High concentrations of anti-caspase-8 antibodies in Chilean patients with type 1 diabetes

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\textbf{ABSTRACT}

\textbf{Introduction:} Deregulation of apoptosis across the Fas–FasL pathway is an increasingly relevant phenomenon in the pathogenic mechanisms associated with autoimmune diseases. Caspase-8 initiates the activation of the apoptotic process and interacts directly with Fas in the membrane of the T lymphocyte.

\textbf{Objectives:} To standardize an Elisa essay to measure the concentration of anti-caspase-8 antibodies in plasma of Type 1 Diabetes (T1D) patients and analyze their possible distribution and association with characteristics of the disease.

\textbf{Methods and subjects:} 124 patients newly diagnosed with T1D and 132 controls: children and youngsters.

ELISA test was standardized to detect anti-caspase-8 antibodies in plasma. It correlated the concentration of this antibody with classical markers of autoimmunity as anti-IA-2 and anti-GAD65, and the clinical characteristics at onset of diabetes mellitus. The statistical analysis was performed using logistic regression.

\textbf{Results:} Patients with T1D showed a higher concentration of anti-caspase-8 antibodies regarding the controls (87.5 ng/ml versus 24.3 ng/ml, \(p<0.0001\), values expressed as median). The proportion of patients with T1D and high concentrations of anti-caspase-8 (percentile 50–75) was significantly different from the control group \((p<0.0001)\). Anti-caspase-8 showed a strong association with positive anti-GAD65 (OR = 3.48, \(p<0.035\)) and ketoacidosis (OR = 10.74, \(p<0.0001\)) events, with glycemia and age at diagnosis as contributing variables.

\textbf{Conclusion:} This is the first report in the literature of levels of anti-caspase-8 antibodies in T1D through ELISA. The high concentration in patients with T1D, and its strong correlation with anti-GAD65 auto-antibodies, suggests a potential role of anti-caspase-8 auto-antibodies as surrogate marker autoimmunity in T1D patients.

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sic and intrinsic pathways, respectively. Caspase-3, -6, and -7 are effector caspases downstream of both pathways (Liadis et al., 2005). Different studies have reported the existence of impaired apoptosis in peripheral T cells in subjects affected by autoimmune diseases (Fujinami et al., 2006; Todaro et al., 2004). In subjects affected by Hashimoto thyroiditis and systemic sclerosis, a resistance to both Fas- and ceramide-induced apoptosis can occur in peripheral T cells, partially as a consequence of an impaired activity of caspase-8 (Stassi et al., 2001; Bossowski et al., 2007, 2008; Otsuki et al., 2006; Ueki et al., 2002; Yamaoka et al., 2008).

In the last years, caspase-8 has been extensively studied as the initiator caspase of the extrinsic apoptosis (Kang et al., 2004; Liadis et al., 2007; Beisner et al., 2005). However, a special attention has been observed related with the essential role in T-cell homeostasis and T-cell immunity (Salmena et al., 2003; Choi and Woo, 2010). These new functions of caspase-8 showed the possible role in cellular functions that are non-apoptotic as the activation of NF-κB, showing a dual role of this protein in cellular proliferation and apoptosis (Lemmers et al., 2007; Su et al., 2005; Maelfait and Beyaert, 2008; Tang et al., 2005). In T1D patients, several auto-antibodies against known and unknown antigens are present in the serum of recently diagnosed patients. In this study, we analyzed the serum concentration of anti-caspase-8 antibodies directed against caspase-8.

Methods and subjects

Subjects

The study was carried out in Santiago, Chile, with new cases of T1D diagnosed by means of standardized methods during 2006–2008. There were 124 new cases of T1D and 132 healthy unrelated control children. The control group represents volunteers who participated from 10 random schools from Santiago comparable for two Hispanic surnames and similar socioeconomic conditions. Information regarding the clinical characteristics of the debut, diagnosis data, allergies, familial history of diabetes and other autoimmune diseases were collected through a clinical questionnaire. Serum samples were obtained only after informed consent had been given. These protocols were ethically approved by the Research Committee of INTA, University of Chile.

GAD65 and IA-2 auto-antibodies

Screening for serological anti-GAD65 and anti-IA-2 auto-antibodies was performed in duplicate by Enzyme Immunoassay (ELISA) from Medizym® Diagnostic (Berlin, Germany). The Medizym® anti-GAD and anti-IA-2 were calibrated against the WHO reference preparation NIBSC 97/550. Both determinations were semi-quantitative methods. Using a cut-off of 10 IU/ml, the Medizym® anti-GAD assay shows a sensitivity of 92.3% and a specificity of 98.8%. On the other hand, the anti-IA-2 assay has a sensitivity of 75% and a specificity of 98%. The values are in the medium range observed for this technique (ELISA) in the Diabetes Antibody Standardization Program (DASP) (Bingley et al., 2003).

