Human Islets of Langerhans Express Fas Ligand and Undergo Apoptosis in Response to Interleukin-1β and Fas Ligation

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IDDM results from a progressive loss of pancreatic β-cells that, in humans, may be triggered by a combination of genetic and environmental factors. Recently, attention has been focused on the hypothesis that the loss of β-cells is initiated by inappropriate induction of apoptosis. We now demonstrate that human islets of Langerhans undergo apoptosis upon exposure to interleukin-1β. The cytokine also sharply increases the number of cells that enter apoptosis on treatment with a stimulatory anti-Fas antibody. Western blotting and immunocytochemistry clearly show for the first time that human pancreatic β-cells normally express Fas ligand. The results suggest that human islet cells are primed to undergo apoptosis by interleukin-1β and that this involves the close association between cell-surface Fas and its ligand. ***Diabetes*** **47:727–732, 1998**

IDDM is caused by insulin deficiency resulting from loss of β-cells in pancreatic islets of Langerhans. This loss of endocrine cells occurs as the final result of complex interactions between inherited and environmental factors, many of which have yet to be defined (1–5). Animal model systems, such as the nonobese diabetic (NOD) mouse (6–8), have provided invaluable information on the likely mechanisms of disease pathogenesis, but such models cannot fully substitute for the use of human material because it is clear that human and rodent islets differ in their sensitivity to agents which induce diabetes (9,10).

Over the past decade, there has been a growing awareness that, under physiological or near-physiological conditions, cell death in vivo most often occurs through the process of cellular self-destruction known as apoptosis (11–13). This has provided a new conceptual framework for understanding the development of IDDM: the β-cell, rather than simply being the passive victim of external killing mechanisms, may, at critical stages, play an active role in its own demise. In this context, there is already evidence that β-cell death leading to IDDM may involve inappropriate triggering of cell-type specific signaling mechanisms that normally control apoptosis (14), such as those involving cell-surface receptors for cytokines or the Fas/tumor necrosis factor receptor (15,16). Inflammatory cytokines in general, and interleukin-1β (IL-1β) in particular, are likely to play an important role in the development of IDDM (1,17). IL-1β has been shown to induce death in mammalian islets and pancreatic β-cell (10,17) and to upregulate Fas in mouse and human islets (18,19). Moreover, Fas has recently been implicated directly in autoimmune diabetes in the mouse (7,20), as well as in the development of Hashimoto's thyroiditis in man (21). In the former case, Chervonsky et al. (7) and Itoh et al. (20) have both reported that NOD mice that are genetically deficient in Fas (i.e., $NOD^{lpr/lpr}$ mice) are completely protected against the development of diabetes, in marked contrast to their Fas-sufficient littermates. Two groups (7,22) have also produced transgenic mouse strains that overexpress Fas ligand (FasL) in islets and demonstrated that some of these show dramatically accelerated development of diabetes, providing further evidence for a central role of interactions between Fas and FasL in the pathogenesis of diabetes in the NOD model.

Although the expression of Fas by islet cells has now been documented in rodents (7,19) and humans (18), the presence of FasL in human islets has not been reported. Indeed, Giordano and coworkers (21,23) failed to detect FasL in normal human islets. Here we report that normal human islet cells express substantial quantities of FasL. In addition, we have found that treatment with IL-1β induces apoptosis in human islets when used alone and produces a further significant increase in apoptosis when used with anti-Fas antibody. These observations indicate that apoptosis induced by ligation of specific cell-surface receptors may play a critical role in the β-cell loss that results in IDDM in humans.

RESEARCH DESIGN AND METHODS

Human islet culture. A total of 15 batches of human islets were used in this study. All were isolated from heart-beating cadaver organ donors by collagenase digestion followed by separation on gradients of bovine serum albumin, as described in detail previously (24). The islets were maintained in culture in RPMI 1640 medium (Gibco) supplemented with L-glutamine (300 μg/ml), sodium penicillin G (100 IU/ml), streptomycin sulfate (100 μg/ml), and 5% fetal calf serum. They were incubated at 37°C in an atmosphere of 95% air/5% CO_2 with 100% humidity. Islets were exposed to IL-1β (2 pg/μl) for periods of up to 96 h; in experiments where islets were also treated with additional reagents, these were introduced 1–2 h before the cytokine. Batches of islets were routinely tested for insulin secretory activity and, in all cases, exhibited a three- to sixfold increase in insulin secretion when the glucose concentration was raised from 4 to 20 mmol/l. The purity of the islet preparations varied between 70 and 95% as judged by dithizone staining.

