High glucose causes apoptosis in cultured human pancreatic islets of Langerhans: A potential role for regulation of specific Bcl family genes toward an apoptotic cell death program.

Massimo Federici, Marta Hribal, Lucia Perego, Marco Ranalli, Zaira Caradonna, Carla Perego, Luciana Usellini, Rita Nano, Paolo Bonini, Federico Bertuzzi, Lionel N.J.L. Marlier, Alberto M. Davalli, Orazio Carandente, Antonio E. Pontiroli, Gerry Melino, Piero Marchetti, Renato Lauro, Giorgio Sesti, and Franco Folli

Type 2 diabetes is characterized by insulin resistance and inadequate insulin secretion. In the advanced stages of the disease, β-cell dysfunction worsens and insulin therapy may be necessary to achieve satisfactory metabolic control. Studies in autopsies found decreased β-cell mass in pancreas of people with type 2 diabetes. Apoptosis, a constitutive program of cell death modulated by the Bcl family genes, has been implicated in loss of β-cells in animal models of type 2 diabetes. In this study, we compared the effect of 5 days' culture in high glucose concentration (16.7 mmol/l) versus normal glucose levels (5.5 mmol/l) or hyperosmolar control (mannitol 11 mmol/l plus glucose 5 mmol/l) on the survival of human pancreatic islets. Apoptosis, analyzed by flow cytometry and electron and immunofluorescence microscopy, was increased in islets cultured in high glucose (HG5) as compared with normal glucose (NG5) or hyperosmolar control (NG5+MAN5). We also analyzed by reverse transcriptase–polymerase chain reaction and Western blotting the expression of the Bcl family genes in human islets cultured in normal glucose or high glucose. The antiapoptotic gene Bcl-2 was unaffected by glucose change, whereas Bcl-xl was reduced upon treatment with HG5. On the other hand, proapoptotic genes Bad, Bid, and Bik were overexpressed in the islets maintained in HG5. To define the pancreatic localization of Bcl proteins, we performed confocal immunofluorescence analysis on human pancreas. Bad and Bik were specifically expressed in β-cells, and Bid was also expressed, although at low levels, in the exocrine pancreas. Bik and Bcl-xl were expressed in other endocrine islet cells as well as in the exocrine pancreas. These data suggest that in human islets, high glucose may modulate the balance of proapoptotic and antiapoptotic Bcl proteins toward apoptosis, thus favoring β-cell death. Diabetes 50:1290–1301, 2001

Type 2 diabetes is characterized by a combination of insulin resistance and alterations in β-cell function (1). The latter may be ascribed, to a certain extent, to the deleterious effect of chronic hyperglycemia, a process referred to as glucotoxicity (2). The amount of β-cells in mammalian adults is tightly regulated, being maintained at ~1% of the weight of the pancreas. Several large studies in autopsies have provided evidence that β-cell mass in type 2 diabetes is ~50% of normal (3–5). The remaining β-cells do not seem capable of secreting as much insulin as normal β-cells to maintain patients in an euglycemic state (5).

Hyperglycemia and metabolic control have been virtually always associated with defects in β-cell function in both type 2 and type 1 diabetes (6–10). Similar abnormalities have been observed in animal models of diabetes, especially rodents, though evidence exists to favor species-specific differences in the regulation of β-cell mass (11,12).

Evidence for a link between hyperglycemia and apoptosis comes from studies in animal models (13–18). Apoptosis is a tightly regulated physiological process, triggered by a variety of metabolic or cytokine-dependent stimuli, consisting in a constitutive program that leads to cell death. Apoptosis manifests in two major execution programs downstream of the death signal: the caspase pathway as well as mitochondrial dysfunction (19). Upstream of irreversible cellular damage reside the Bcl family members, which are proteins with both proapoptotic and antiapoptotic properties that play a pivotal role in whether...
a cell lives or dies. Transcriptional-dependent apoptosis requires the upregulation of death genes or the downregulation of survival genes (20). Interestingly, in mice heterozygous for null alleles of IGF and insulin receptor substrate-2, a novel animal model of type 2 diabetes, the development of hyperglycemia is associated with pancreatic β-cell failure and apoptosis in pancreatic islets (21).

