Surface and intracellular Fas expression associated with cytokine-induced apoptosis in rodent islet and insulinoma cells

P Augstein, G Wachlin¹, S Berg, J Bahr, C Salzsieder, B Hehmke, P Heinke and E Salzsieder

Institute of Diabetes 'Gerhardt Katsch' Karlsburg e.V., Karlsburg, Germany

¹Department of Pathophysiology of the Ernst-Moritz-Arndt University of Greifswald, Germany

(Requests for offprints should be addressed to P Augstein, Institute of Diabetes 'Gerhardt Katsch' Karlsburg e.V., Greifswalder Str.11e, 17495 Karlsburg, Germany)

Abstract

During the process of insulinitis in the pathogenesis of type I (insulin-dependent) diabetes mellitus, proinflammatory cytokines induce expression of the death receptor Fas on the surface of pancreatic β -cells and thereby contribute to the enhanced susceptibility of β -cells for apoptosis. The aim of this study was to compare cell-surface and intracellular Fas expression associated with cytokine-induced apoptosis in commonly used β -cell models such as isolated islets and insulinoma lines derived from mouse and rat. The cell line NIT-1 responded to the interleukin (IL)-1 β +interferon (IFN)- γ stimulus with translocation of Fas to the cell surface. Likewise, islet cells from non-obese diabetic (NOD) mice and BB/OK rats expressed increasing amounts of the Fas receptor on their surfaces after exposure to IL-1 β in combination with IFN- γ and tumour necrosis factor- α . Moreover, islets obtained from BB/OK rats at an age near the onset of diabetes had an increased surface expression of Fas compared with young rats. In contrast, western blot analysis of cell lysates from cytokine-exposed islets and insulinoma cells revealed total Fas expression levels comparable to those of untreated controls. In conclusion, islets from BB/OK rats and NOD mice, in addition to NIT-1 insulinoma cells, responded to cytokine exposure with surface expression of the Fas receptor, whereas in cell lysates the levels of expression of Fas were found to be independent of cytokine exposure. Taken together, the findings indicate that cytokine-treated β -cells might possess two pools of Fas protein, one of which is inducible by cytokines and accounts for surface Fas expression, whereas the other is constitutively expressed in cytoplasmic compartments. The underlying mechanisms, including possible interactions between these two sources of cellular Fas expression, need to be investigated in future studies.

Journal of Molecular Endocrinology (2003) **30**, 163–171

Introduction

Type I (insulin-dependent) diabetes mellitus (IDDM) is characterised by selective autoimmune destruction of β -cells, resulting in a reduced β -cell mass at onset of the disease. The most prominent histological feature of IDDM is the mononuclear infiltration of pancreatic islets (Gepts 1965). This process of insulinitis results in β -cell death in areas where insulin-producing β -cells are surrounded by infiltrating mononuclear cells (Bach 1994, Benoist

& Mathis 1997, Rabinovitch 1998). Here, a complex system of immune effector cells, cytokines and other proinflammatory products of these cells such as nitric oxide (NO) attacks the β -cells and impairs both their function and their viability (Mandrup-Poulsen *et al.* 1993, Eizirik *et al.* 1996, Mauricio & Mandrup-Poulsen 1998, Rabinovitch 1998, Eizirik & Darville 2001). Likewise, pro-inflammatory cytokines (interleukin (IL)-1 β , tumour necrosis factor (TNF)- α) and type 1 cytokines (interferon (IFN)- γ) secreted by islet infiltrating cells

induce the expression of the death receptor Fas (CD95) on the surface of β -cells and, thereby, render β -cells susceptible to the Fas/Fas ligand (FasL) pathway of apoptosis (Stassi *et al.* 1995, 1997, Moriwaki *et al.* 1999, Suarez-Pinzon *et al.* 1999, Zumsteg *et al.* 2000). Indeed, the expression of Fas and apoptosis of β -cells have been demonstrated in pancreas biopsies taken from newly diagnosed patients with IDDM and were both absent in β -cells from healthy controls (Moriwaki *et al.* 1999). *In vitro*, Fas expression by β -cells was demonstrated both at the mRNA level in mouse and rat islets (Yamada *et al.* 1996, Zumsteg *et al.* 2000, Darville & Eizirik 2001) and in the β -cell lines NIT-1, β -TC1, RINm5F (Yamada *et al.* 1996, Zumsteg *et al.* 2000, Darville & Eizirik 2001), and at the protein level in human and mouse β -cells and in NIT-1 cells after exposure to IL-1 β alone or in combination with IFN- γ or TNF- α (Zumsteg *et al.* 2000). Consequently, Fas-mediated apoptosis has been believed to be involved in the pathogenesis of autoimmune processes resulting in β -cell death (Eizirik & Mandrup-Poulsen 2001).