Caspase-8 antibodies (ELISA standardization)

Plasma samples were obtained after centrifugation and stored at −20 °C until required. ELISA measurement of antibodies against caspase-8 was carried out by coating 96-well flat bottomed immunoplates with 0.02 μg/well of recombinant human monoclonal caspase-8 (Sigma, C1095, St. Louis, MO) diluted in 0.1 M phosphate-buffered saline (PBS) pH 7.4 in a final volume of 200 μl. After overnight incubation at 25 °C, plates were washed three times with 300 μl/well of PBS and blocked with 380 μl/well of PBS containing 20% (w/v) non-fat milk. After 2h at 25 °C, plates were washed as described above and 200 μl of serum diluted in PBS containing 0.05% (w/v) Tween 20 was added. Plates were incubated for 1h at 25 °C, washed four times with 300 μl/well of PBS containing 0.05% Tween 20. Then 50 μl/well of horseradish peroxidase conjugated goat polyclonal anti-human IgG, 1:6000 (Sigma, A6029, St. Louis, MO) was added. Plates were incubated for 1h at 25 °C and washed three times with 300 μl/well of PBS. Then 50 μl/well of o-phenylenediamine dihydrochloride (OPD) containing 0.001% (v/v) H2O2 and 100 μl/well of 3N HCl were added as stop solution. Optical density (OD) of wells was read at 492 nm. Results were expressed as delta OD, corresponding to the difference between the mean OD of duplicate wells and the wells that have been made throughout the procedure except to add serum. The calibration curve was carried out with serial dilutions from 2 × 10⁵ pg/ml of recombinant human monoclonal anti-caspase-8 (Sigma, C4106, St. Louis, MO) in PBS containing 0.5% (w/v) non-fat milk and 0.05% (w/v) Tween 20. We used eight concentration points to establish a linear curve for anti-caspase-8 from 0 to 20,000 pg/ml by serial dilution technique. The intra-assay coefficient of variation (CV) was 3.22% and the inter-assay CV was 2.74%. The limit of detection was 0.51 ng/ml, the limit of quantification was 1.7 ng/ml. In accordance with the distribution of the caspase-8 antibody profile, we used the percentile distribution to compare between T1D and controls.

Statistical analysis

The results are expressed as means, standard deviations, median, interquartile range (IQR) or percentiles 25, 50 and 75. Differences between study groups were assessed through the two-sample Wilcoxon rank-sum (Mann–Whitney) test and Two-sample t-test. Anti-caspase-8 antibody concentration was categorized by the percentile distribution. Differences in frequencies of categorical variables between study groups were assessed through Chi-square test, Fisher’s exact test and test for trend across ordered groups. The association between higher levels of anti-caspase-8 antibodies (greater than 75th percentile) and clinical/immunological characteristics in T1D children was assessed through logistic regression techniques. Values were considered to be statistically significant when p < 0.05. Data analyses were performed using the STATA 10 statistical package (Stata Statistical Software 1984–2007).

Results

Patients with T1D have an age of diagnosis of 9.4 ± 4.7 years, glyceremia of 456 ± 259, ketoacidosis of 56% and duration of breastfeeding of 5 ± 4.5 months. Clinical and demographic information is presented in Table 1. A majority of the subjects included in this study were below 15 years old in both groups. The patients were tested for anti-GAD65 and anti-IA-2 auto-antibodies, as well as anti-caspase-8 antibodies. We first compared the serum anti-caspase-8 antibody concentrations between T1D patients and control subjects. The anti-caspase-8 antibody levels in the T1D group (median: 87.5 ng/ml) were strongly higher compared with the control group (median: 24.3 ng/ml) (p < 0.0001). The main result of the study was the higher mean antibody level detected between the diabetic patients. In Fig. 1, we summarized the distribution of antibody levels in T1D patients and controls.

Serum anti-caspase-8 antibody profiles were analyzed by means of percentile distributions (Table 2). The results show that the proportion of T1D patients was significantly different from that of control subjects with relation to percentile distribution (p < 0.0001). Until P50–P75 the proportion of T1D cases increased
Table 1
Clinical characteristics in T1D cases and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>T1D cases (n = 124)</th>
<th>Controls (n = 132)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)*</td>
<td>10.8 ± 4.3</td>
<td>11.3 ± 2.4</td>
<td>0.2628</td>
</tr>
<tr>
<td>Gender (male/female) (%)</td>
<td>50.9/49.1</td>
<td>46.3/53.7</td>
<td>0.536</td>
</tr>
<tr>
<td>Anti-GAD65 (%)</td>
<td>67.3</td>
<td>1.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Anti-IA-2 (%)</td>
<td>56.9</td>
<td>0.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Anti-caspase-8 antibodies (ng/ml)</td>
<td>87.54 (67.42)</td>
<td>24.28 (129.43)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Results are given as mean ± standard deviation, percentages or as median (interquartile range).

* Two-sample t-test with unequal variances.

Table 2
Anti-caspase-8 antibody concentration in T1D cases and controls according to percentile distribution.