Few studies have investigated the relationship between glucose level and the development of apoptosis in islets of Langerhans (22,23). Despite some evidence for glucose toxicity leading to cell death (19,24), the molecular mechanisms underlying this phenomenon are still unclear.

In this study, we tested the hypothesis that high glucose concentrations adversely affect survival of cultured human pancreatic islets through the modulation of Bcl gene expression. We show that high glucose causes apoptosis of human pancreatic islet cells in vitro, possibly by unbalancing the expression of several Bcl family members.

RESEARCH DESIGN AND METHODS

Subjects and islet culture. Human pancreatic islet cells were obtained by collagenase digestion and density-gradient purification from pancreata of living multirgan donors as previously described (25,26). Islet preparations with a purity >70%, which could not be transplanted into human recipients for low yield, thus adversely affecting their diabetes-curing potential in vivo (27), were used in this study, after approval by the local ethical committees. Some 10,000 purified islets from six preparations were incubated in M199 containing 10% fetal bovine serum (FBS) for polymerase chain reaction (PCR) studies in the presence of 5.5 mmol/l glucose for 1 day (normal glucose 1 [NG1]) or 5 days (normal glucose 5 [NG5]), or in the presence of glucose 16.7 mmol/l for 5 days (high glucose [HG5]). Some 10,000 purified islets from 4 preparations were cultured in NG1, NG5, and HG5 and analyzed for apoptosis by flow cytometry and fluorescence microscopy; 10,000 purified islets from 3 preparations were cultured in NG1, NG5, and HG5 and analyzed for apoptosis by flow cytometry and fluorescence microscopy; all incubations were performed at 37°C/5% CO2.

Insulin and proinsulin secretion. Three preparations of human pancreatic islet cells were cultured for 5 days in presence of 5.5 mmol/l glucose (NG5), 16.5 mmol/l glucose (HG5), or 5.5 mmol/l glucose plus 11 mmol/l mannitol (NG5 + MAN5) in M199 containing 10% FBS. All incubations were performed at 37°C/5% CO2. After 5 days, the supernatants were collected and stored. The islets were washed two times with M199 without FBS, and incubated with M199 containing 0.5% FBS for 2 h at 37°C. At the end of the incubations, the supernatants were collected and stored. Then, the islets were incubated with M199 containing 0.5% FBS, 30 mmol/l glucose, and 0.5 mmol/l isobutylmethylxanthine (IBMX) for 30 min. After the incubation, the supernatants were collected, and the islets were lysed with 150 mmol/l NaCl, 20 mmol/l Tris, pH 7.5, 1% Triton X-100, 1% aprotinin, 1 mmol/l phenylmethylsulfonfluoride (PMSF), and 1 mmol/l Benzanilide. Protein concentrations were quantified with Bio-Rad Protein Protein assay. Insulin and pro-insulin were assayed in both supernatants and islet lysates by RadioImmunoAssay (Linco Research, St. Louis, MO).

Evaluation of apoptosis by flow cytometry and immunofluorescence. At the end of culture under NG5, HG5, or NG5 + MAN5 conditions, single-cell suspensions were obtained by gently dissociating equal amounts of size-matched islets (400 islets each experimental point from seven different preparations for NG5 and HG5, and three preparations for NG5 + MAN5) with 3 mmol/l EGTA, 20 mg/ml bovine serum albumin (BSA), 2.8 mmol/l glucose, and 0.83 μg/ml trypsin (25). Thereafter, cells were fixed in a methanol-aceton solution (4:1) as described (26,29). The percentage of cells undergoing apoptosis was quantified in fixed cells after staining with hypotonic fluorochrome solution (50 μg/ml propidium iodide solution and RNase [13 kU/ml]) (Sigma, St. Louis, MO.). To count the number of hypodiploid events, cells were analyzed with use of a flow cytometer (FacsScallibur; Becton Dickinson, Mountain View, CA) using CellQuest software (Becton Dickinson). This method to assess apoptosis has been evaluated in different cell types, including human pancreatic islet cells (26). To visualize the basis of fluorescence emission of DNA fragments (26,28–31). Each experimental point was assessed twice by flow cytometry and intrasample variation was <1% in each sample.