The spontaneously diabetic BB rat and the non-obese diabetic (NOD) mouse are widely accepted animal models for *in vivo* investigations of the mechanisms of β -cell death and also for *in vitro* experiments in isolated pancreatic islets. Autoimmune β -cell destruction in both animal models shares many features with human IDDM, including inflammation of pancreatic islets (Kikutani & Makino 1992, Rossini *et al.* 1995, Buschard 1996, Benoist & Mathis 1997). Remarkably, apoptosis of β -cells has been demonstrated in infiltrated islets of NOD mice and BB rats (O'Brien *et al.* 1997, Augstein *et al.* 1998, Lally *et al.* 2001). However, whereas cytokine-induced Fas surface expression has been reported on islets of NOD mice (Stassi *et al.* 1997, Suarez-Pinzon *et al.* 1999, Thomas *et al.* 1999, Amrani *et al.* 2000, Liu *et al.* 2000, Zumsteg *et al.* 2000), Fas protein has not yet been detected on BB rat islets. Moreover, transformed β -cell lines attained great importance in experimental diabetes research because these cell lines have the advantage of growing permanently and expanding, depending on the experimental conditions used. In general, both primary and permanent β -cells have been used to investigate the mechanisms of Fas/FasL mediated apoptosis in IDDM.

The aim of our present study was to examine the expression of Fas by islet and insulinoma cells from mice and rats in response to proinflammatory and type 1 cytokines at the protein level. Thus we compared the Fas staining patterns of islet cells from BB rats and NOD mice with those of RIN 5AH-T2 (Gazdar *et al.* 1980) and NIT-1 cells (Hamaguchi *et al.* 1991), by analysing the surface and intracellular expression of Fas receptor at the protein level.

Materials and methods

Islet isolation and culture

NOD mice were purchased from the Møllegaard Breeding Centre (Møllegaard & Bomholtgård, A/S, Denmark) and housed at the animal facilities of the Institute of Diabetes 'Gerhardt Katsch' Karlsburg. Islets were isolated according to the method of Thomas *et al.* (1998) by cannulation of the bile duct and infusion of ice-cold collagenase P (1.5 mg/ml; Roche) solution *in situ*. BB/OK rats (8–12 and 70–90 days old) were obtained from the Department of Laboratory Animal Science (Medical Faculty, University of Greifswald) and islets were isolated by means of fractionated collagenase digestion (Serva, Heidelberg, Germany; 8 mg/15 ml Hanks's balanced salt solution) (Witt *et al.* 1989). The islets were purified by density gradient centrifugation (Hehmke *et al.* 1986) and pre-cultured (37 °C, 5% CO₂) in RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 10% FCS and antibiotics. Islets from NOD mice were exposed to the cytokine combination IL-1 β +IFN- γ (50+500 U/ml; Dunn, Asbach, Germany) and islets from BB/OK rats to the combination IL-1 β +TNF- α +IFN- γ (10+500+500 U/ml; Biosource, Camarillo, California). After islets had been exposed to cytokines for 24 h, their light microscopical appearance (LMA index) was graded from 1 to 3 (3=very good) and then they underwent lysis for western blot analysis or disintegration into single islet cells by fractionated dispase digestion (Witt *et al.* 1989) for analysis of viability and surface expression of Fas (Augstein *et al.* 2002).

Insulinoma cell lines

The insulinoma cell line NIT-1 deriving from NOD/LT mice (Hamaguchi *et al.* 1991) was

