Apoptosis of pancreatic β-cells in type 1 diabetes: The role of Caspase-3 in β-cell apoptosis

Tatsuo Tomita1*

1Departments of Integrative Bioscience and Pathology, Oregon Health and Science University, 611 SW Campus Drive, Portland, Oregon 97239-3097, USA

ABSTRACT

Type 1 diabetes (T1D) results from autoimmune destruction of pancreatic β-cells after asymptomatic period over years. Insulitis activates antigen presenting cells, which trigger activating DC4+ helper-T cells, releasing cytokines. Cytokines activate CD8+ cytotoxic –T cells and lead to β-cell destruction. Apoptosis pathway consists of extrinsic and intrinsic pathway. Extrinsic pathway includes Fas pathway to CD4+-CD8+ interaction whereas intrinsic pathway includes mitochondria-driven pathway at balance between anti-apoptotic Bcl-2 and Bcl-xL and pro-apoptotic proteins. Activated cleaved caspase-3 (ACC) is the converging point between extrinsic and intrinsic pathway. ACC may be used as a marker for β-cell apoptosis: in T1D islets weakly insulin-positive β-cells are present with increased ACC positive β-cells whereas β-cell absent islets, which exclusively consist of α-cells and PP-cells with decreased ACC-cells, represent the end-stage of regenerating β-cells. Weakly insulin-positive β-cells could be rejuvenated to supply endogenous insulin. Apoptosis takes place only when pro-apoptotic protein exceeds anti-apoptotic proteins. Since concordance rate of T1D in the identical twins is about 50%, environmental factors are involved in development of T1D, opening a door to means to prevent autoimmune β-cell destruction for therapeutic application by transfecting β-cells with Casp3\(^{-/-}\) genes or pan-caspase inhibitor therapy. Prudent glucose control prevents ongoing hyperglycemia-induced β-cell apoptosis.

Introduction

Type 1 diabetes (T1D) results from severe insulin deficiency by loss of insulin-producing β-islet cells and it develops mostly in young and accounts for 5 to 10% of the diabetic subjects1,2. T1D develops as the consequence of progressive β-cell destruction by autoimmune processes after an asymptomatic period over several years2. MHC class II are expressed at the surface of antigen presenting cells (APC) (e.g. dendritic cells, macrophages and B-lymphocytes) including DR, DQ and DP and some specific combination of alleles for DQA1 and DQB1 genes and DRB1 genes (DRB1*03 and DRB1*04) significantly increase the development of T1D2-5. The earliest sign of autoimmunity against β-cells are often detectable months or years of clinical T1D and the most common autoantibodies in pre-diabetic subjects are directly against glutamic acid decarboxylase (GAD65), tyrosine phosphatase-like protein (IA-2) and insulin6. Up to 90 % of newly diagnosed T1D subjects have autoantibodies to one or more of these antigens6 and autoimmunity detection rates increase to 98% when combining the detection of these three antibodies plus antibodies against the newly discovered β-cell autoantigen ZnT87,8.
In identical twins, the concordance rate is below 50% for T1D as compared to a higher concordance rate for type 2 diabetes (T2D), suggesting that environmental or non-genetic factors contribute for T1D as also in the case for T2D, the latter has the concordance rate of about 90% in the identical twins. Epidemiological data suggest that autoimmune process is triggered early in life, which may indicate that the pool of self-reactive naïve T-cells stay in the control of the immune system for several years with some antibody positive subjects never develop insulitis nor proceed to overt T1D. The environmental factors include viral infection, including that of Coxsackievirus B (CVB), rubella, mumps, rotavirus and cytomegalovirus, and toxins, dietary factors during infancy, vaccination and others. With immunocytocchemical staining of 29 cases of pancreata from the T1D subjects who died within 18 months of diagnosis, Wilcox et al reported the following findings: CD8+ cytotoxic cells were most abundant population in insulitis; CD68+ macrophages were also present in insulitis whereas CD4+ helper cells were present in the islet infiltrates, but were less numerous than CD8+ or CD68+ cells whereas CD20+ cells increased during late insulitis since CD20+ cells were recruited late during insulitis. β-cell damage and destruction are mediated through CD8+ and CD4+ but natural killer cells (NK cell) do not appear to be required for β-cell death. Insulitis is defined by at least 15 CD45+ cells/islet present in three or more islets and CD8+ cells are predominant. Only 10%–30% of islets show insulin at any time, even at the time of T1D diagnosis, and there is different stage of islet destruction within the same pancreas. The first detection of autoantibodies peaks at one to three years of age in children who develop early T1D and the second incidence peak is seen around puberty and shows more heterogenous autoantibody profile than in early forms of T1D. T1D follows a slow-progressing autoimmune process before presenting typical clinical symptoms through a cascade of complicated immunological sequences as reported by Eizirik and his associates as follows:

**Autoimmune destruction of pancreatic β-cells**

A) After the early stages of insulitis, activated local APCs recruit and activate CD4+ helper T-cells via migration to the pancreatic lymph nodes to present β-cell antigen and release of chemokines/ cytokines (Figure 1A).

B) CD4+ helper T-cells in turn stimuliates APC secretion of cytokines and nitric oxide (Figure 1B).

C) Cytokines induce secretion of chemokines by endothelial cells which enhance to recruit immune cells into the islets and activate CD8+ cytotoxic T-cells together with cytokines (Figure 1C).

D) β-cells also secrete chemokines in response to viral infection or cytokines, enhancing recruitment and activation of immune cells. Activated CD8+ cells in turn induce β-cell apoptosis (Figure 1D).