In another set of experiments, islets from four preparations, treated for 4 days under normal glucose or high glucose conditions, were analyzed as described above and allowed to attach on poly-x-lysine–coated Lab-Tek slides (Nunc, Rochester, NY) overnight at 37°C/7% CO2. Some 106 cells were plated in each well. After a further 24 h of treatment under normal or high glucose conditions, dissociated cells were fixed and permeabilized as described (28). Apoptosis was assessed by co-immunostaining with terminal deoxynucleotidyl-mediated dUTP nick end-labeling (TUNEL) (Boehringer, Indianapolis, IN). Human β-cells were immunostained using mouse anti-human insulin (Linco Research), and detected using goat anti–mouse pig phycocerythrin-conjugated antibody (Sigma). The number of apoptotic cells was calculated by counting the number of TUNEL-positive cells in each field of view. Data were expressed as the mean of two to three independent experiments; each experiment was repeated at least three times. Statistical significance was determined by One-Way ANOVA with Bonferroni post hoc test.

RESULTS

Insulin and proinsulin secretion. In another set of experiments, islets from four preparations, treated for 4 days under normal glucose or high glucose conditions, were fixed for 2 h in 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer followed by osmium tetroxide, dehydrated in graded ethanol up to propylene oxide, and finally embedded in an Epon-Araldite mixture, and in Spurr resin. Next, 80-mm ultrathin sections were mounted on copper grids, incubated with mouse monoclonal anti-human insulin antibody diluted 1:10 in phosphate-buffered saline (PBS) and 1% BSA. Then, the grids were incubated with goat anti–mouse IgG coated with 10-nmol colloidal gold particles. Finally, the sections were rinsed with PBS and distilled water, fixed in 2.5% glutaraldehyde, and stained with uranyl acetate and lead citrate. Apoptosis of endocrine cells was evaluated on the basis of ultrastructural characteristics, as previously described (33,34).

Immunoelectron microscopy was carried out on 4% paraformaldehyde-fixed, London-Rhesus-White embedded islets that had been cultured for 5 days under normal or high glucose conditions. Islet ultrathin sections were mounted on copper grids, incubated with normal goat serum, and then incubated with rabbit anti–human insulin antibody diluted 1:10 in phosphate-buffered saline (PBS) and 1% BSA. Then, the grids were incubated with goat anti–mouse IgG coated with 10-nmol colloidal gold particles. Finally, the sections were rinsed with PBS and distilled water, fixed in 2.5% glutaraldehyde, and stained with uranyl acetate (35). All experiments were examined with a Philips electron microscope.

Bel family mRNA analysis by semiquantitative reverse transcriptase–polymerase chain reaction. At the end of incubations in 5.5 mmol/l glucose for 1 day (NG1) or 5 days (NG5), or in the presence of glucose 16.7 mmol/l (HG5), total RNA was extracted from 10,000 islets with RNAzol B method (Biogenesis, Bournemouth, U.K.), a modification of the single-step method (36). Reverse transcriptase (RT) was performed on 1 μg total RNA using 5 U of Moloney murine leukemia virus RT (Life Technologies) and 10 mmol/l dithiothreitol, 0.5 mmol/l dNTPs and 10 μg/ml RNase-free DNase. The final mixture was heated for 5 min at 85°C. Total reaction volume was 20 μl in a buffer containing 50 mmol/l Tris-HCl (pH 8.3), 7.5 mmol/l KCl, 10 mmol/l dithiothreitol, and 3 mmol/l MgCl2. The first-strand cDNA was diluted to 200 μl, and 1 μl of the product was used in each PCR in a final volume of 50 μl. PCR assays were performed using the PCR SuperMix High Fidelity (Life Technologies) and a titration between 28 and 46 cycles of RT-PCR products revealed that 40 cycles was optimal condition. Sequences of the oligonucleotides used in the RT-PCR analysis were as follows: Bad forward primer 5′-CCAGATCCAGAGTTTGAGC; Bad reverse primer 5′-TCCCTGCTCCCAAGTTTGGC; Bcl-2 forward primer 5′-TATAAAGCTGTGATTCTGAGGGG; and Bcl-2 reverse primer 5′-CTCTCCACACACTAGTCC; Bid forward primer 5′-ACAGAAGGGGCTGTTG; Bid reverse primer 5′-GCCAGCAGGAAGAAAAGATTGCG; Bcl-xL forward primer 5′-GGGCGGTTTCTTCTCCAG; Bcl-xL reverse primer 5′-CGGGCTTCTCCACCTCCTCC; Bik forward primer 5′-CGGGCTTCTCCACCTCCTCC; Bik reverse primer 5′-CGGGCTTCTCCACCTCCTCC; Bik forward primer 5′-CGCCAGAGGAAGAAAAGATTGCG; and Bik reverse primer 5′-GCCAGCAGGAAGAAAAGATTGCG. PCR assays were performed using the FluoroImager (Bio-Rad, Richmond, CA) and the Image-
FIG. 1. A–E: Validation of RT-PCR for amplification of Bcl and housekeeping genes in human islets cultured in high glucose versus normal glucose. Cycle titration curves of RT-PCR amplification for housekeeping genes, cyclophyllin (A) and α-tubulin (B), and Bcl family genes, Bcl-xL (C), Bad (D), and Bid (E), in human islets treated under normal glucose (NG5, ●) or high glucose (HG5, ○) conditions. Means ± SD of two separate experiments of relative expression of the genes are shown in graph.
Quant program to quantify ethidium bromide signal. Cyclophilin and α-tubulin, well-known housekeeping genes in pancreatic islets, were used to normalize the template in each reaction (37). The results were expressed as mRNA levels relative to NG1 control islets, which were chosen as basal value to normalize the template in each reaction (37). The results were expressed as fold changes relative to NG1 control islets (38,39). The filters were blocked with Tris-buffered saline (TBS) 1× containing 5% nonfat milk, and incubated with the primary antibodies overnight at 4°C. Antibodies against Bad, Bid, and Bcl-x were from Santa Cruz.