purchased from ATCC (Rockville, MD, USA) and cultured according to the supplier's recommendations. Rat insulinoma cells of the line RIN 5AH-T2 generated from a NEDH rat transplantable insulinoma (Gazdar *et al.* 1980), kindly donated by Dr B. Ziegler (Department of Pathophysiology, University of Greifswald, Germany), were cultured in RPMI 1640 supplemented with 7.5% FCS. NIT-1 and RIN 5AH-T2 cells were cultured in 25 cm² tissue culture flasks (Greiner, Frickenhausen, Germany) at 37 °C in a 5% CO₂/95% air mixture. The β -cell lines were passaged at weekly intervals by trypsinization (Invitrogen Life Technologies) and inoculated at a density of 2×10^5 cells per well in a 24-well tissue culture plate (TPP, Techno Plastic Products AG, Trasadingen, Switzerland) and pre-cultured for 48 h (NIT-1) and 72 h (RIN 5AH-T2). Afterwards, NIT-1 and RIN 5AH-T2 cells were exposed to IL-1 β +IFN- γ (100+1000 U/ml; Dunn, Asbach, Germany). After the LMA index had been read, cells were harvested by trypsin treatment and their viability determined as described previously (Augstein *et al.* 2002).

Flow cytometric analysis of Fas expression

Surface Fas expression was analysed on viable NIT-1 cells and islet cells from NOD mice using the hamster anti-Fas antibody Jo2 (2 μ g/ml) recognising mouse Fas (Yamada *et al.* 1996, Suarez-Pinzon *et al.* 1999, Zumsteg *et al.* 2000) and hamster immunoglobulins (2 μ g/ml) as negative control (BD Pharmingen, Heidelberg, Germany). Jo2 immunoreactivity was detected by biotinylated anti-hamster IgG (Dianova, Hamburg, Germany) and Streptavidin-phycoerythrin (BD Pharmingen). Surface expression of Fas on rat islet cells was detected by the rabbit Fas antibody AAP-221 (StressGen, Victoria BC, Canada), which was raised against an extracellular epitope of Fas and visualised by PE-labelled anti-rabbit IgG (Dianova).

To analyse the total expression of Fas, NIT-1 and RIN 5AH-T2 cells were fixed and permeabilised using Permeafix (Ortho Diagnostics Systems, Neckargemünd, Germany) or BD FACS Permeabilizing Solution 1 (BD Pharmingen) as recommended by the supplier. After being washed with PBS containing 5% FCS and EDTA (0.2 mmol/l), cells were incubated with a polyclonal rabbit anti-Fas antibody M-20 (2 μ g/ml;

sc-716; Santa Cruz Biotechnology, Heidelberg, Germany). After incubation for 45 min and washing, cell-bound Fas antibody was detected by fluorescein isothiocyanate-labelled anti-rabbit IgG. Normal rabbit IgG (Santa Cruz Biotechnology) was used as negative control. The samples were analysed on an EPICS/XL flow cytometer (Coulter, Krefeld, Germany) equipped with System II Software, version 3.0 (Coulter).

Hypodiploid nuclei

Islets or insulinoma and islet cells were harvested, washed twice with PBS and resuspended in a hypotonic propidium iodide (PI) solution (50 μ g/ml; 0.1% sodium citrate and 0.1% Triton X-100) as described by Thomas *et al.* (1999). Fluorescence of PI was analysed by flow cytometry in the log mode after exclusion of doublets and degraded DNA from cell debris. Hypodiploid nuclei (%) were revealed by analysis of the sub-G₁ area. In addition, the cells in the cell cycle compartments G₀/G₁, S and G₂/M were estimated.

Western blotting

After exposure to cytokines, NIT-1 and RIN 5AH-T2 cells, and islets from NOD mice, were lysed on ice (1×10^7 cells/50 μ l radioimmunoprecipitation buffer; PBS containing 1% Igepal, 0.5% deoxycholic acid and 0.1% SDS) supplemented with a freshly added protease inhibitor cocktail (Complete Roche) according to the supplier's procedure. After the cells had been lysed for 30 min, samples were sonicated, incubated again for 30 min on ice and centrifuged at 10 000 *g*. BB/OK rat islets were lysed on ice for 30 min (10 mmol/l Tris-HCl pH 7.4 supplemented with 0.1 mmol/l EDTA, 10 mmol/l NaCl, 0.5% NP40, 2 mmol/l MgCl₂, 1 mmol/l phenyl methylsulphonyl fluoride and 50 μ g/ml DNase), and then centrifuged at 23 900 *g*. Protein concentrations of supernatants were determined by Micro BCA (Pierce, Rockford, USA) and samples were separated by SDS-PAGE 12% (BioRad, Munich, Germany). The proteins were blotted onto polyvinylidene difluoride membranes using a tank blotting system (BioRad). The membranes were subsequently blocked in 100 mM Tris-buffered saline (pH 7.5) containing 0.9% NaCl and 3% BSA. Afterwards, the membranes were incubated