Fas pathway: Fas (CD 95), a member of TNF superfamily is activated via binding of Fas L (CD 178) and Fas and Fas L are detected respectively at the surface of β-cells and T-cells infiltrating islets. Once activated, Fas trimerezes and recruits the Fas-associated death domain (FADD), which recruits pro-caspase-8 leading to its activation by autolocalavage. Activated caspase-8 subsequently cleaves the effector caspase-3 and/or activates the BH3 protein Bid (Figure 1E).

F) The perforin/granyme system: Perforin and granzyme are contained in granules inside the CD8+ T-cells. Perforin is involved in pore formation across the membranes via Ca2+ dependent mechanisms. The pore enables the entry of serine protease granzyme inside the cell, causing the cleavage of and activation of several targets, such as effector caspase-3 and the BH3 protein Bid (Figure 1F).

G) Interleukin-1β (IL-1β) activates NFkB and the kinases PKC, p38 and JNK: IL-1β binding to its receptor induces the formation of multiproteic complex at its cytoplasm domain including IL-IRAcP, Tollip, MyDB8 and IRAK family, namely IRAK-1 and IRAK-4 (Figure 1G).

H) Tumor necrosis factor α (TNF-α) activates caspase-8, NF-kb and the MAPK p38 and JNK: Activation of TNF receptor 1 (TNF-R1) upon TNF-α binding leads to its trimerization and recruitment of adaptor protein TNF-associated death domain protein (TRADD), which in turn recruits TRAF-2 and serine threonine kinase Rip. TRAF-2 activates NF-kb and MAPK pathways. TNF-α phosphorylates and activates p38 and JNK. Caspase-8 activates effector caspase-3 in the same way in the Fas pathway (Figure 1H).

I) Interferon Y (IFN-Y) activates Stat-1 and the kinase ERK: IFN-Y binding to its receptor induces its oligomerization and cytoplasmic recruitment of two members of Janus kinase (Jak) family, Jak-1 and Jak-2, which recruit Stat-1 and trigger its activation by phosphorylation. Stat-1 then homodimerizes and migrates to nucleus where it regulates expression containing Y-activated sequence (GAS) elements such as caspases, Fas and iNOS (Figure 1I). In this theory, activated caspase-8 cleaves and activates effector caspase-3 and BH3 protein Bid in the E) Fas pathway and H) TNDα pathway and in part in I) IFNY pathway.

**Extrinsic and intrinsic apoptosis pathway**

There are two main apoptosis pathways, the extrinsic (receptor-mediated) and the intrinsic (mitochondrial-driven) pathway. Above described theory refers mostly extrinsic pathway and is not distinctively clarified into
Figure 1. Schematic representation of the auto-immune attack to the beta-cells in T1D.

(A) At the early stage of insulitis, activated local antigen presenting cells (APC) recruit and activate CD4+ helper T-cells via migration to the pancreatic lymph node, presentation of beta-cells antigens and release of chemokines/cytokines. (B) CD4+ helper T-cells, in turn, stimulates APC secretion of cytokines and nitric oxide. (C) Cytokines induce the secretion of chemokines by endothelial cells which enhance the recruitment of immune cells into the islets and, together with cytokines, activate CD8+ cytotoxic T-cells. (D) The beta-cell themselves also secrete chemokines in response to viral infection or cytokines, further enhancing the recruitment and activation immune cells. Activated CD8+ cytotoxic T-cells, in turn, induce beta-cell apoptosis via (E) the Fas pathway and (F) the granzyme/perforin system. Cytokines also bind to receptors at the surface of beta-cells: (G) Interleukin-1β (IL-1β) activates NF-κB and the kinase d PKC, p38 and JNK. (H) Tumor necrosis factor α (TNFα) activates caspase-8 and the MAPK p38 and JNK. (i) Interferon γ (IFNγ) activates Stat-1 and the kinase ERK. From reference 3. From reference 3. Pirot, P., Cardozo, AK, Eizirk, DL, Arq Bras Endocrine Meatv 2008;52(2):156-165.
extrinsic or intrinsic pathway, respectively and each pathway will be described below despite some redundancy between the above and below description on apoptosis (Figure 2)\textsuperscript{19}.

It is the extrinsic pathway that is activated upon ligation of the cells surface death receptors (s), which in turn activate(s) a downstream effector mechanism orchestrated by the caspase family of cysteine proteases (Figure 2)\textsuperscript{19}. The prototype example of death signaling via the extrinsic pathway is the Fas death receptor (FDR), which instigates assembly of the death-inducing signaling complex (DISC), a multi-protein complex comprising the cytoplasmic aspects of the Fas receptor, the adaptor protein FADD and precaspase-8 (Figure 2)\textsuperscript{19}. Caspase-3 is the converging point of the apoptotic pathway (Figure 2)\textsuperscript{19} and its peptide inhibitors have been shown to prevent islet apoptosis and improve islet graft function\textsuperscript{21}. Apoptosis induced by ligation of cell surface receptors like Fas L or TNF receptors, "death receptors" represents a pathway controlled by caspases\textsuperscript{22,23}. Ligand binding of the receptor causes assembly of series of proteins of the death-inducing signaling complex, which then activates apical caspase, procaspase-8\textsuperscript{19,24}. The resulting events proceed in cascade that caspase-8 induces activation of caspase-3\textsuperscript{31}. Apoptosis manifests in two major execution programs, downstream of the death signal, the caspase pathway\textsuperscript{25} and upstream of irreversible cellular damage reside the Bcl family members, which are proteins with both anti-apoptotic and pro-apoptotic properties, playing a pivotal role in the life and death of cells (Figure 2)\textsuperscript{19}. Anti-apoptotic members of the Bcl family, including Bcl-2 and Bcl-xL blunt intrinsic death signaling by blocking the recruitment of pro-apoptotic members to the mitochondria\textsuperscript{26}. The cumulative data support the notion that high glucose might modulate the balance of the pro-apoptotic caspase family and anti-apoptotic Bcl proteins toward apoptosis, thus leading to β-cell death\textsuperscript{23,27}.