After extensive washes in TBS 1×, filters were incubated for 1 h at room temperature with the appropriate horseradish peroxidase–conjugated secondary antibodies. Bound antibodies were visualized using chemiluminescence substrate (Enhanced Chemiluminescence System) according to the manufacturer’s instructions (Amersham Life Sciences, Little Chalfont, U.K.).

**RESULTS**

High glucose causes apoptosis of human pancreatic islets. To investigate whether a high glucose environment affects survival of islet cells, we took advantage of an opportunity to analyze pancreatic islets isolated from human donors. Flow cytometry analysis revealed that human islets cultured for 5 days in high glucose (HG5) showed higher apoptotic events as compared with both islets cultured at normal glucose concentration (NG5) or in appropriate hyperosmolar control (40.1 ± 3.6, 22.5 ± 3.2, and 24.2 ± 2.0% for HG5, NG5, and NG5+MAN5, respectively; P < 0.01 for HG5 vs. NG5 and P < 0.05 for HG5 vs. NG5+MAN5, respectively, by ANOVA) (Fig. 2A). To evaluate whether increased apoptosis induced by high glucose affected β-cells within human pancreatic islets, immunofluorescence analysis of dissociated islet cells was carried out in HG5- versus NG5-treated islets. Apoptosis was assessed by TUNEL assay to identify DNA fragmentation, and β-cells were identified using specific anti-insulin antibodies. Merging of TUNEL- and insulin-positive signal revealed increased β-cells apoptosis in HG5-treated samples (48.1 ± 8.5 and 20.2 ± 5% in HG5 and NG5, respectively, P < 0.05, by ANOVA) (Fig. 2B); non-β-cell apoptosis showed a tendency to increase in HG5 versus NG5, although this finding was not statistically significant (40.0 ± 19.0 and 24.0 ± 12.0 in HG5 and NG5, respectively, P = 0.2). Insulin (red) and DNA fragmentation (green) specific fluorescence analysis at confocal microscope revealed intense apoptosis in β-cells treated in HG5 with respect to NG5 (Fig. 2C–H). By electron microscopy, NG5 islet preparations contained well-granulated β-cells (Fig. 3A and B), whereas HG5 β-cells were almost devoid of insulin-containing granules (Fig. 3C), and showed apoptosis features (Fig. 3D).

Insulin and proinsulin secretion and content were analyzed in islets cultured in NG5, HG5, and NG5+MAN5 (Table 1). Chronic insulin release was increased by threefold in HG5 as compared with NG5 and NG5+MAN5 treatments (P < 0.01). However, insulin release stimulated by 30 mmol/l glucose + 5 mmol/l IBMX was reduced by four- to fivefold in HG5-treated islets as compared with NG5 and NG5+MAN5 islets (P < 0.01). Insulin content in islets after 5 days of culture was greatly reduced in HG5 with respect to NG5 and NG5+MAN5 (P < 0.01). Proinsulin release and content were significantly different in HG5 with respect to NG5 and NG5+MAN5 treatments (P < 0.01).