Western blotting. Lysates were prepared from human islet samples and cultured Jurkat cells by incubation in lysis buffer (20 mmol/l Tris, 20 mmol/l NaCl, 0.2% Tri-

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FasL, Fas ligand; IL, interleukin; PVDF, polyvinylidene difluoride; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

ton-X100, 2 mmol/l phenylmethylsulphonyl fluoride, 1 µl/ml aprotinin, pH 8.2) for 1 h at 4°C. Samples were then centrifuged at 12,000*g* for 10 min at 4°C, and the protein content of the supernatant was estimated using the bicinchoninic acid method (Pierce Chemicals). Fifty micrograms of protein per sample was electrophoresed on SDS-PAGE minigels and electroblotted onto PVDF (polyvinylidene difluoride) membrane. FasL was detected using a monoclonal mouse anti-human FasL antibody (Transduction Laboratories) and an anti-mouse alkaline phosphatase-conjugated secondary antibody (Sigma) and developed using the chemiluminescent substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.1.^{3,7}] decan}-4-yl) phenyl phosphate (CSPD) Tropix Westernlight.

Immunohistochemistry. Immunohistochemistry was performed on sections of paraformaldehyde-fixed, paraffin-embedded human pancreas. After dewaxing and serial alcohol rehydration, sections were permeabilized with methanol (2 min), and then washed (4 × 1 min) with phosphate-buffered saline and blocked for 30 min at room temperature (10% goat serum in phosphate-buffered saline, pH 7.2), then incubated for 2 h at 37°C with primary antibodies (mouse anti-FasL [1:100] and guinea-pig anti-insulin [1:30]) diluted in blocking buffer. Sections were washed three times in blocking buffer before the addition of anti-mouse fluorescein and anti–guinea-pig rhodamine-conjugated secondary antibodies (1:60 in blocking buffer; Sigma) for a further 2 h at 37°C. Sections were viewed under fluorescence illumination to demonstrate rhodamine and fluorescein staining. In some experiments, colorimetric detection of FasL expression was also achieved using immunoperoxidase to visualize the protein. In all cases, controls lacking primary antibody or containing a primary antibody raised against an irrelevant protein were negative.

Quantification of apoptosis. Islets were harvested from the tissue culture plates and dispersed by trypsin digestion followed by brief centrifugation (500*g*/3 min). The extent of apoptosis was determined by fluorescence microscopy after staining a sample of the cells with acridine orange (10 µg/ml in phosphate-buffered saline). One thousand cells per sample were counted, and the number of intact cells displaying the condensed and fragmented nuclear morphology characteristic of apoptosis was quantified and expressed as a percentage of the total.

Statistical analysis. In all experiments, the statistical significance of differences between experimental conditions was determined by analysis of variance. Results were considered significant when $P < 0.05$.

RESULTS

Probing of human islet proteins with an anti-FasL monoclonal antibody revealed the presence of a single immunoreactive band of apparent molecular weight 37 kDa (Fig. 1). This comigrated with a single immunoreactive protein present in human Jurkat leukemic cells used as a positive control for FasL (Fig. 1). FasL was expressed in all islet samples analyzed and was not affected significantly by the length of time in culture (up to 96 h) or by the presence of IL-1β (Fig. 1, *lanes B–E*). We analyzed human islets from six separate donors for the presence of FasL, and all were found to express this protein in abundant amounts. Furthermore, immunodetection of serial sections of pancreas, obtained independently, revealed identical staining patterns for both FasL and insulin, confirming that expression of FasL was localized to β-cells (Fig. 2). No specific staining was evident in the exocrine tissue. In parallel immunocytochemical studies using an anti-Fas monoclonal antibody, no specific staining was observed in pancreas sections, suggesting that human islet cells do not normally express Fas.