One of immunocytochemical markers for apoptosis is cleaved caspase-3 (ACC): The caspase-3 protein is a member of the cysteine-aspartic acid protease (caspase) family and plays central roles in the execution-phase of cell apoptosis\textsuperscript{20}. Caspases exist as zymogens in the soluble cytoplasm, endoplasmic reticulum, mitochondrial membrane space, and nuclear matrix\textsuperscript{22}. Caspases are inactive proenzymes that undergo proteolytic processing at conserved aspartic residues to produce two subunits, large mass of ~20 kDa and small mass of~10 kDa, that dimerize to form the active enzyme\textsuperscript{19}. This activated enzyme cleaves and activates caspase-6 and -7, and the protein itself is processed and activated by caspases- 8, 9 and -10\textsuperscript{29}. One of these cleaved caspases is present on activated caspase-3, a ubiquitously distributed caspase which is a main effector caspase of the apoptotic cascade within cells\textsuperscript{29-33}. Caspase-3 is active over a broad pH range that is more basic than many of other executioner caspases, indicating that caspase-3 will be fully active under normal and apoptotic cell condition\textsuperscript{29}. Caspase-3 is activated in the both extrinsic and intrinsic apoptosis pathway. In the extrinsic pathway caspase-3 plays a dominant role by activating caspase cascade of apoptotic pathway. In the intrinsic pathway, cytochrome c from the mitochondria works in combination with caspase-9, apoptosis activating factor (Apar-1) and ATP to process procaspase\textsuperscript{30,31}. 
Immunocytochemical study

The commercially available polyclonal anti-ACC detects endogenous levels of the large (17/19 kDa) cleaved caspase-3 resulting from cleavage adjacent to Asp 175 and does not recognize the full length or other cleaved caspases (Cell Signaling Technology Publication, Beverly, MA, USA, 2006)\(^{34}\) and its immunopositivity staining is specifically in the islet cell nucleus alone\(^{35,36}\). Recently, an involvement of caspase-3 in both T1D and T2D was implicated: in T1D, Fas(CD 95)-Fas L (CD 178) may be critical for β-cell destruction, as apoptosis in β-cell clone expressing the human Fas β-cell line is mediated by elevated caspase-3 like activity in tissue culture\(^{37}\) and the frequency of β-cell apoptosis in T2D pancreatic tissues from autopsy is increased using deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)\(^{38}\).

We performed ACC immunocytochemical staining in 8 cases of T1D pancreata compared with 8 controls to study the converging point of extrinsic and intrinsic pathway\(^{39}\). T1D islets showed more ACC-positive cells at 16% of the total islet cells, with large and small islets positive at 14% and 17%, respectively at 3.3 and 3.6 and 2.4 times that of the corresponding control total, large and small islet values, respectively (Table 1)\(^{39}\). The T1D islets were a mixture of small and large regenerating islets consisting of non-β-cells of α-, δ- and pancreatic polypeptide (PP)-cells with moderately increased ACC-positive cells (Figure 3A-E, right column) (Table 1)\(^{39}\). Among generally small islets from T1D individuals (Case 6), which consist of insulin cells (25%) and more α-cells (75%), δ-cells (10%) and there were 13% ACC-positive β-cells at three times more than control islets (Table 1, Figure 3A-D)\(^{39}\). The presence of increased ACC-cells in these islets suggest that these islets were in the process of increased β-cell apoptosis in the remaining β-cells. One type of large islets consists exclusively of α-cells and PP-cells (50% each) with practically no remaining β-cells and less ACC-cells, suggesting that these large islets correspond to the burnout, end-stage apoptosis-completed stage in response to long-standing compensation of insulin secretion (Figure 4E-H) (Case 8)\(^{39}\). Another type of large islets consists of remaining weakly insulin-staining minor β-cells, major α-cells and relatively decreased δ-cells (16%), which represent remaining β-cells with less insulin and lesser IAPP immunostaining, thus suggesting apoptotic β-cell containing less insulin and even lesser IAPP, (Case 7, Table 2) (Figure 4A-D)\(^{41}\). The extra-large islets from Case 7 in Table 2 revealed weakly insulin-positive granular β-cells containing abundant cytoplasms with lesser IAPP-positive cells (17%), major α-cells (39%) and increased δ-cells (28%) plus irregular sickle-shaped densely IAPP-positive β-cells (Figure 4E-H)\(^{41}\). The pancreas was obtained at autopsy from T1D subjects of ages 18 to 75, and the immunocytochemical presence of ACC suggests that ACC mediated apoptosis was in the ongoing process even at the subjects’ death with a delicate balance of β-cell apoptosis and regeneration. These data also suggest that β-cell apoptosis and regeneration process is progressive from the preclinical stage to the end-stage of T1D and T2D\(^{40,41}\). The ACC positive β-islet cells were more in type 1 diabetic islets than in type 2 diabetic islets\(^{39,41}\).

