ORIGINAL ARTICLES

Apoptosis: an Indicator of Type 1 Diabetes in Children

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ABSTRACT

Beta cell apoptosis is a fundamental process involved in the pathogenesis of type 1 diabetes, it is precisely controlled by a host of factors that function as an apoptotic cascade, and the final effectors phase of type 1 diabetes has become an area of intense research due to the unequivocal evidences showing cell death in type 1 diabetes. In the study aimed to explore markers of apoptosis and oxidative stress in a group of children with type 1 diabetes and evaluate their role in disease initiation. The study population consisted of 103 (24 old diabetics + 29 new onset diabetics + 25 non diabetic Patients’ relatives + 25 healthy controls) children were included in the study and the serum levels of caspase 9, Bcl2, lipid peroxide and nitric oxide were estimated and compared between the studied groups. The study found a statistically significant higher values of caspase 9 and Bcl2 in old diabetics compared to new diabetics (p=0.000), (p=0.007) respectively, with a significant correlation of serum caspase-9 levels with the level of HbA1c. Bcl2 level was significantly higher in relatives group vs. controls (p = 0.02). Lipid peroxide level was significantly higher in the newly diagnosed patients vs. controls (p=0.003). In conclusion, the study proved the importance of serum Bcl2, caspase 9 and oxidative stress markers as indicators of apoptosis and their involvement in pathogenesis of type 1 diabetes, with the need of further studies to assess the role of the antiapoptotic Bcl2 protein in disease prediction.

Key words: Apoptosis – Type 1 diabetes mellitus- Caspase 9 - Bcl2

Introduction

Type 1 diabetes (T1DM) is caused by selective apoptotic destruction of beta cells in the islets of the pancreas, with consequent insulin deficiency and chronic hyperglycemia. (Thomas et al., 2009; Lei et al., 2013) Apoptosis has long been appreciated as an important form of cell death in biological processes and various pathologies. Our current understanding of the regulation of apoptosis is incomplete despite decades of research. Knowing that ROS (reactive oxygen species) play a central role in cell signaling has spurred much recent interest in the role of redox mechanisms in apoptotic signaling and control. (Circu and Aw, 2010) Apoptosis is precisely controlled by a host of factors that function as an apoptotic cascade (Taylor et al., 2008). Key enzymes that execute cell demise at the late stages of apoptosis are a group of cysteine proteases, known as effector caspases, including caspase-3, -6, and -7 (Riedl and Salvesen, 2007; Salvesen and Riedl, 2008). Activity of these effector caspases is controlled by initiator caspases, including caspase-8 and -9 (Riedl and Salvesen, 2007). Initiator caspases are activated via different mechanisms and regulate different apoptotic pathways. Bcl-2 family proteins, including pro-apoptotic (e.g., Bid, Bak, and Bax) and antiapoptotic (e.g., Bcl-2 and Bcl-xL) members play a key role in this process (Garrido et al., 2006). Caspase-9 is of high interest due to its entry point into the apoptotic pathway and its involvement in disease. As an initiator caspase in the intrinsic pathway of apoptosis, targeting caspase-9 provides flexibility between the upstream apoptotic signals and the cleavage cascade, making this a critical target point for cell survival or cell death. This role is reflected by its specific involvement in a variety of diseases. First they inactivate proteins that protect living cells from apoptosis, such as Bcl-2. Second, they can directly disassemble cell structures. (Huber, 2012)

The oxidative stress is recognized as a key causative factor in the development of major diabetes complications (Valko et al., 2007). Yet, a growing body of evidence has accumulated indicating that excessive production of reactive oxygen species accompanied by diminished antioxidant capacity may be involved in the disease initiation as well (Kawasaki et al., 2004). Determination of oxidative stress markers, in addition to clinical and laboratory indices, has recently been advocated as a part of the evaluation process of type 1 diabetic patients (Dalle-Donne et al., 2006). Mechanism of cell death induced by oxidative stress is under intense investigation by many groups. (Naderi et al., 2003; Padgett et al., 2013). Understanding of the final effectors phase of type 1 diabetes has become an area of intense research due to the unequivocal evidences showing cell death in type 1 diabetes and recent realization of the importance of apoptosis in a variety of physiological or
pathological conditions. (Lee et al., 2004) So the study aimed to explore markers of apoptosis and oxidative stress in a group of children with T1DM and evaluate their role in disease initiation.