Regulation by high glucose of Bcl gene transcripts in cultured islets. Glucose is a well-known modulator of gene expression, and recently published articles suggest a role for glucose toxicity in inducing apoptosis in embryos (18,24). Triggering of early phases of apoptosis program is dependent on reciprocal interactions between Bcl family proteins. Therefore, we investigated the possibility that high glucose concentration in the diabetic range (HG5) could modulate Bcl family gene expression in human pancreatic islets compared with physiological fasting glucose levels (NG5) by altering the ratio between anti-apoptotic versus proapoptotic genes. Since there is very little information on expression of Bcl family genes in human pancreatic islets, we first looked for a panel of both antiapotptotic and proapoptotic Bcl genes by RT-PCR. We compared the expression levels in NG1, NG5, and HG5 islets for each Bcl gene. Bcl-2 was highly expressed in NG1 islets, and its expression did not change after 5 days of culture with respect to NG5 and NG5+MAN5 (P < 0.01) (Fig. 4B). No differences were observed in Bcl-xl mRNA levels from islets cultured in NG1 or NG5, indicating that the reduction in Bcl-xl expression observed in HG5 islets was due to glucose exposure rather than the duration of culture (Fig. 4B). Bcl-xl protein levels decreased to 60% in islets cultured in HG5 versus NG5 conditions (Fig. 4B).

Next, we studied whether in the context of enhanced apoptosis, expression of Bad, Bid, and Bik proapoptotic genes is modulated by high glucose conditions. Bad, Bid, and Bik are expressed in NG1 islets at low levels. Bad was slightly, but not significantly, increased in islets under NG5 conditions. Bad expression greatly increased after culture under HG5 conditions (61 ± 12%, P < 0.01) (Fig. 4C). Bad protein levels increased (80%) in islets cultured in HG5 versus NG5 conditions (Fig. 4C). Expression of Bid was only modestly increased in NG5 cultured islets, but its expression markedly increased when islets were cultured...
under HG5 conditions (300 ± 98%, P < 0.001) (Fig. 4D). Bid protein levels increased (160%) in islets cultured under HG5 versus NG5 conditions (Fig. 4D). Expression of Bik was low in both NG1 and NG5 cultured islets, and significantly increased in HG5-treated islets (40 ± 6%, P < 0.01) (Fig. 4E).

**Immunolocalization of proapoptotic and antiapoptotic genes.** To evaluate whether there was a specific expression pattern of the Bcl genes in endocrine pancreas, we investigated expression of the proapoptotic genes Bad, Bid, and Bik, and the antiapoptotic gene Bcl-xl in human pancreatic sections. To this end, double-immunofluorescence analysis was performed using specific antibodies recognizing the four Bcl family proteins. An anti-insulin antibody was used to localize islets of Langerhans in the context of pancreas structure. Bcl-2 distribution was not analyzed in this study, because its pancreatic localization has been previously reported to be present in both the endocrine and the exocrine component (41). The staining with an antibody against Bid (Fig. 5A) indicated that this
protein is expressed in both endocrine and exocrine cells with a prevalent pattern expression in endocrine cells (as revealed by the similar Bid and insulin staining in the islets of Langerhans) (Fig. 5B). Bad protein appeared to be almost exclusively located in endocrine β-cells as demonstrated by double staining with an insulin antibody (Fig. 5C and D). However, Bik (Fig. 5E) and Bcl-xl (Fig. 5G) were expressed in both exocrine and endocrine cells, as demonstrated by double staining with an insulin antibody (Fig. 5F and H).