Butler and his associates studied type 1 diabetic islet using ACC and TUNEL immunofluorescence: ACC immunostaining revealed 5.8% for type 1 diabetic islets compared to 2.7% for control islets at twice as much for type 1 diabetic islets of the control islets\(^{42}\). Our data on ACC-positive cells were 15.7 % for type 1 diabetic islets and even lesser IAPP , (Case 7, Table 2) (Figure 4A-D)\(^{41}\). The pancreas was obtained at autopsy from T1D subjects of ages 18 to 75, and the immunocytochemical presence of ACC suggests that ACC mediated apoptosis was in the ongoing process even at the subjects’ death with a delicate balance of β-cell apoptosis and regeneration. These data also suggest that β-cell apoptosis and regeneration process is progressive from the preclinical stage to the end-stage of T1D and T2D\(^{40,41}\). The ACC positive β-islet cells were more in type 1 diabetic islets than in type 2 diabetic islets\(^{39,41}\).

Table 1. Cleaved caspase-3 Immunostaining.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Large islets</th>
<th>Small islets</th>
<th>Total islets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case</td>
<td>Positive Islet Positive Positive</td>
<td>Positive Islet Positive Positive</td>
<td>Islet Positive</td>
</tr>
<tr>
<td>Cells</td>
<td>%</td>
<td>Cells</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>18/M</td>
<td>7.3</td>
<td>37.3</td>
</tr>
<tr>
<td>2</td>
<td>35/F</td>
<td>9.2</td>
<td>62.1</td>
</tr>
<tr>
<td>3</td>
<td>43/F</td>
<td>2.8</td>
<td>44.9</td>
</tr>
<tr>
<td>4</td>
<td>50/F</td>
<td>21.8</td>
<td>73.3</td>
</tr>
<tr>
<td>5</td>
<td>61/F</td>
<td>7.7</td>
<td>43.1</td>
</tr>
<tr>
<td>6</td>
<td>75/F</td>
<td>3.6</td>
<td>50.6</td>
</tr>
<tr>
<td>7</td>
<td>75/F</td>
<td>5.1</td>
<td>51.6</td>
</tr>
<tr>
<td>8</td>
<td>75/F</td>
<td>4.4</td>
<td>44.3</td>
</tr>
<tr>
<td>Mean</td>
<td>7.7*</td>
<td>50.9</td>
<td>14.4*</td>
</tr>
<tr>
<td>se</td>
<td>2.1</td>
<td>3.75</td>
<td>2.80</td>
</tr>
</tbody>
</table>

Control (n = 8)

| Mean | 2.9* | 72.4 | 4.0* | 1.7* | 25.0 | 6.8* | 7.5* | 58.5* | 4.7 (30)* |
| se | 0.29 | 5.32 | 0.39 | 0.13 | 1.20 | 0.43 | 0.19 | 2.91 | 0.40 (30) |

Numbers in parenthesis are the numbers of islets examined. *p values calculated with the corresponding control values are: p < 0.001.
4.7% for control islets at three times more ACC-positive cells in type 1 diabetic islets than control islets. Butler's group reported TUNEL immunofluorescence at 0.2% for type 1 diabetic islets as compared to 0% for control islets. Observing immunofluorescence photomicrographs, the positive staining was not clear and hazy for both ACC immunostaining and TUNEL including positive staining in the perinuclear cytoplasm, not as clear as our ACC immunoperoxidase staining. Another study using TUNEL reported 0.5% positive cells for normal β-cells. Immunopositive ACC is present in the apoptotic cells and ongoing apoptotic cells, which may explain a higher percentage of apoptotic cells revealed by ACC immunoperoxidase staining than TUNEL staining. Thus, ACC immunoperoxidase staining recognizes protein levels more than TUNEL, the latter recognizes DNA levels.

With 10 cases of T1D islets, immunocytochemical study was performed for insulin, glucagon, somatostatin (SRIF) and islet amyloid polypeptide (IAPP), the latter is a 37 amino acid polypeptide and is concomitantly co-secreted with insulin from β-cells into the blood stream in response to glucose-stimulated secretion. The islet from Case 6 is generally small, consisting of insulin-cell poor (25%), glucagon-cell rich (75%) and somatostatin-cell rich (10%) with increased cleaved caspase-3 positive cells (13%). The large islet from Case 8 has practically no insulin-cells (<1%) and consists of major glucagon and pp-cells (a combined 100%) with a normal number of cleaved caspase-3 positive cells (4%).

Figure 4. Insulin-poor small islet (A-E) and insulin-absent large islet (E-H) from T1D subjects in Table 1. The islet from Case 6 is generally small, consisting of insulin-cell poor (25%), glucagon-cell rich (75%) and somatostatin-cell rich (10%) with increased cleaved caspase-3 positive cells (13%). The large islet from Case 8 has practically no insulin-cells (<1%) and consists of major glucagon and pp-cells (a combined 100%) with a normal number of cleaved caspase-3 positive cells (4%). (*): Residual insulin-cells A and E: Insulin, B and F: Glucagon, C and G: PP, D and H: Cleaved caspase-3 immunostained. From reference 34, Tomita, T, Islets 2010; 2(1):24-29, with permission.
(SRIF) cells were 20---30% at twice the ratio of the control islets (Table 2)\textsuperscript{41}. In Cases 6,7,9 and 10, α-cells were the major cells at 41—46% and β-cells were at 30—34% (Table 2)\textsuperscript{41}. The four cases (Cases 6,7,9 and 10) revealed a mixture of extra-large, large and medium sized islets. The extra-large islets contained more than 100 islet cells, which were rare in the control islets (Table 2)\textsuperscript{41}. In large and medium sized islets, all β-, α- and δ-cells contained scanty cytoplasm and α-cells were more numerous than β-cells in majority of islets (Figure 4A, C and D)\textsuperscript{41}. About 10% of type 1 diabetic islets were extra-large islets in Cases 6,7,9 and 10, in which majority were α-cells (Figure 4G)\textsuperscript{41} and β-cells were weakly insulin-stained than the control β-cells (Figure 4A and D)\textsuperscript{41}. IAPP immunostaining was even weaker and less granular than insulin staining and some deeply IAPP positive staining was noted in sickle-shaped cytoplasm, often without nucleus, which appeared to be degenerating or dying β-cells (Figure 4H)\textsuperscript{41}. SRIF cells were relatively increased with abundant cytoplasm (Figure 5H)\textsuperscript{40}. Although β-cells from T1D subjects were much less numerous than the control islets, these residual β-cells could be activated or rejuvenated to produce and supply at least a part of endogenous insulin for better glucose homeostasis.