<table>
<thead>
<tr>
<th>Anti-caspase-8 concentration (ng/ml)</th>
<th>T1D cases (n = 124)</th>
<th>Controls (n = 132)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentile &lt; 25</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Percentile 25–50</td>
<td>55.79 (31.75)</td>
<td>0 (24.27)</td>
</tr>
<tr>
<td>Percentile 50–75</td>
<td>87.54 (36.56)</td>
<td>24.27 (55.04)</td>
</tr>
<tr>
<td>Percentile ≥ 75</td>
<td>124.1 (167.7)</td>
<td>82.31 (210.7)</td>
</tr>
</tbody>
</table>

Values are median (interquartile range) unless otherwise specified.

Table 3
Crude association between anti-caspase-8 antibodies (75th percentile) and clinical/immunological characteristics in T1D children (Woolf approximation).

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-GAD65</td>
<td>3.26</td>
<td>1.04–10.26</td>
<td>0.035</td>
</tr>
<tr>
<td>Anti-IA-2</td>
<td>1.01</td>
<td>0.43–2.39</td>
<td>0.976</td>
</tr>
<tr>
<td>Ketoacidosis</td>
<td>10.0</td>
<td>2.8–35.6</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

CI: confidence interval.

Discussion

Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease targeting pancreatic beta-cells. The process of β cell destruction, marked by the production of auto-antibodies, occurs over many years and ultimately results in metabolic abnormalities first manifested as impaired glucose tolerance (Knip and Siljander, 2008).
evaluation of isolated antibodies even in the population at risk of developing diabetes has a limited clinical value and for this reason, the screening of a battery of them improves the predictive power (Pihoker et al., 2005). The first antibodies described in association with the development of T1D were islet cell auto-antibodies (ICA). Subsequently, antibodies to insulin (IAA), glutamic acid decarboxylase (GAD) or GAD), protein tyrosine phosphatase (IA-2) and zinc transporter-8 (ZnT8A) have been described. They are detectable before clinical onset and define the subgroup of patients with latent autoimmune diabetes in adults (Bingley, 2010).

T1D is marked by the production of pancreatic islet β cell-specific auto-antibodies and selective destruction of the insulin-producing β cells by auto-reactive T cells (Pirot et al., 2008). In this study we provide some suggestive evidence of the presence of anti-caspase-8 antibodies in recently diagnosed T1D patients, related with a high OR for anti-GAD65 antibodies and higher frequency of ketoacidosis events.

The report of anti-caspase-8 antibodies in serum of patients as been described in others pathologies as silicosis, systemic sclerosis (SSc) and systemic lupus erythematosus (SLE) were the antibody profile were analyzed using the method of western blotting and desorption/ionization protein chip analysis (Ueki et al., 2002). The immune markers routinely are limited to auto-antibodies, which have some intrinsic techniques limitations. However, because T cells are central pathogenic actors of T1D, the quest for their measurement appeared to offer a path towards new autoimmune markers, among them anti-CD3 monoclonal antibodies, may involve direct effects on pathogenic T cells, the induction of populations of regulatory cells, or both, the mechanism of action of the anti-CD3 monoclonal antibody need to be clarified (Herold et al., 2002). Other observations have highlighted interest in CD8 (+) T cell in T1D diabetic patients, both approaches aimed at highlighting the potential role of T cells as a trigger of autoimmune processes (Martinuzzi et al., 2008).

Our current results highlight the complexities of studying autoimmune processes in T1D. In our view, this is the first report on anti-caspase-8 antibody levels related with classical autoimmunity markers such as anti-GAD65 auto-antibodies and clinical aspects such as ketoacidosis. Although the relationship between β-cell apoptosis and autoimmunity remains to be fully established, there is emerging evidence that T cell induced apoptosis is a dominant effector mechanism in T1D patients. The pathological role of anti-caspase-8 auto-antibodies in the occurrence of immune disorders are unclear, but it is speculated that this auto-antibodies could enter the cells when the permeability is increasing by means of apoptotic signals. However, these speculative phenomena remain to be clarified (Nagata et al., 2010).

In summary, we know that the number of positive antibodies, rather than the individual antibody, is thought to be most predictive of progression to overt T1D. The determination of auto-antibodies (as anti-caspase-8 in this case) or others immune markers in T1D could facilitate studies concerning to the pathophysiology underlying this disease. It is probably that antibodies against caspase-8 should only be considered as a surrogate marker for T1D in the complex immunological picture of the disease. In this context, the clinicians and researchers should be aware of the tests available, their limitations and their clinical relevance and applicability.

Competing interest

The authors declare that they have no competing interests.

Acknowledgements

This work was supported by FONDECYT Grant 1060790 (Prof. Francisco Pérez-Bravo). We thank all families participating in this investigation.

Authors’ contribution: FPB, AOA, ECP, FDB analyzed the data and drafted the manuscript. ECD and ECP provided subjects. AOA performed the serum analysis. LLM performed the statistical analysis. FPB advised on the technical protocol. All authors contributed to the conceptual design and analytical support. All authors read and approved the final manuscript.

References