with rabbit polyclonal antibodies M-20 (0.4 μ g/ml; Santa Cruz Biotechnology), recognising an epitope at the C terminus (Fladmark *et al.* 1997, Gottlob *et al.* 1998, Hill *et al.* 1999, Micheau *et al.* 1999, Nomura *et al.* 1999), and AAP-221 (2 μ g/ml; StressGen, Victoria BC, Canada), recognising the Fas residues 29–44 (Watanabe-Fukunaga *et al.* 1992), or rabbit immunoglobulins as negative control, followed by alkaline phosphatase-conjugated antibodies. The specificity of M-20 and AAP-221 binding for Fas was confirmed in competition studies using the corresponding Fas control peptide at a fivefold excess. In addition, a recombinant Fas/Fc chimera (R&D Systems, Wiesbaden, Germany) containing the extracellular domain of mouse Fas (Met¹–Arg¹⁶⁹) was used to verify AAP-221 binding. The immunoreaction was visualised by the Sigma Fast BCIP/NBT buffered substrate. The blots were scanned and analysed by measuring the average counts of pixels within the object using Molecular Imager Software (BioRad). For calculation of the levels of expression of Fas in cytokine-treated samples, the levels in controls were set as 100%. Molecular weight was calculated using the Gel imaging and densitometry software Scan Pack 3.0 (Whatman Biometra, Göttingen, Germany).

Nitric oxide synthesis

Production of NO was measured as accumulation of nitrite in conditioned media as determined by the Griess reaction. In brief, 150 μ l cell-free medium was mixed with an equal volume of the Griess reagent (one part 0.1% naphthylethylene diamine dihydrochloride and one part 1% sulphanilamide in 5% H_3PO_4) and incubated for 10 min at room temperature. Nitrite concentration was determined in triplicate within a concentration range that corresponds to the linear part of the standard curve. The absorbance was measured at 540 nm on an immunoreader (ELx800UV; BioTek Instruments, Winooski, USA).

Statistical analysis

Results are expressed as means $\pm$ S.E. Statistical significance was assessed using unpaired Student's *t*-test. When multiple tests were performed, a Bonferroni–Holm correction was used to adjust the

Figure 1 Time-course analysis of cytokine-induced effects (IL-1 β +IFN- γ : 100+1000 U/ml) on nuclei of the insulinoma cell lines (A) RIN 5AH-T2 and (B) NIT-1 analysed by quantitation of hypodiploidy (sub-G₁, cross-hatched bars), of G₀/G₁ (dotted bars), S (solid bars) and G₂/M (clear bars) cell-cycle compartments. The untreated controls are shown as means from all investigated time points. Data are given as means and were obtained in at least eight (RIN 5AH-T2) and five (NIT-1) individual experiments for each time point. (C) Islets from NOD mice and BB/OK rats were analysed after 24 h exposure to IL-1 β +IFN- γ (50+500 U/ml; *n*=12) and IL-1 β +TNF- α +IFN- γ (10+500+500 U/ml; *n*=7), respectively. Significant changes (*P*<0.05) are shown for the sub-G₁ (*), G₀/G₁ (#), S (+) and G₂/M (°) compartments in comparison with untreated controls.

level of significance. Differences were considered to be statistically significant at a level of *P*<0.05.

Results

Cytokine-induced apoptosis

Time-course analysis of cytokine-induced apoptosis in NIT-1 and RIN 5AH-T2 insulinoma cells demonstrated the appearance of hypodiploid nuclei (sub-G₁; *P*<0.05) after 24 h exposure to IL-1 β and IFN- γ , which was associated with reduced numbers of cells belonging to cell-cycle compartments G₀/G₁, S and G₂/M (Fig. 1A, B). At this

Figure 2 Time-dependent effects of IL-1 β +IFN- γ (100+1000 U/ml) on (A) RIN 5AH-T2 and (B) NIT-1 cells monitored by viability (x), LMA index (□) and NO production detected as nitrite accumulation (○) in the culture media. Data are given as mean $\pm$ S.E. from at least eight (RIN-5AH T2) and 10 (NIT-1) individual experiments. * P <0.05, ** P <0.01 compared with untreated controls.

time point, increased nitrite generation was accompanied by an impaired LMA index and reduced viability (Fig. 2A, B). Similarly, the cytokine combinations IL-1 β +IFN- γ and IL-1 β +IFN- γ +TNF- α induced damage of nuclear DNA, as revealed by increased percentages of hypodiploid nuclei in islet cells of NOD mice and BB/OK-rats (Fig. 1C). In addition, cytokine-induced accumulation of nitrite in the islet culture medium was accompanied by a deterioration in LMA index (Table 1).