Because IL-1β has been reported to upregulate expression of Fas in human β-cells (18), we investigated whether the coexpression of Fas and FasL might represent a mechanism for "self-killing" of β-cells by apoptosis, after cytokine exposure. Accordingly, islets were incubated with IL-1β alone or in the presence of an agonistic antibody that activates Fas (Fig. 3). The antibody did not induce cell death when provided to islets under control conditions, consistent with the lack of Fas expression in these circumstances. However, when presented to islets in combination with IL-1β, a significant increase in apoptosis was seen. Moreover, we observed in these studies that IL-1β alone was able to induce apoptosis

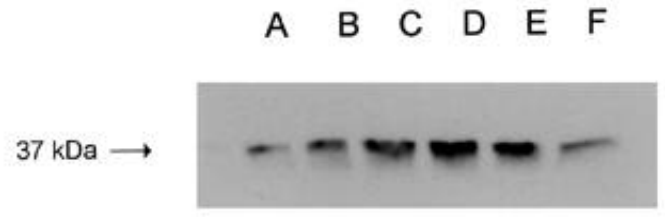

FIG. 1. Detection of FasL in human islets. Isolated human islets were lysed, and 50 µg of protein/lane was electrophoresed on SDS-PAGE minigels, transferred to PVDF membrane, and probed with anti-FasL monoclonal antibody. Jurkat cell lysate was run in parallel as a positive control (*lane F*) and yielded a single band of apparent molecular weight 37 kDa. Human islet lysates prepared from islets snap frozen on receipt (*lane A*), islets maintained in tissue culture under control conditions for 96 h (*lanes B* and *D*), and islets cultured for 96 h with 2 pg/µl IL-1β (*lanes C* and *E*) all contained a single band that comigrated with the FasL band seen in the Jurkat sample. Culture with IL-1β did not alter the mean staining intensity of the FasL band in the islet samples.

in human islets (Fig. 3). Using the data from all 15 islet preparations, IL-1β alone induced a mean increase in islet cell apoptosis of 3.1-fold (control, 1.26 ± 0.15%; +IL-1β, 3.94 ± 0.40%; $P < 0.001$), and although the magnitude of the effect was variable between preparations, there were no batches of islets in which the response did not occur. Examination of the time course of IL-1β–induced apoptosis revealed that the response was maximal within 48 h (Fig.4) and that the effect was completely blocked by coincubation with the caspase-specific inhibitor, Z-VAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Fig.4), confirming the mode of cell death as apoptosis (25,26).

In rodent islets, the effects of IL-1β are mediated principally by induction of nitric oxide synthase, with the subsequent generation of nitric oxide from arginine within the islet cells (10,27–29). Nitric oxide induces apoptosis of rodent β-cells (30–33), an effect that has recently been reported to involve the generation of cGMP and activation of protein kinase G (34). By contrast, we found that in human islets, IL-1β–induced apoptosis was insensitive to the presence of the arginine analog L-NMMA (N^{ω}-monomethyl-L-arginine; an inhibitor of nitric oxide synthase) and was also unaffected by KT5823, a selective inhibitor of protein kinase G (Table 1), suggesting that the signaling pathway(s) initiated by IL-1β was independent of nitric oxide and cGMP. However, in accord with data from HIT-T15 (34), 8-bromo-cGMP did promote apoptosis in human islets, and the magnitude of this response was comparable to that seen with IL-1β (Table 1). The response to the cGMP analog was significantly attenuated by KT5823, confirming the involvement of protein kinase G in the response (Table 1).

To examine whether induction of apoptosis by IL-1β alone was due to interaction between constitutively expressed FasL and upregulated Fas, we used the antagonistic anti-Fas antibody ZB4. At a dilution that abrogated the effects of IL-1β in human thyroid (10 µg/ml) (21), ZB4 inhibited apoptosis induced by the combination of IL-1β and anti-Fas in human islets (Fig. 5), although this inhibition was only partial. ZB4 did not inhibit apoptosis induced by IL-1β alone. This may suggest that in human islets, the apoptotic response to IL-1β is mediated by at least two pathways, only one of which is Fas-dependent. However, it cannot be excluded that the degree