These experiments suggested that Bad and Bid were predominantly expressed in endocrine β-cells, whereas Bik and Bcl-xl had a broader expression within the pancreas. Since islets are composed of at least four different cell types (α, β, δ, and PP), we investigated whether the apoptotic Bcl genes were expressed in a specific subset of

**TABLE 1**
Insulin and proinsulin secretion and content of human islets of Langerhans cultured in NG5, HG5, and NG5+MAN5

<table>
<thead>
<tr>
<th></th>
<th>NG5</th>
<th>HG5</th>
<th>NG5+MAN5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic insulin release (nmol/l/mg/day)</td>
<td>925.50 ± 129.10*</td>
<td>2,843.00 ± 337.02</td>
<td>1,060.40 ± 186.50*</td>
</tr>
<tr>
<td>Basal insulin secretion (nmol/l/mg/min)</td>
<td>0.0574 ± 0.007</td>
<td>0.043 ± 0.007</td>
<td>0.064 ± 0.004</td>
</tr>
<tr>
<td>Stimulated insulin release (nmol/l/mg/min)</td>
<td>0.49 ± 0.035†</td>
<td>0.129 ± 0.035</td>
<td>0.68 ± 0.07†</td>
</tr>
<tr>
<td>Insulin content (nmol/l/mg/min)</td>
<td>101 ± 25§</td>
<td>23.9 ± 5.7§</td>
<td>284.1 ± 16.7§</td>
</tr>
</tbody>
</table>
| Chronic proinsulin release (nmol/l/mg/day)  | 0.26 ± 0.14||\n| Proinsulin content (nmol/l/mg/min)          | 15.10 ± 0.02|| \n
Data are means ± SD of three separate experiments in three different human islet preparations and analyzed by ANOVA.

*NG5 vs. HG5 and NG5+MAN5 vs. HG5 (P < 0.01); †NG5 vs. HG5 and NG5+MAN5 vs. HG5 (P < 0.01); ‡NG5 vs. HG5, NG5+MAN5 vs. HG5, NG5 vs. NG5+MAN5 (P < 0.01); §NG5 vs. HG5 and NG5+MAN5 vs. HG5 (P < 0.001); ||NG5 vs. HG5, NG5+MAN5 vs. HG5, NG5 vs. NG5+MAN5 (P < 0.001).
FIG. 4. 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.
FIG. 5. A-H: Immunofluorescence analysis of Bcl family genes in normal human pancreas. Insulin-positive signal was used as a marker of islets of Langerhans. Sections were stained with the following antibodies: anti-Bid (A); anti-Bad (C); anti-Bik (E); and anti–Bcl-xl (G). The same sections were double stained with anti-insulin antibodies to identify islets of Langerhans (B, D, F, and H). Note the robust expression of Bid and Bad in islets, and expression of Bik and Bcl-xl in both endocrine and exocrine cells (original magnification 150×).
cells. To this end, double-immunofluorescence confocal analysis was performed using anti-insulin antibodies as a marker for β-cells, antiglucagon as a marker for α-cells, and antichromogranin as a general marker for endocrine cells. Double staining with the anti-insulin, and anti-Bid or -Bad antibodies revealed an almost complete colocalization between the two signals (yellow-orange signal in Fig. 6C and F), demonstrating that both Bid and Bad proteins were expressed in β-cells. However, the Bik and Bcl-xl staining showed a different pattern of expression in islets with respect to insulin signal due to lower level of expression in β-cells (data not shown). To further define Bik- and Bcl-xl–specific cellular localization, we performed double-staining analysis with antiglucagon and antichromogranin antibodies. We observed a partial colocalization of Bik with glucagon, indicating expression of this gene in α-cells, and a partial colocalization of Bcl-xl with chromogranin, indicating expression of Bcl-xl in endocrine cells of islets other than β-cells (yellow-orange signal in Fig. 6J and N).

