves many different molecules. It is therefore possible that different molecular defects, among different subjects, affect the same apoptotic pathway. Taken together, our data suggest that a defective expression and function of caspase-3 in peripheral lymphocytes of type 1 diabetic subjects may play an important role in AICD resistance, thus contributing to the development of autoimmune phenomena in genetically susceptible individuals.
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