Surface Fas expression

NIT-1 and islet cells derived from NOD mice expressed on their surfaces increased levels (P <0.01) of the Fas receptor after exposure to IL-1 β +IFN- γ (Fig. 3). Islets from BB/OK rats cultured in the presence of IL-1 β +IFN- γ +TNF- α

Table 1 Biological activity of cytokines in pancreatic islets from BB/OK rats and NOD mice is demonstrated by impaired hypodiploidy and LMA index, and by accumulation of nitrite in the culture medium. IL-1 β +IFN- γ were used for treatment of NOD mouse islets (50+500 U/ml, n =14). BB/OK rat islets were exposed to the combination of IL-1, TNF- α and IFN- γ (10+500+500 U/ml, n =6). Data are given as mean $\pm$ S.E. * P <0.05, ** P <0.01 compared with untreated control

	BB/OK rats	NOD mice
Hypodiploidy (%)		
Controls	10.50 $\pm$ 2.93	16.79 $\pm$ 2.80
Cytokine-treated	25.40 $\pm$ 7.71*	26.34 $\pm$ 3.34**
LMA index		
Controls	3.00 $\pm$ 0.00	2.96 $\pm$ 0.04
Cytokine-treated	1.80 $\pm$ 0.48*	1.79 $\pm$ 0.11**
Nitrite (pmol/islet)		
Controls	1.50 $\pm$ 0.20	1.51 $\pm$ 0.21
Cytokine-treated	6.80 $\pm$ 0.27**	2.73 $\pm$ 0.35**

Figure 3 Flow cytometric analysis of surface Fas expression in primary islet cells from NOD mice and BB/OK rats and in transformed NIT-1 cells after 24 h of cytokine exposure. IL-1 β +IFN- γ were used for Fas induction in NIT-1 cells (100+1000 U/ml; n =7) and NOD mouse islets (50+500 U/ml; n =8). BB/OK rat islets were exposed to the combination of IL-1 β , TNF- α and IFN- γ (10+500+500 U/ml; n =16). Data are given as mean $\pm$ S.E. ** P <0.01 compared with untreated controls.

showed increased surface expression of Fas (Fig. 3) detected with a polyclonal rabbit Fas antibody (Fig. 4). Moreover, untreated islet cells obtained from BB/OK rats near the onset of diabetes, at 70–90 days of age, reflected an increased percentage (P <0.01) of surface Fas expression (12.34 $\pm$ 1.47; n =12) compared with young, 8–12-day-old animals (3.34 $\pm$ 0.68; n =18).

Figure 4 Western blot binding of the polyclonal Fas antibody AAP-221 to a recombinant Fas/Fc chimera (55 kDa) containing the extracellular domain of the mouse Fas protein (A, lane 1), to HeLa cells (B, lane 3) and to thymocytes derived from BB/OK rats (C, lane 1), NOD mice (C, lane 2) and Wistar rats (C, lane 3). Reactivity with BB/OK rat islets (B, lanes 1 and 2). The antibody detects Fas in untreated (B, lane 1) and IL-1 β -exposed islets (B, lane 2). Molecular weight standards are given in kDa.

Total Fas expression

Using western blot analysis, total Fas expression was assessed in lysates of cytokine-treated and -untreated islets and insulinoma cells. The specificity of Fas antibody M-20 binding was confirmed by competitive inhibition of M-20 immunoreactivity after incubation with the Fas peptide used for raising the antibody (Fig. 5). In lysates from NOD mouse and BB/OK rat islets, in addition to NIT-1 and RIN 5AH-T2 cells, the antibody M-20 recognised a protein migrating at about 50 kDa (Fig. 5) that was detected at comparable levels in cytokine-treated islets and in untreated controls. Densitometric analysis of western blots obtained with Fas antibody M-20 demonstrated that total Fas protein expressed in cytokine-treated NIT-1 cells and RIN 5AH-T2 cells amounted to $82.8 \pm 1.5\%$ ($n=5$) and $86.2 \pm 1.3\%$ ($n=4$) compared with that of the controls respectively. Detection of total Fas expression by flow cytometry confirmed the high percentage of Fas positivity in cytokine-treated insulinoma cells (NIT-1: 78.6 ± 4.9 ; RIN 5AH-T2: 92.9 ± 0.5), which was increased in untreated controls (NIT-1: 81.0 ± 5.5 , $P<0.05$; RIN 5AH-T2: 96.6 ± 0.6 , $P<0.05$).