**DISCUSSION**

In this study, we addressed the question of whether high glucose affects human islet cell survival in vitro. We found that human islets cultured for 5 days in high glucose concentration within the diabetic range show an increased cell death compared with islets cultured in normal glucose concentration and islets cultured in hyperosmotic medium. We observed that increase in cell death was due to increased apoptosis, a pathophysiological process tightly regulated during embryonic and adult life (42,43). Excessive cell death through apoptosis or necrosis may contribute to acute organ failure as well as chronic diseases involving the loss of postmitotic cells (44). Several studies in the past decade have shown that chronic elevation of blood glucose concentrations in both humans and experimental animal models leads to β-cell dysfunction in terms of insulin secretion and insulin synthesis (45–47). Both in vivo and in vitro studies on rodent islets have suggested that hyperglycemia is associated with features of apoptosis when the architecture of the islet is maintained (11–17). However, due to the intrinsic rarity of pancreatic specimens, no studies have analyzed the effect of high glucose on human islet survival. To reproduce in vitro conditions resembling the diabetic milieu, we analyzed the effects of glucose toxicity on entire islets rather than isolated β-cells. The latter approach might be useful to analyze cell-specific mechanisms of damage, but using entire islets mirrors “in vivo” conditions more closely than studying single purified cells or cultured insulinoma cells (48). A major difficulty in assessing apoptosis lies in the necessity to use a quantitative assay. Flow cytometry combined with propidium iodide staining of fixed cells is a quantitative methodology because it allows estimation of both apoptosis and secondary necrosis through the identification of hypodiploid events, a feature absent in necrosis (26–30). Electron or fluorescence microscopy might help to identify structural features of apoptosis, such as nuclei degeneration, chromatin aggregation, or even cell-specific apoptotic events when multicellular structures are under study, as in the case of islets of Langerhans. We have identified an increased apoptosis in the islets exposed to high glucose concentrations, which appeared to involve the β-cells as confirmed by both electron microscopy and immunofluorescence studies. Our results showing increased apoptosis in human islets exposed to diabetes-like glucose concentrations (16.7 mmol/l) are in agreement with those observed in animal models of type 2 diabetes, such as Psammomys Obesus and Goto-Kakizaki rats in which circulating glucose concentrations were comparable to those of the present study (11,15). By contrast, a study has reported that exposure of rat islets to high glucose in the absence of serum resulted in cell survival (22). Another study has observed that islets from Ob/Ob mice, an animal model characterized by insulin resistance, survived better at 11 than 5 mmol/l glucose, but apoptosis occurred when islets were exposed to 17 and 27 mmol/l glucose (23). The disparities between our study and previous observations could be due to differences between species in the source of islets (i.e., human islets in our study versus murine islets in other studies), differences in culture conditions (i.e., culture in the presence or absence of serum), or differences in other unknown variables. Interestingly, it has been suggested that human islets are more susceptible than rodent islets to glucose-induced cellular dysfunction (12). For instance, noncurative amounts of human islets transplanted in diabetic nude mice, thereby exposed to hyperglycemia, are characterized by a severe reduction in graft insulin content (49). Moreover, β-cell mass progressively falls when hyperglycemia persists after islet transplantation, and normalization of plasma glycemia induced by insulin treatment has a beneficial effect on the outcome of transplanted islets to diabetic mice (50–52). However, these studies have not analyzed apoptotic response to metabolic derangement. Interestingly, electron microscopy analysis of islets cultured under NG5 or HG5 conditions allowed us to identify several endocrine cells that appeared almost totally degranulated after exposure to high glucose. These data strengthen the concept that hyperglycemia might direct dysfunctional cells to develop an apoptotic pattern.

Our results show that high glucose–induced apoptosis is associated with the modulation of several genes belonging to the Bcl family. Activation of Bcl-like proapoptotic proteins represents the no-return point in both the extrinsic and intrinsic apoptotic pathways (19,42–44). The members of the Bcl family are divided into two subclasses: some of them possess antiapoptotic function, whereas the others, all carrying the so-called BH3 domain, exert proapoptotic function (19,42–44). It is known that Bcl proteins may be regulated by mechanisms beyond posttranslation modification or conformational changes. In fact, it has been suggested that constitutively active proapoptotic Bcl members may be transcriptionally regulated. To avoid toxicity in healthy cells, they would be transcriptionally silent or expressed at very low levels, but in response to selected death stimuli, cells would initiate their transcription (44). Evidence exists that two proapoptotic molecules, EGL-1 in *C. elegans* and Hrk in human cells, are upregulated in response to death stimuli (20,53). Moreover, the antiapoptotic genes Bcl-2 and Bcl-xl are transcriptionally responsive (20). Our observation of increased expression of the proapoptotic BH3-only members of the Bcl family such as Bad, Bid, or Bik in the context of increased apoptosis might imply that hyperglycemia could cause apoptosis.
FIG. 6. A–N: Immunofluorescence analysis of Bcl family genes in human islets of Langerhans. Human pancreatic sections were stained with the following antibodies: anti-Bid (A); anti-Bad (E); anti-Bik (G); anti–Bcl-xl (L); anti-insulin (B and D); antiglucagon (H); and antichromogranin (M). Note the expression of proteins in the cytoplasmic compartment of cells. Merged confocal sections showed almost complete colocalization of Bid and Bad with insulin (C and F, respectively), a partial colocalization of Bik with glucagon (I), and a partial colocalization of Bcl-xl with chromogranin (N) (original magnification 330×).
through the modulation of key genes in the cell-death constitutive program. Imbalance in the expression of the proapoptotic Bad versus the antiapoptotic Bcl-xl is known to lead to alteration of the mitochondrial homeostasis, and subsequent activation of irreversible steps of apoptosis, as Caspase-9 and Caspase-3 processing (19,44). Interestingly, we also demonstrate a decrease in Bcl-xl expression, although Bcl-xl appears to be expressed at very low levels in the islets and colocalizes predominantly with chromogranin-positive cells rather than with β-cells (data not shown). It has been shown that small variations in Bcl-xl levels might influence β-cell function and survival. However, only very low levels of Bcl-xl have been found in mouse islets by immunocytochemistry (54). Nevertheless, it is possible that Bcl-xl plays a minor role in β-cell survival pathways.