Lam et al studied 47 cases of T1D pancreata compared to 59 cases of nondiabetic controls for β-cell mass, β-cell turnover using Ki-67 and insulin/glucagon co-expression by immunocytochemical staining and TUNEL: residual

<table>
<thead>
<tr>
<th></th>
<th>Large Islets</th>
<th>Medium Islets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Islet %</td>
<td>% Ins % Glu % SRIF % IAPP % IAPP/Ins</td>
</tr>
<tr>
<td>Case 1. 18/M</td>
<td>51.2</td>
<td>15.7</td>
</tr>
<tr>
<td>Case 2. 35/F</td>
<td>75.1</td>
<td>0</td>
</tr>
<tr>
<td>Case 3. 39/M</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Mean (n = 3)</td>
<td>58.2</td>
<td>5.2</td>
</tr>
<tr>
<td>SE</td>
<td>8.2</td>
<td>5.2</td>
</tr>
</tbody>
</table>

|       | Case 4. 43/F | 57.6 | 37.6 | 46.6 | 16.5 | 16.7 | 44.4 | 27.8 | 41 | 43 | 16.4 | 20.8 | 52 |
| Case 5. 50/F | 82 | 38.3 | 46.3 | 15.4 | 18.3 | 49.9 | 30.5 | 43 | 39.5 | 17.2 | 31 | 67.6 |
| Case 6. 52/F | 80.9 | 44.2 | 43.3 | 13.4 | 24.9 | 68.5 | 30.1 | 40.3 | 44.7 | 14.4 | 11.7 | 74.6 |
| Ex large | 123.5 | 42.5 | 46.8 | 10 | 19.7 | 48.8 | |
| Case 7. 61/F | 83.1 | 36.4 | 35.4 | 27.7 | 23.7 | 59.2 | 30.7 | 39.2 | 33.1 | 27.9 | 21.8 | 57 |
| Ex large | 133.5 | 32.2 | 39.2 | 28.2 | 16.5 | 51.5 | |
| Case 8. 65/F | 39 | 30.7 | 41.3 | 27.3 | 13.3 | 46.7 | 22.4 | 28 | 49.1 | 22.4 | 15 | 60.3 |
| Case 9. 75/F | 87.1 | 32.8 | 51 | 16.6 | 15.5 | 52.4 | 21 | 35.8 | 39.7 | 24.4 | 26.8 | 74.7 |
| Ex large | 115.4 | 32.2 | 53.9 | 14.2 | 12.2 | 43.1 | |
| Case 10. 77/F | 71.8 | 39.8 | 48.9 | 10.7 | 20.4 | 52.1 | 22.3 | 39.7 | 46.8 | 13.6 | 16.7 | 43 |
| Ex large | 121 | 41 | 47 | 11.7 | 26.7 | 65.3 | |

| Mean (n = 7) | 71.6 | 36.1 | 44.7 | 19.6 | 19 | 53.3 | 26.4 | 38.1 | 42.2 | 19.5 | 20.5 | 61.2 |
| SE | 6.5 | 2.4 | 2 | 3.5 | 1.6 | 3.1 | 1.6 | 1.8 | 2 | 2.1 | 4.4 |

| Ex large (n = 4) | Mean | 123.2 | 37 | 46.7 | 16 | 18.7 | 52.2 |
| SE | 3.8 | 2.7 | 3 | 4.1 | 3 | 4.7 |

Ins, Insulin; Glu, Glucagon; SRIF, Somatostatin; IAPP, Islet amyloid polypeptide; Ex large, Extra large islet; NC, Not calculable.
insulin-producing β-cells were detected in some cases but not in all cases and several T1D pancreata had substantial numbers of β-cells. β-cell proliferation was prominent early in life but it dramatically declined after infancy and β-cell proliferation by Ki-67 and β-cell proliferation was equivalent and β-cell death by apoptosis by TUNEL was rare in both T1D and control islets44. This study also supports the remaining insulin-positive β-cells as seen in our cases and rare detection of apoptotic β-cells using TUNEL44. Bressenot et al studied apoptosis in MDA-MB231 monolayer cells and HT29 human colon adenocarcinoma cell line using antibody against active caspase-3, active caspase-7 and cleaved poly-ADP-ribose polymerase 1 (c-PARP) immunofluorescent method: apoptosis-induced cells showed a significant higher number of active caspase-3-labeled cells, and active caspase-3 and c-PARP immunofluorescent perfectly colocalized in monolayer cells. A restricted expression of c-PARP was obvious in the greater part of active caspase-3 expressing cells45. There was absent caspase-7 immunofluorescence in some active caspase-3 positive cells and the relevance of each antibody for apoptosis needs to be further scrutinized45.