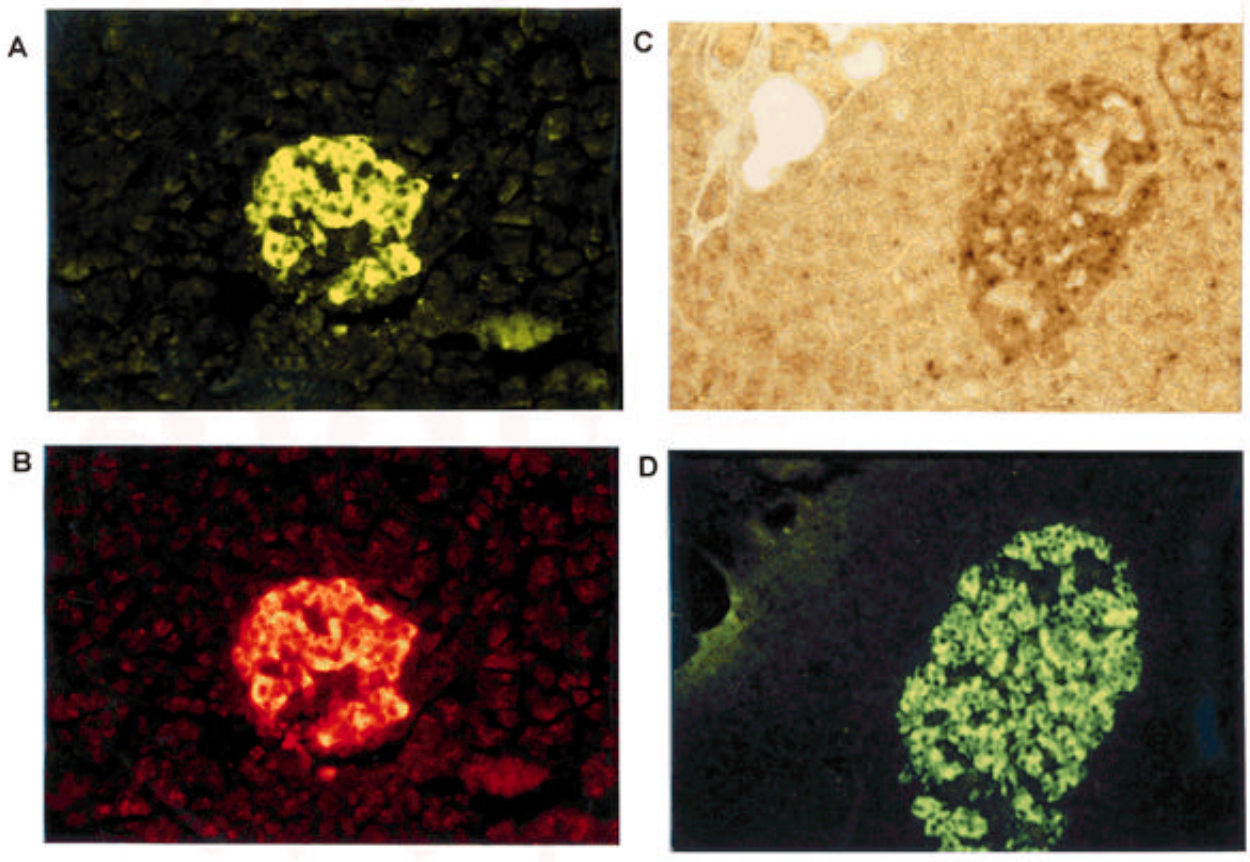

FIG. 2. Immunohistochemical detection of FasL and insulin expression in sections of human pancreas. Sections of human pancreas were probed with anti-FasL antibody (*A*) or anti-insulin antibody (*B*). Fluorescently labeled secondary antibodies were then applied (*A*: anti-mouse-fluorescein; *B*: anti-guinea pig-rhodamine). Positive staining for FasL was detected in islets but not in exocrine tissue (*A*). Comparison of the staining pattern obtained with each antiserum confirmed that FasL is expressed in insulin-positive β-cells. Control sections in which the primary antibody was omitted or in which the primary antibody was directed against Fas did not exhibit any specific immunostaining. *C* and *D* show serial sections of human pancreas in which FasL was detected colorimetrically with immunoperoxidase (*C*) and insulin was detected with a secondary antiserum labeled with fluorescein isothiocyanate (*D*).

of penetration of the ZB4 antibody (and, indeed, the stimulatory anti-Fas antibody) to cells within the islets was restricted and that this contributed to its failure to block the response to IL-1β alone.