Our results obtained by confocal fluorescence microscopy analysis revealed that Bad and Bid are specifically localized in β-cells, with Bid being expressed at low levels also in the exocrine pancreas. Recent observations have led to the hypothesis that Bad is expressed in islets (55), but this is the first demonstration of a β-cell-specific expression pattern in human islets. In a genetic model of type 2 diabetes characterized by β-cell failure and hyperglycemia (the Irs2−/− and Irs2−/−/Igf-I−/− knockout mice), Bad was found to be overexpressed when mice developed hyperglycemia (21). The phosphatidylinositol 3-kinase/Akt survival pathway, which is reduced by glucose toxicity in other tissues (56), is crucial in the control of Bad activity (26,56). Thus, it is intriguing to hypothesize glucose-toxic damage acting on both transcriptional and posttranslational level of Bad activity. Embryos from mice carrying a null mutation for Bax, a proapoptotic member of the Bcl family, show resistance to hyperglycemia-induced apoptosis, whereas in wild-type mice, high glucose induces an increase in Bax expression, which correlates with apoptosis (24).

Our finding of specific Bid expression in β-cells is attractive since Bid, implicated in apoptosis through alteration of mitochondrial membrane potential, is activated by the Fas/FasL dyad, a main pathway involved in generation of mitochondrial membrane potential, is activated by attractive since Bid, implicated in apoptosis through alteration (24).

In summary, our study provides phenomenological evidence for increased apoptosis in human islets cultured in hyperglycemia, to a level mirroring somewhat overt diabetes. Our results suggest a correlation between the induction of apoptosis and the modulation of Bcl family gene expression to favor cell death. Future studies should gain further insight into the function and regulation of these genes in β-cell death, to provide plausible targets for novel pharmacological β-cell–sparing approaches.

ACKNOWLEDGMENTS
The authors were supported by the following grants and institutions: TELETHON Grant E.739 and Ministero della Sanità 2000 (F.F.); European community no. QLG1-CT-1999-00674 (G.S.); a grant of Progetto di Ricerca Finalizzata RF98.49 from Ministero della Sanità (M.F.); a grant of Progetto di Ricerca Finalizzata 98.8 from Ministero della Sanità (L.N.J.L.M.); Progetto di Ricerca Finalizzata RF98.34 from Ministero della Sanità, Cofin MURST grants 1999 (R.L.); Cofin MURST grants 1999 (O.C.); TELETHON Grant A.118 (A.M.D.); a postdoctoral fellowship by the University of Milan (L.P.); and a postdoctoral fellowship by European community no. QLG1-CT-1999-00674 (M.L.H.).

We thank Drs. Giacomo Dellantonio, Francesca Sutti, and Elena Dalcin for help with preliminary experiments; Prof. Carlo Capella (Department of Surgical Pathology, Università dell’Insubria, Varese, Italy) for reviewing electron microscopy data; and Dr. Tara J. Zoli-Folli for English editing.

REFERENCES

1300 DIABETES, VOL. 50, JUNE 2001

Pharmacology