Discussion

In this comparative study we used isolated islets from diabetes-susceptible NOD mice and BB/OK rats and the two insulinoma cell lines NIT-1 (Hamaguchi *et al.* 1991) and RIN 5AH-T2 (Gazdar *et al.* 1980) as models to investigate the Fas staining

pattern associated with cytokine-induced apoptosis. IL-1 β and IFN- γ have been known for a long time to mediate inhibitory effects on β -cell function and proliferation associated with β -cell cytotoxicity (Mandrup-Poulsen *et al.* 1993, Mandrup-Poulsen 1996). In accordance with previously published studies, our models for cytokine-induced apoptosis were characterised by DNA damage and cell death (Dunger *et al.* 1996a,b, Delaney *et al.* 1997). Time-course analysis revealed an association of increasing hypodiploidy and impaired distribution of cell-cycle compartments in both insulinoma cell lines. Equally, islets from BB/OK rats show an augmented proportion of hypodiploid nuclei after 24 h of exposure to cytokine. IL-1 β and IFN- γ have been reported to depress DNA synthesis in islets and insulinoma cells (Sandler *et al.* 1989, Sjöholm 1992, Rane & Reddy 2000), and in fact we observed that the G₀/G₁, S and G₂/M compartments contributed to the increase of the sub-G₁ fraction in cytokine-treated islet and insulinoma cells.

After 24 h exposure, in cases in which cytokines mediated significant cytotoxic effects, we investigated the pattern of expression of the Fas receptor. In agreement with findings reported previously, both primary islet cells and insulinoma cells derived from NOD mice responded to exposure to IL-1 β and IFN- γ by surface expression of Fas (Stassi *et al.* 1995, Suarez-Pinzon *et al.* 1999, Thomas *et al.* 1999, Zumsteg *et al.* 2000). Lower levels of cytokine-induced surface Fas expression were detected in NIT-1 cells, indicating that differences exist in the cytokine responsiveness or regulation of

Figure 5 Representative western blots showing the reactivity of NIT-1 cells (lanes 1–4), NOD mouse islets (lanes 5, 6), BB/OK rat islets (lanes 7, 8) and RIN 5AH-T2 cells (lanes 9, 10) with the rabbit Fas antibody (M-20) recognising an epitope at the C terminus. The specificity of M-20 binding is shown in lanes 1 and 2. Here, the antibody binding was completely abolished by competitive inhibition with the Fas peptide used for raising the antibody. IL-1 β in combination with IFN- γ was added to NIT-1 cells (100+1000 U/ml, lanes 2, 4), NOD mouse islets (50+500 U/ml, lane 6) and RIN 5AH-T2 cells (50+500 U/ml, lane 10). BB/OK rat islets were exposed to IL-1 β , TNF- α and IFN- γ (10+500+500 U/ml, lane 8). Cytokine-exposed samples are compared with untreated controls (lanes 1, 3, 5, 7, 9).

Fas expression of transformed and primary β cells. Interestingly, we observed a reduction of Fas expression in NIT-1 cells that had been passaged frequently and kept in culture for longer periods of times (P. Augstein, unpublished observations). Probably, surface expression of Fas in NIT-1 cells is influenced by their oncogenic transformation and could be affected by similar mechanisms that enable tumour cells to escape from cytotoxic elimination mechanisms such as downregulation of death receptors involved in apoptotic pathways (Cefai *et al.* 2001).

Importantly, we were able to demonstrate surface expression of Fas on islet cells from BB/OK rats and showed thereby that cytokine-induced Fas mRNA transcription (Darville & Eizirik 2001) results in *de novo* expression of Fas protein at the surface of BB/OK rat islet cells. In BB/OK rat islets, the combination of the cytokines IL-1 β , TNF- α and IFN- γ induced a level of surface expression of Fas comparable to that observed in NOD mice in this study. In support of our present findings, in a parallel study IL-1 β +IFN- γ -treated rat insulinoma cells (RIN 5AH-T2) were reported to express Fas on their surfaces at levels similar to those observed in BB/OK rat islet cells (Kuttler *et al.* 2001). Moreover, islets obtained from BB/OK rats at an age near the onset of diabetes and known to be affected by insulinitis (Hahn *et al.* 1991) had a significantly increased level of spontaneous expres-