DISCUSSION

The concept that autoimmune diabetes may be initiated by inappropriate expression of Fas on pancreatic islets has received considerable recent support, and a number of animal models have been defined in which this is likely to be the case. For example, Yamada et al. (19) have demonstrated Fas-mediated killing of mouse islet cells, and Chervonsky et al. (7) and Itoh et al. (20) have reported a similar phenomenon in transgenic mice expressing FasL under the control of an insulin promoter. Indeed, these two groups provided evidence for direct "self-killing" of islet cells by ligation of Fas with FasL expressed on the same β-cells. They suggested that this mechanism may underlie the development of IDDM in the NOD mouse model and speculated that a similar process could be involved in the early etiology of IDDM in man.

We now provide direct evidence that human islets of Langerhans express FasL and show that under conditions when Fas is upregulated (i.e., after treatment of islets with IL-1β), apoptosis of islet cells is increased. The demonstration

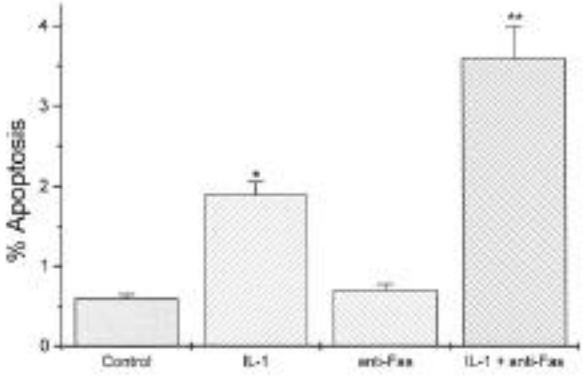

FIG. 3. IL-1β induces apoptosis in human islets and confers sensitivity to an agonistic anti-Fas antibody. Islets were exposed to 2 pg/μl IL-1β, 100 ng/ml anti-Fas antibody (antibody CH11; Longthorne and Williams, 1997) or a combination of the two for 4 days. The islets were then harvested and dispersed before staining with acridine orange. One thousand cells per sample were scored for nuclear morphology, and the percentage showing apoptotic characteristics was determined. The results show the mean ± SE of four separate experiments using batches of human islets prepared from four different donors. *P <0.001 compared with untreated controls; **P < 0.001 compared with IL-1β alone.

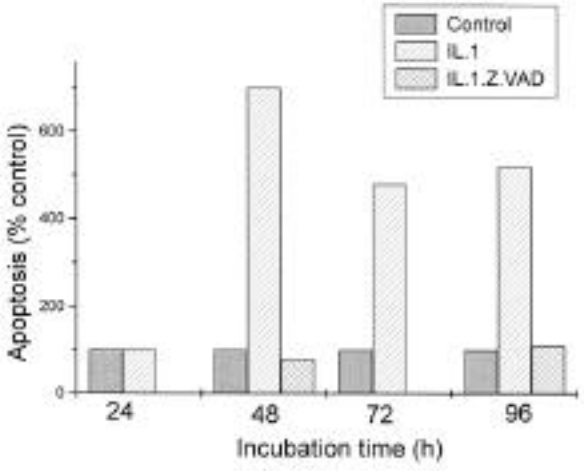

FIG. 4. Time course of IL-1β–induced apoptosis. Human islets were cultured for periods up to 4 days in the absence or presence of 2 pg/µl IL-1β. The caspase inhibitor Z-VAD-fmk (50 µmol/l, pre-incubated for 1 h; Enzyme System Products) was included with IL-1β treatment in samples for removal at two time points. The percentage of apoptotic cells was determined at the time intervals shown by acridine orange staining and fluorescence microscopy.