**Molecular aspect on β-cell apoptosis**

The Bcl-2 family governs mitochondrial outer membrane permeabilization and can be either anti-apoptotic (Bcl-2, Bcl-xL, Bcl-w) or pro-apoptotic (Bad, Bid, Bik, Bak) and there is a total of 25 genes known in the Bcl-2 family46,47. Bcl-2 family exerts anti- and pro-apoptotic effect by activation or inactivation of an inner mitochondrial permeability transition pore, which is involved in the regulation of matrix Ca^{2+}, pH and voltage. Bcl-2 family proteins can induce by pro-apoptotic members or inhibit by anti-apoptotic members through the release of cytochrome c into the cytosol, which activates caspase-9 and -3, leading to apoptosis49. The members of the Bcl-2 family share one

---

**Figure 5: A-E: Bcl family gene regulation in human islets cultured in high versus normal glucose.** Expression of Bcl-2, Bcl-xl, Bad, Bid, and Bik mRNA was detected by RT-PCR and quantified by FluorImager analysis of ethidium bromide signal. In each experiment, band densities were normalized against cyclophyllin, and the results are expressed as mRNA level to NG1 control islets (NG1 = 100%). 

A: Bcl-2. B: Bcl-xl (HG5 vs. NG5, **P < 0.01). C: Bad (HG5 vs. NG5, **P < 0.01). D: Bid (HG5 vs. NG5, ***P < 0.001). E: Bik (HG5 vs. NG5, **P < 0.01). One representative gel is also shown. Islets from six donors were analyzed. Means ± SD of relative expression of the genes are shown in bar graph. Statistical analysis was performed by ANOVA.
or more of the four characteristic domains (BH1, BH2, BH3 and BH4) and the anti-apoptotic Bcl-2 and Bcl-xL conserve all four BH domains\(^49\). All pro-apoptotic proteins contain a BH3 domain necessary for dimerization with other proteins of Bcl-2 family (Bax and Bak). The BH3-only of the Bcl-2 family of proteins only contains a single BH3-domain and plays a key role in promoting apoptosis. The BH3-only family members are Bim, Bid and Bad\(^49\).

In the thymus, the elimination of potentially autoreactive T lymphocytes occurs by positive and negative selection, the latter involving activation-induced cell death (AICD) ensures an efficient elimination of T lymphocytes by apoptosis and this is a major mechanism in the regulation of peripheral tolerance and subsequently can determine the development of autoimmunity\(^50\). To evaluate the role of caspase-3 in T1D AICD, caspase-3 expression was analyzed in peripheral blood lymphocytes from 37 new onset T1D subjects and from 36 normal control subjects in the resting conditions and after anti-Fas triggered apoptosis. Caspase-3 mRNA expression was reduced in the resting lymphocytes in 18/37 T1D subjects and 1/36 controls\(^50\). The T1D subjects studied from both Fas mediated AICD and caspase-3 mRNA expression revealed that a reduced caspase-3 mRNA expression in the resting lymphocytes in all T1D subjects showing resistance to Fas mediated apoptosis\(^50\). Caspase-3 protein analysis confirmed mRNA data and showed an impaired expression of caspase-3 active form in T1D subjects compared to the controls\(^50\). The defective expression and function of caspase-3 in peripheral lymphocytes in T1D subjects may contribute to the development of AICD resistance in T1D\(^50\).
With the right atrial appendages from diabetic and nondiabetic subjects retrieved during elective coronary bypass surgery, sliced appendages (300–500μm) were subjected to 90 min stimulated ischemia and 120 min reoxygenation51. The tissues were sectioned at 10 μm and were processed to TUNEL for apoptotic analysis. The apoptotic percentages were 12.5 ± 2.9 % and 15.0 ± 2.8 % for T1D and T2D groups, respectively, relative to 3.7 ± 0.8 % (p<0.05) in the nondiabetic groups51. The level of caspase activity in the cellular lysates was detected by fluorescence signal and the amount of acapase-3-like activity was measured using the effector caspase inhibitor zVAD-fmk. The activities of initiator caspasases in fresh nondiabetic tissues were similar in T1D, T2D and nondiabetic groups, but the activity was significantly increased in T1D and T2D groups relative to nondiabetic group after ischemia and re-oxygenation51. The caspase-3 inhibitor completely abolished apoptosis in the nondiabetic group but in T1D and T2D groups caspase-3 inhibition reduced apoptosis by 50%51. Thus, appendage tissues from T1D and T2D subjects revealed increased apoptosis after ischemia and re-oxygenation and caspase activities significantly increased in T1D and T2D tissues relative to nondiabetic group especially after ischemia and re-oxygenation and this 50% reduction of apoptosis by caspase-3 inhibitor was partially blocked51.

For a genetic aspect to study the role of caspase-3 in β-cells in vivo, the caspase-3 knockout (Casp3-/-) mice were tested: the knockout mice were protected from autoimmune diabetes by multiple-low-dose streptozotocin (MLDS) injection with 40 mg/kg of body weight, ip for 5 days, in which insulitis lymphocytic infiltrates in pancreatic islets was completely absent56. In Casp3-/- mice blood glucose was above 25 mmol/l compared to Casp3+/+ mice whose blood glucose was below 10 mmol/l52. At 14 days post MLDS, pancreatic islets from Casp3-/- mice revealed less β-cells and predominant α-cells compared to Casp3+/+ islets consisting of major β-cells and less α-cells in the outer margin of the islet52. β-cell antigen-specific T-cell activation and proliferation were observed only in the pancreatic draining lymph node of RIP-GP/p14 Casp3+/+ mice but not in Casp3-/+ mice50. A genetic approach to transfer Acsps3+/+ to Casp3-/+ may provide a future genetic engineering to stop the development of T1D53. c-Myc is a potent inducer of both cell proliferation and apoptosis and sensitizes cells to apoptotic triggers by augmenting the death receptor pathway and priming the mitochondria to release cytochrome c55. C-Myc has a predominantly pro-apoptotic role in pancreatic β-cells53. Woo and her associates further used the caspase-3 knockout mice containing inducible c-Myc transgene (c-Myc-Casp3-/-). C-Myc-activated Casp3-/- mice were protected from multipole-low-dose streptozotocin-diabetes, revealing that caspase-3 deletion confers protection from c-Myc-induced apoptosis and MLCD-diabetes53. Islet transplantation can provide insulin independence in T1D subjects but most islet recipients revert to insulin dependence because up to 70% of transplanted islets lose adequate insulin secretion within the first week of transplantation52,54. Most islets do not function due to the host immune rejection, nonspecific inflammatory response and poor revascularization55,56.