sion of Fas compared with islets from young BB/OK rats without insulinitis (Hahn *et al.* 1991), indicating an association of continuing β -cell destruction and surface Fas expression in this model of autoimmune diabetes. A similar correlation of Fas expression and β -cell death has been demonstrated in NOD mice by analysing Fas expression of transplanted syngenic islets in non-diabetic and diabetic NOD mice (Suarez-Pinzon *et al.* 1999). Taking all these data together and in context with reports from the literature (Suarez-Pinzon *et al.* 1999, Thomas *et al.* 1999, Darville & Eizirik 2001), it can be concluded that *de novo* surface Fas expression in all models investigated results from exposure to proinflammatory and type 1 cytokines, followed by transcription of Fas mRNA and translocation of Fas receptor to the β -cell surface.

By western blotting, we investigated total Fas expression using the Fas antibody M-20, and detected a protein migrating at 50 kDa in cytokine-treated and untreated lysates from all models, indicating that Fas appears to be expressed in intracellular compartments of β -cells independent of cytokine exposure. Moreover, this implies that intracellular Fas expression is regulated by mechanisms that differ from those accounting for Fas protein presented at the β -cell surface. Indeed, Peter *et al.* (1995) monitored Fas expression by metabolic labelling of Fas and demonstrated that

Fas is a long-living protein with a low metabolic turnover rate. Even in cell lines showing high Fas expression at the cell surface, low expression rates of the Fas gene were evident (Peter *et al.* 1995). Furthermore, Fas from the cell surface was found to be smaller and desialyated (Peter *et al.* 1995, Keppler *et al.* 1999).

In conclusion, Fas protein expression was induced specifically at the surface of IL-1 β + IFN- γ -treated islet cells from diabetes-prone NOD mice, and in transformed NIT-1 cells. Also, islet cells from BB/OK rats exposed to IL-1 β , TNF- α and IFN- γ responded with translocation of Fas receptor to the cell surface, demonstrating that Fas mRNA transcription in rat β -cells is indeed accompanied by expression of Fas protein. Moreover, islet cells obtained from BB rats near the onset of diabetes showed an increased level of Fas on their surfaces compared with 8–12-day-old rats, implying a correlation with current β -cell destruction *in vivo*. Western blot analysis revealed that total Fas expression is present in untreated islet and insulinoma cells from mice and rats, suggesting (1) that Fas is expressed in cytoplasmic compartments independent of cytokine exposure, and (2) that different mechanisms account for regulation of cell-surface and intracellular Fas expression. Moreover, the intracellular Fas protein pool might also contribute, to some degree, to the provision of Fas receptor for translocation to the cell surface. Consistent with this concept, the amount of total Fas protein was slightly decreased in our cytokine-treated insulinoma cells as detected by western blot and flow cytometric analysis. Future studies are necessary to reveal the regulation and interconnections of the Fas protein recruited for surface and intracellular expression.

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft AU 151/1-1, 1-2 and from the Ministerium für Bildung, Wissenschaft und Kultur Mecklenburg-Vorpommern IDK 97 007 80/SOM and IDK 97 007 80/HSP III. The authors thank Dr A. Uber and Mrs K. Niemann for helpful discussions and assistance with densitometric analysis of western blots.