of FasL expression in human islets was achieved by direct immunodetection of the protein using both Western blotting and immunohistochemistry. The protein was detected in islet preparations from six separate organ donors, suggesting that it is a normal product of the human endocrine pancreas. This situation differs from that in rodents, where FasL expression is reportedly absent from the endocrine pancreas under normal circumstances (35–37). Using sections of human pancreas, we have confirmed that the islet cells that are immunoreactive for FasL are also stained by an anti-insulin antibody. Thus, FasL appears to be expressed on human β-cells. Indeed, examination of immunostained pancreas sections revealed that FasL expression was not restricted to a subpopulation of islets but confirmed that the majority of human islets express FasL (results not shown). These data contrast with the results of recent studies in which Giordano and colleagues (21,23) failed to detect FasL expression in human islets. However, these authors did not present the results of Western blotting experiments with human islets, despite demonstrating that FasL could be detected in thyroid cells by this method when the mRNA was, at best, only weakly detected by reverse transcription–polymerase chain reaction. In this context, a negative result in immunocytochemical experiments (21,23) must be interpreted cautiously because a number of commercially available anti-FasL sera are poorly reactive on fixed tissue sections, even when expression levels are relatively high. Our results, using two independent methods to visualize the protein, reveal that FasL is present in human islet β-cells and show that it does not require IL-1β for expression.

The finding that human islet β-cells can express FasL is extremely significant in light of evidence from rodents and from humans that Fas can be upregulated in islets under conditions when immune-cell infiltration of the pancreas is increased (7,18,19). We now confirm that culture of human

TABLE 1
Effects of inhibition of nitric oxide and cGMP-mediated signaling on IL-1β–induced apoptosis in human islets

Experimental condition	Apoptosis (% of control ± SE)	*n*
Control	100	3
+ IL-1(2 pg/µl)	314 ± 44*	3
+ IL-1 + 0.5 µmol/l KT5823	352 ± 99	3
+ IL-1 + 100 µmol/l L-NMMA	299 ± 48	3
+ 1 mmol/l 8-bromo-cGMP	330 ± 20*	5
+ 8-bromo-cGMP + KT5823	79 ± 8†	5

Human islets were cultured for 4 days in the absence or presence of test reagents as shown. The islets were then dispersed with Trypsin, and 1,000 cells from each sample were scored by acridine orange staining to determine the percentage apoptosis. The replicate experiments (*n*) were carried out on individual batches of human islets prepared from separate donors. *$P < 0.05$ vs. control; †$P < 0.01$ vs. 8-bromo-cGMP alone.

islets in the presence of IL-1β results in the development of Fas sensitivity, as evidenced by the ability of an agonistic anti-Fas antibody to induce islet-cell apoptosis after IL-1β exposure. The failure of this antibody to promote apoptosis in islet cells that had not been treated with IL-1β confirms that Fas was not expressed by the cells under control conditions (18). These data also support the results of Stassi et al. (18) that human β-cells are induced to express Fas upon culture with IL-1β and suggest that after cytokine treatment, human islet cells are primed to undergo apoptosis by the simultaneous presence of both Fas and FasL. Because IL-1β is known to be released in the vicinity of pancreatic β-cells during the early stages of autoimmune diabetes (17), it provides a mechanism by which β-cell loss could be initiated in human

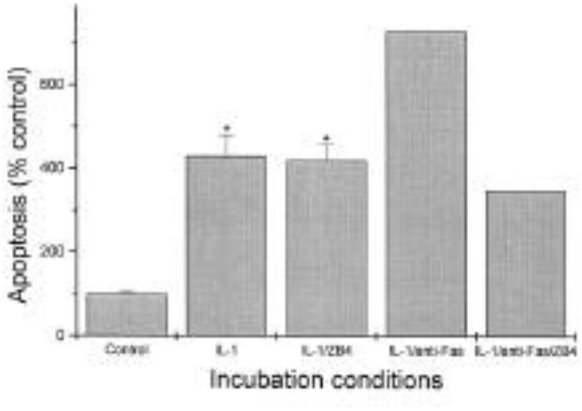

FIG. 5. Effects of an antagonistic anti-Fas antibody (ZB4) on IL-1β– and anti-Fas–induced apoptosis in human islets. Human islets were cultured for 4 days under control conditions or in the presence of 2 pg/µl IL-1β, 100 ng/ml anti-Fas antibody, IL-1β + anti-Fas, IL-1β + 10 µg/ml ZB4, or IL-1β + anti-Fas + ZB4. ZB4 was added 2 h before the addition of anti-Fas, which was introduced 1 h before the IL-1β. The percentage of apoptotic cells was determined by acridine orange staining and fluorescence microscopy. The data represent the mean ± SE of four experiments with islets from four different donors (where error bars are indicated) or from two separate donors.*$P < 0.05$ compared with control.

patients. Even if direct "self-killing" does not occur, the presence of Fas and FasL on neighboring islet endocrine cells and expression of FasL on activated T-cells would still be sufficient to mediate cell death upon ligation of the receptor.