Patients And Methods:

The study included 53 children with T1DM, divided into two groups: group 1): included 24 children with T1DM with disease duration more than 2 months, they were 12 males and 12 females with mean age 12.77±3.5. group 2) were 29 diabetics with recent onset, less than 2 month, 11 males and 18 females with mean age 9.3±2.7. Patient’s relatives without diabetes included 25 subject (11 males and 14 females; mean age 9.7±3.4) were examined within the same period. The results of the studied markers in diabetic children and their relatives were compared with a group of 25 healthy children, they were unrelated individuals who did not have a history of types 1 or 2 diabetes in the first-degree family line, (16 girls and 9 boys, mean age 10.24±4.2), subjects were examined within the same period for apoptosis markers and oxidative stress parameters. Approval from the local Medical Ethics Committee was obtained for the study.

Diabetics included in the study, were diagnosed according to the criteria of ISPAD clinical practice guidelines, (Craig et al., 2009), they were recruited from the Pediatric Diabetic Clinic of Children Hospital, Ain Shams University.

Peripheral venous blood samples were obtained from patients and controls. The samples were collected in sterile tubes and allowed to coagulate for 1 hour at room temperature. Then, serum samples were aliquoted in smaller containers that were marked with the patient’s name, date and stored at −80 °C until assaying. Serum bcl-2 concentrations were determined using a commercially available, non-isotropic, enzyme-linked immunosorbent assay (Oncogene Research Products, bcl-2 ELISA, Cat#QIA23). Caspase-9 levels were measured by the enzyme-linked immunosorbent method using the commercial ELISA kit for human Caspase-9 (Bender MedSystems, Austria) in accordance with the manufacturer’s instructions.

Serum NO levels were measured with Griess reagent. The first step is the conversion of nitrate using nitrate reductase. The second step is the addition of Griess reagent, which converts nitrite to a purple azo-compound. Protein interference was avoided by treatment of the reacted samples with zinc sulphate and centrifugation for 5 minutes at 10,000g; the azochromophor spectrophotometry was performed at 450 nm; sodium nitrate was used as the standard and results were expressed in mmol/L. The lipid peroxide content of serum was determined by measuring the thiobarbituric acid-reactive substances (TBARS) (Ohkawa et al., 1979)

Glycated Hemoglobin (HbA1c) was determined by automated high-performance liquid chromatography. Statistical analysis was carried out with SPSS (Statistical Package for Social Science) program version 10 for Windows (SPSS Inc, Chicago, IL, USA). The quantitative data were presented in the form of mean and standard deviation. Kruskal Wallis Test was used for group comparison. Nonparametric relationships were examined with Mann-Whitney U tests. Correlations were calculated using the Spearman Correlation Coefficient. P values <0.05 were deemed statistically significant.

Results:

The main clinical characteristics of diabetes group are shown in table 1. Although new onset group and controls were age matched, long standing diabetics were older in average; their mean age differed significantly in comparison with the other groups.

- Comparison between diabetic groups as regards their glycemic control revealed a significant increase in HbA1c in the old diabetics (9.07 ± 1.9 pg/mL vs. 6.25 ± 0.9 pg/mL, P = 0.03). (Table 1)

The mean serum levels of caspase 9, Bcl2, lipid peroxide and nitric oxide of the studied groups are shown in table 2.

- Lipid peroxide level was significantly higher in the newly diagnosed patients vs. controls (p=0.003), but it was significantly lower in old diabetic children when compared to both groups of relatives (P = 0.018) and newly diagnosed ( P = 0.000).

-As regards the level of nitric oxide, there was a non significant difference between the studied groups, however, it was also higher in new and old diabetic compared to their relatives and the controls.