Transfection of rat insulinoma cells (INS-1E cells) with chemically synthetic small RNA (siRNA) reduced caspase-3 transcripts by 50%52. Adv (replication deficient adenoviral) vector encoding shRNA transduced islets showed relatively prolonged levels of gene silencing and protected islets from cytokine-induced apoptosis56. Return to normoglycemia was achieved by transplanting Adv-caspase-3-shRNA transfected human islets under the kidney capsule in the MLDS mice53. INS-1E cells were further transfected with X-linked inhibitor of apoptosis (Adv-hXIP) which protects Ins-1E cells from inflammatory cytokines through decreasing the activities of caspase 3/7, 8 and 9, which resulted in reduced apoptotic β-cells66. Prolonged normoglycemia was achieved by transplanting Adv-XIP transfected human islets under the kidney capsule of the MLDS mice56. XIAP (X-linked inhibitor of apoptotic protein) of a potent endogenous inhibitor of downstream effector caspases 3,7 and 9 prevents apoptosis induced by both extrinsic and intrinsic signals57,58.

Emamaulee and her associates transplanted 500 and 250 islets under the kidney capsule in the MLDS mice and control mice59. Those treated with zVAD-fmk (10 mg/kg body weight) MLDS mice from day 0 to 5 posttransplant showed improved blood glucose levels compared with controls59. Ninety percent of zVAD-fmk -treated MLDS mice became euglycemic with 250 islets versus 27% of the control mice59. Short-term zVAD-fmk treatment significantly reduced posttransplant apoptosis in islet graft and thus pan-caspase inhibitor therapy is beneficial in promoting early marginal mass islet engrafting posttransplant59.

Glucose toxicity

To study glucose toxicity as a deleterious effect of chronic hyperglycemia on β-cells in vitro, Federici et al57 cultured 400 isolated human islets per batch for 5 days in a low glucose (5.5 mmol/l) and a high glucose medium (16.6 mmol/l) for studying possible high glucose effects on Bcl-2 family gene expression by reverse transcription polymerase chain reaction57.

1) Bcl-2 was highly expressed in both low and high glucose media and expression did not change between a high and a low glucose condition (Figure 5A)57.

2) however, Bcl-Xl was reduced by 45% in the high glucose cultured islets compared with that of a low glucose condition, supporting that a reduction
in Bcl-xL gene expression was due to high glucose exposure (Figure 5B)27.

3) Bad, Bid and Bik were expressed in low glucose medium at low levels. Bad gene expression was greatly increased with a high glucose medium and Bad RNA level increased 80% as well compared with that of low level glucose (Figure 5B and C)27. Bad gene expression was markedly increased with high glucose medium and so were Bik gene (Figure 5D and E)27. Thus, anti-apoptotic Bcl-2 was unaffected by high glucose but pro-apoptotic genes, Bad, Bid and Bik markedly increased in high glucose cultured islets (Figure 5)27. These data support that chronic high glucose incubation in vitro modulates the balance of pro-apoptotic and anti-apoptotic Bcl proteins toward apoptosis, thus leading to eventual β-cell death27.

Using MIN6N8 cells, which are SV40 transformed insulinoma cells derived from NDO mice, Kim et al extensively studied the effects of chronic hyperglycemia in the tissue culture: when MIN6N8 cells were cultured in different concentrations of glucose for varying time periods: a high glucose (33.3 mmol/l) induced marked genomic DNA fragmentation in a time- and dose-dependent manner and caused a significant increase of TUNEL-positive cells compared to a low glucose (5.5 mmol/l) cultured cells, concomitant cleavage of poly (ADP-ribose) polymerase (RARP) similar to caspase-3 cleavage (Figure 6A)60. Pretreatment with a specific caspase-3 inhibitor (z-DEVD-CHO) completely reduced a high glucose-induced RARP cleavage (Figure 6C)60 and apoptosis. Culturing with a high glucose significantly increased Fas and P53 expression whereas decreased Bcl-2 and Bcl-xL expression (Figure 6B)60. A significant mitochondrial release of cytochrome c into cytosol was observed after 2 days (Figure 6C)60. Translocation of Bax was confirmed by immunostaining: Bax interacted with VDAC (Figure 6E)60 and Bax oligomerization substantially increased 2 days in a high glucose medium (Figure 6F)60. Thus, chronic exposure to a high glucose increased through Bax oligomerization, cytochrome c release and caspase-3 activation, leading to β-cell apoptosis60. Glucose toxicity on β-cell apoptosis was studied with chronic exposure to a high glucose, which markedly reduced glucokinase (GCK, hexokinase IV) expression in a time- and dose-dependent manner61. Immunocytochemistry on tissue culture cells showed that glucose reduces GCK expression dose dependently decreasing by 40% in a high glucose medium. Chronic exposure to a high glucose for 4 days abolished to stimulate insulin content and inhibited ATP production62.