A second important aspect of the present results is that they provide direct evidence that IL-1β can induce apoptosis of human pancreatic islet cells in the absence of other cytokines. Induction of apoptosis by IL-1β is known to occur in rodent β-cells (30,33,38), and there is evidence that IL-1 β alone can be cytotoxic to human islets (39), although other work has suggested that, in humans, islet cell death may require the presence of multiple cytokines (40–42). We now provide unequivocal evidence that enhancement of apoptosis occurs in human islets exposed to IL-1β alone. This conclusion has also recently been drawn by Stassi et al. (23). In our experiments, but in contrast to Stassi et al. (23), the response to IL-1β was not sensitive to an inhibitor of nitric oxide synthase or to the selective protein kinase G inhibitor KT5823. Thus, the effect was not secondary to generation of nitric oxide and/or cGMP, which is consistent with data showing that IL-1β does not promote nitric oxide synthase expression in human islets, except in combination with other cytokines (43). Despite this, it should be emphasized that the results do not exclude the possibility that under conditions where islet nitric oxide levels are increased (i.e., when islet cells are exposed to multiple cytokines), a rise in nitric oxide and cGMP could lead to further enhancement of apoptosis. In support of this, we have shown that exposure of human islets to the cGMP analog 8-bromo-cGMP results in a direct increase in apoptosis to a level very similar to that measured in response to IL-1β. This response was inhibited by KT5823, the selective inhibitor of protein kinase G. These results suggest that the cGMP-dependent pathway for induction of apoptosis described recently in the clonal islet cell line HIT-T15 (34) also operates in human islets. Activation of this pathway, in combination with the Fas-mediated mechanism induced by IL-1β, may then lead to an accelerated rate of islet cell death during cytokine-mediated autoimmune attack in vivo.

In the present study, we were not able to prevent the IL-1β–mediated induction of apoptosis in human islets by addition of the antibody ZB4. This antibody blocks interaction with Fas and has recently been reported to attenuate the apoptotic response to IL-1β in human thyroid cells (21). One interpretation of these data is that IL-1β promotes apoptosis by two separate mechanisms in human islets. One of these involves upregulation of Fas and engagement of FasL, whereas the second is independent of Fas. However, an alternative possibility should also be considered, namely, that the architecture of the islet precludes penetration of antibody molecules under the organ culture conditions used here. Thus, ZB4 may have been physically unable to intervene in the interaction between Fas and FasL occurring on cells located within the core of the islet. Whatever the explanation, it is certainly the case that, in vivo, islets will be exposed to a cocktail of cytokines during immune attack and that coexpression of Fas and FasL under these conditions provides a situation in which apoptosis will be enhanced.

The conclusions arising from our study are also relevant to recent suggestions that enhancement of FasL expression in pancreatic β-cell grafts might be a useful therapeutic vehicle to promote the prolongation of graft survival after islet transplantation (35,37). This idea derives from evidence that tissues such as the eye and Sertoli cells of the testis achieve an immune-privileged status by virtue of their capacity to express FasL (44–46). This is assumed to provide protection from infiltrating immune cells by ligation of Fas, which then causes apoptosis of activated T-cells. Thus, it has been suggested that ectopic expression of FasL in organs destined for transplantation could lead to a reduction in their propensity for immune rejection (46). This approach has already been tested for islet cells, and the results have proved equivocal (7,22,35–37). In two recent studies, overexpression of FasL in the islets of transgenic mice actually resulted in sensitization to immune-mediated destruction rather than in protection (7,22). The authors attributed this to T-cell–mediated induction of Fas expression on islet cells that were now also bearing FasL. Considering that we report here that human islets express FasL constitutively, it follows that they may be especially prone to undergo apoptosis under conditions favoring upregulation of Fas, such as during the heightened immune responsiveness that follows islet transplantation.

In summary, we propose that cytokine-induced upregulation of Fas on human β-cells is likely to be a central component of the autoimmune mechanism of islet cell death during the early phase of progression toward IDDM. The presence of Fas will increase the sensitivity of β-cells both to activated T-cells and to islet endocrine cells expressing FasL.

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