- There was a significantly higher Bcl2 level in old diabetic patients in comparison with both the newly diagnosed (6.68 ±6.66 vs. 2.9 ± 5.00 P = 0.007), and control groups (p-value= 0.004), mean Bcl2 level was also significantly higher in relatives group 5.17± 5.6 vs.2.09 ± 1.8 for controls (p = 0.02). Caspase 9 level was also higher in old diabetic patients in comparison with newly diagnosed (p-value=0.000) and also in comparison with the relatives groups (p-value=0.000) and a near to significance difference between old and controls (p= 0.07), the mean caspase 9 level was higher in control group (5.62 ± 3.6) than relatives (1.74 ± 3.6), p = 0.001. There was significantly positive association between HbA1c and caspase 9 level (r = 0.8, p = 0.004)
Table 1: Comparison between old and new diabetic patients as regards the clinical characteristics

<table>
<thead>
<tr>
<th></th>
<th>Old</th>
<th>New</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>24</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Mean age± SD(years)</td>
<td>12.70±3.5</td>
<td>9.3±2.7</td>
<td>0.000**</td>
</tr>
<tr>
<td>Female/Male (n)</td>
<td>12/12</td>
<td>11/18</td>
<td></td>
</tr>
<tr>
<td>Positive Family history(n)</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Blood Glucose(mg/dl)</td>
<td>226.92</td>
<td>226.67</td>
<td>0.326</td>
</tr>
<tr>
<td>HbA1c (gm%)</td>
<td>9.06±1.9</td>
<td>6.25±0.9</td>
<td>0.03*</td>
</tr>
<tr>
<td>Disease duration (range)</td>
<td>2-15 years</td>
<td>0.5-~2 months</td>
<td></td>
</tr>
</tbody>
</table>

Hba1c = Glycated hemoglobin A1c

* Significant difference in comparison with control, P-value Mann-whitney U < 0.05
** Highly significant difference in comparison with newly diagnosed, P-value Mann-whitney U < 0.01

Table 2: Apoptosis and Oxidative stress parameters in the studied groups.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Caspase9</th>
<th>Nitric Oxide</th>
<th>Bcl2</th>
<th>Lipid Peroxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Old cases</td>
<td>7.95±5.1±c</td>
<td>49.63±13.47</td>
<td>6.68±6.7±c</td>
<td>8.1±1.1±c</td>
</tr>
<tr>
<td>New cases</td>
<td>2.09±4.17</td>
<td>48.71±15.92</td>
<td>2.91±5.00</td>
<td>14.62±4.5</td>
</tr>
<tr>
<td>Relatives</td>
<td>1.74±2.6</td>
<td>46.05±5.5</td>
<td>5.17±5.6</td>
<td>11.97±4.98</td>
</tr>
<tr>
<td>Controls</td>
<td>5.62±3.65</td>
<td>44.53±3.3</td>
<td>2.09±1.8</td>
<td>9.30±0.75</td>
</tr>
<tr>
<td>Sig.</td>
<td>0.000**</td>
<td>0.667</td>
<td>0.018*</td>
<td>0.001**</td>
</tr>
</tbody>
</table>

Data: means ± SD. *significant difference was found between the studied groups as regard Bcl2 level, P (Kruskal Wallis)< 0.05,
** highly significant differences between groups as regard caspase9 and lipid peroxide level, P (Kruskal Wallis)< 0.001,
* significant difference in comparison with control, P-value (Mann-whitney U) ≤0.05;
* significant difference in comparison with newly diagnosed, P-value (Mann-whitney U) ≤0.05 .
c significant difference in comparison with relatives group, P-value (Mann-whitney U) ≤0.05 .