Main molecular studies of apoptotic Bcl proteins had been performed in artificially forced overexpression experiments in vitro62-64. Caspases are activated in a hierarchy order, in which initiator caspases (caspase-8 and -10) function to cleave effector caspases (caspase-3 and -7), the latter in turn degrade intercellular protein substrates and lead to the classical morphological changes of apoptosis (Figure 2)19. Extracellular events present during the inflammatory response through the release of cytokines, including INF-α, IL-1β and interferon-Y by infiltrating leukocytes or direct cytotoxic T-cell engagement, can initiate apoptosis18. These intrinsic cues function via surface molecule in the death receptor pathway, where specific ligand-receptor binding such as TNFα-TNF receptor binding, Fas -Fas L binding leads to receptor clustering, adaptor molecule recruitment and formation of death-inducing signaling complex (DISC) (Figure 2)19. Caspase-8 associates with DISC complex, where it is activated, released and leads to effector activation for caspase-319. Intracellular cues such as DNA damage, hypoxia, nutrient deprivation or reactive oxygen species (ROS) function via the mitochondrial pathway, which is tightly modulated by the Bcl-2 proteins (Figure 6)31,60. In healthy cells, pro-apoptotic Bcl-2 proteins (Bim, Bid, Bad, Bak and Bak 2) are present in the mature form, while anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-xL) are constitutively active and reside in the outer membrane of mitochondria (Figure 6)31,60. Following an intrinsic cue, proapoptotic Bcl proteins become activated and translocate to the mitochondria, where they bind to inactivate anti-apoptotic Bcl-2 proteins or form pores in the mitochondrial membrane, which facilitates the release of cytochrome c into the cytosol (Figure 2)19. When cytochrome c accumulates in the cytosol, it complexes with procaspase-9 and Apaf-1 to form the “apoptosome”, which in turn activates caspase-3 (Figure 2)19,61-63. Both intrinsic and extrinsic signaling cascades converge at the point of caspase-3 activation, which is often considered as the “point of no return” in apoptosis18. Apoptosis can only occur when the concentration of pro-apoptotic Bcl proteins exceeds that of anti-apoptotic proteins at the mitochondrial membrane of the intrinsic pathway27. Using transgenic mice, Daniel el al found: glucose-induced changes in the mitochondrial membrane potential were significantly reduced in Bad−/− β-cells and the average (Ca 2+) I response to 11 mM glucose was significantly lower in Bad−/−β-cells65,66. An intact BH3 domain is required for glucose-stimulated insulin secretion by its binding to Bcl-2 and Bcl-xL. Treatment with BAD SAHBA (stabilized α-helix of Bcl-2 domain) restored the secretion defect in Bad−/− islets, underscoring the sequence specificity of the BAD SAHB effect66. α-helical destabilization of the Bcl-2-BH4 domain abolishes its ability to inhibit the IP3 receptor67. They further identified GCK as a novel and direct physiological target of the BAD BH3 domain in β-cells and that phosphorylation within the BH3 domain drives the metabolic functionality of BAS and severs as a physiological switch of its apoptotic and metabolic effects66.
The early event of β-cell dysfunction includes endoplasmic reticulum stress and oxidative stress leading to β-cell exhaustion and eventual apoptosis. Pancreatic islets represent only 2% of the pancreas but produce the most important hormone for survival, insulin for glucose homeostasis, in which a human adult needs about 40 Unit of insulin (about 13 mg) per day. Endoplasmic reticulum (ER) is an organelle responsible for protein folding and assembly and transport of synthesized protein in cisternae. The ER is well developed in β-cells for synthesizing large amounts of insulin. Rough ER (RER) is in charge of protein synthesis and the newly synthesized proteins are folded into three-dimensional structure in RER and is sent to Golgi complex or membrane via small vesicles. ER stress, the cellular responses to the disturbance of normal function of ER, is the most common cause for protein miss-folding. In T1D, increased insulin synthesis in residual β-cells exceeds the folding capacity of ER, resulting in the accumulation of unfolded protein. The unfolded proteins including insulin, are prone to aggregate with each other in a crowded environment and directed to degradative pathway, which further exacerbate existing T1D.

Thus, In the all intricate cascade of islet apoptosis, the therapeutic application of BAD SAHBs and other BAD SAHB that activates glucose-stimulated insulin secretion, but does not affect the survival function of Bcl-xL, may serve as a prototype therapeutic application in diabetes and islet transplantation.

Conclusion

As mentioned so far, there are a milliard of cascade of biological processes for β-cell destruction by an autoimmune reaction in T1D while β-cells are replaced by non-β-cells at a delicate balance of islet cell apoptosis and regeneration in long time frame. Since other environmental factors are involved for the development of T1D in addition to genetic factors, there are many ways to theoretically and eventually prevent or retard autoimmune reactions in the complicated processes of T1D development. Restore insulin secretion on the remaining non-functioning β-cells in T1D may lead to rejuvenate and activate production and secretion of more endogenous insulin. It is also evident that an adequate glucose homeostasis in T1D is a prudent way to protect the remaining β-cells in T1D subjects against hyperglycemia-induced β-cell apoptosis. There is a future hope for therapeutic application to slow or even prevent β-cells apoptosis pathways in T1D at the molecular level by deleting Casp3-/- to Casp3-/- and silencing Casp3-/- gene or using pan-caspase inhibitor treatment to prevent and slow progressive apoptosis in pancreatic β-cells.

References


34. Cleaved caspase-3 (Asp 175) antibody (2006) vol 9661, Cell Signaling Technology, Beverly, MA, Cat # 9661.