Discussion:

Apoptosis is a multi-step process which starts with an extracellular death signal (induction phase), activating one or more signal transduction pathways (effector phase) that converge into few final death pathways (degradation phase). These final pathways are usually executed by proteases called caspasas and by caspase-activated DNAse. (Dotta et al., 2005)

Pivotal roles are played by the facilitators of this process, the caspasas, with caspase-9, being of particular interest due to its role in initiating the cascade. Its activation mechanism and regulatory checkpoints are targets for treatment of a variety of apoptosis-related diseases, beta-cell apoptosis in T1DM is thought to be the main cause of the disease, and an improved understanding of the involvement of apoptosis is needed. (Huber, 2012)

Although caspase-9 is an intracellular protein, data from the literature shows that measurement of caspasas in serum could be useful in monitoring different diseases (Roth et al., 2011). The present study measured caspase 9 level in the serum of the studied groups and found significant higher level in old diabetics when compared with the newly diagnosed and controls. Previous studies investigated the role of caspasas in diabetes mellitus but with different methodology. Liadis et al. (2005) found that caspase-3-mediated cell apoptosis is a requisite step for T-cell priming, a key initiating event in type 1 diabetes.

Allen et al., 2003 investigated the role of caspasas in high glucose-induced cell apoptosis, they measured the activity of caspasas 3, 8, and 9 in cytolsates. Caspase-3 and 9 activity was significantly higher than in controls. However, caspase-8 activity was increased but did not reach significance compared with controls.

In agreement with our results, Meier et al. (2005) reported that the high frequency of apoptosis in beta cells in longstanding type 1 diabetes implies ongoing beta cell provision in these people.

The Bcl-2 family has a double-edged effect in diabetes. These proteins are crucial controllers of the mitochondrial pathway of beta cell apoptosis induced by pro-inflammatory cytokines or lipotoxicity. In parallel, some Bcl-2 members also regulate glucose metabolism and beta cell function. (Gurzov and Eizirik, 2011) In this study there were significantly higher Bcl2 level in old diabetic patients in comparison with both the newly diagnosed and control groups. Interestingly Bcl2 level was higher in the relative group than controls, that may be postulated to its anti-apoptotic role. Allison et al., (2000) and Thomas et al., (2009) reported that overexpression of Bcl-2 held great promise for protection of beta cells from apoptosis in T1DM, however, others have shown that Bcl-2 affords no protection (Barbu et al., 2002; Tran et al., 2003).

Increasing evidences provide support that oxidative stress and apoptosis are closely linked physiological phenomena and are implicated in pathophysiology of some of the chronic diseases (Kannan and Jain, 2000) The redox status of a cell can have obscure and elaborate effects on apoptosis. While addition of exogenous ROS is sufficient to trigger the apoptotic cascade, the executioners of apoptosis, for instance the caspasas, are extremely sensitive to redox alterations and require a reducing environment in which to function. Perhaps one of the most potent regulators of apoptosis is NO, having the unique ability to both induce and block cell death. This discovery has significant implications for the regulation of apoptosis by NO. (Curtin et al., 2002)

(Astanenie et al., 2005; Ramakrishna and Jailkhani, 2007) observed increased levels of nitric oxide in Type I DM, and reported that, existence of increased total antioxidant power in the presence of normal lipid peroxidation in plasma of type I diabetic patients indicates the existence of oxidative stress. Our results are in
accordance with those of previous finding show increased NO levels in the diabetic group, but with no statistical significance, however lipid peroxide level was significantly higher in newly diagnosed cases compared to controls. This was in agreement with Davi et al. (2003) who reported enhanced lipid peroxidation as an early event in T1DM patients, they also reported that enhanced lipid peroxidation in recently diagnosed diabetic children and young adults was improved later on parallel to the improvement in glycemic control of these patients this could be explained by that some antioxidants are probably lost due to renal hyperfiltration in the early phase of.

Conclusion:

Considerable evidence indicates that increased oxidative stress and induction of apoptosis may play an important role in the development of diabetes mellitus, old type 1 diabetic patients had increased caspase 9 and Bcl2 level, with significant positive association between caspase 9 and the severity of disease. To our knowledge, there have been no previous reports on the presence of elevated circulating markers of apoptosis in the sera of patients with diabetes, that suggest their involvement in disease progression, furthermore the increased Bcl2 level in non diabetic patients’ relatives, can be considered as a predictor of the disease.

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Reference


Ramakrishna, V. and R. Jailkhani, 2007. Evaluation of oxidative stress in Insulin Dependent Diabetes Mellitus (IDDM) patients Diagnostic Pathology, 2: 22.