References

- Amrani A, Verdaguer J, Thiessen S, Bou S & Santamaria P 2000 IL-1 α , IL-1 β , and IFN- γ mark beta cells for Fas-dependent destruction by diabetogenic CD4(+) T lymphocytes. *Journal of Clinical Investigation* **105** 459–468.
- Augstein P, Stephens LA, Allison J, Elefanty AG, Ekberg M, Kay TW & Harrison LC 1998 Beta-cell apoptosis in an accelerated model of autoimmune diabetes. *Molecular Medicine* **4** 495–501.
- Augstein P, Dunger A, Salzsieder C, Heinke P, Kubernath R, Bahr J, Fischer U, Rettig R & Salzsieder E 2002 Cell surface trafficking of Fas in NIT-1 cells and dissection of surface and total Fas expression. *Biochemical and Biophysical Research Communications* **290** 443–451.
- Bach JF 1994 Insulin-dependent diabetes mellitus as an autoimmune disease. *Endocrine Reviews* **15** 516–542.
- Benoist C & Mathis D 1997 Cell death mediators in autoimmune diabetes – no shortage of suspects. *Cell* **89** 1–3.
- Buschard K 1996 Diabetic animal models. *APMIS* **104** 609–614.
- Cefai D, Schwaninger R, Balli M, Brunner T & Gimmi CD 2001 Functional characterization of Fas ligand on tumor cells escaping active specific immunotherapy. *Cell Death and Differentiation* **8** 687–695.
- Darville MI & Eizirik DL 2001 Cytokine induction of Fas gene expression in insulin-producing cells requires the transcription factors NF- κ B and C/EBP. *Diabetes* **50** 1741–1748.
- Delaney CA, Pavlovic D, Hoorens A, Pipeleers DG & Eizirik DL 1997 Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. *Endocrinology* **138** 2610–2614.
- Dunger A, Augstein P, Schmidt S & Fischer U 1996a Identification of interleukin 1-induced apoptosis in rat islets using *in situ* specific labelling of fragmented DNA. *Journal of Autoimmunology* **9** 309–313.
- Dunger A, Cunningham JM, Delaney CA, Lowe JE, Green MH, Bone AJ & Green IC 1996b Tumor necrosis factor- α and interferon- γ inhibit insulin secretion and cause DNA damage in unweaned-rat islets. Extent of nitric oxide involvement. *Diabetes* **45** 183–189.
- Eizirik DL & Darville MI 2001 Beta-cell apoptosis and defense mechanisms: lessons from type 1 diabetes. *Diabetes* **50** (suppl 1) S64–9.
- Eizirik DL & Mandrup-Poulsen T 2001 A choice of death – the signal-transduction of immune-mediated beta cell apoptosis. *Diabetologia* **44** 2115–2133.
- Eizirik DL, Flodstrom M, Karlsen AE & Welsh N 1996 The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells. *Diabetologia* **39** 875–890.
- Fladmark KE, Gjertsen BT, Doskeland SO & Vintermyr OK 1997 Fas/APO-1(CD95)-induced apoptosis of primary hepatocytes is inhibited by cAMP. *Biochemical and Biophysical Research Communications* **232** 20–25.
- Gazdar AF, Chick WL, Oie HK, Sims HL, King DL, Weir GC & Lauris V 1980 Continuous, clonal, insulin- and somatostatin-secreting cell lines established from a transplantable rat islet cell tumor. *PNAS* **77** 3519–3523.
- Gepts W 1965 Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes* **14** 619–633.
- Gottlob K, Fulco M, Levrero M & Graessmann A 1998 The hepatitis B virus HBx protein inhibits caspase 3 activity. *Journal of Biological Chemistry* **273** 33347–33353.
- Hahn HJ, Lucke S, Kloting I & Besch W 1991 Prospective investigations of long-term normoglycaemic BB/OK-rats: serial determination of glucose tolerance, insulinitis, B-cell volume density and pancreatic insulin content. *Experimental and Clinical Endocrinology* **98** 185–192.

- Hamaguchi K, Gaskins HR & Leiter EH 1991 NIT-1, a pancreatic beta-cell line established from a transgenic NOD/Lt mouse. *Diabetes* **40** 842–849.
- Helmke B, Kohnert KD & Odselius R 1986 The use of a new dextran gradient medium for rapid isolation of functionally intact neonatal rat pancreatic islets. *Diabetes Research* **3** 13–16.
- Hill LL, Ouhitit A, Loughlin SM, Kripke ML, Ananthaswamy HN & Owen-Schaub LB 1999 Fas ligand: a sensor for DNA damage critical in skin cancer etiology. *Science* **285** 898–900.
- Keppler OT, Peter ME, Hinderlich S, Moldenhauer G, Stehling P, Schmitz I, Schwartz-Albiez R, Reutter W & Pawlita M 1999 Differential sialylation of cell surface glycoconjugates in a human B lymphoma cell line regulates susceptibility for CD95 (APO-1/Fas)-mediated apoptosis and for infection by a lymphotropic virus. *Glycobiology* **9** 557–569.
- Kikutani H & Makino S 1992 The murine autoimmune diabetes model: NOD and related strains. *Advances in Immunology* **322** 51285–51322.
- Kutler B, Wanka H & Morgenstern O 2001 Inhibition of cytokine-induced Fas-expression by the NO-synthase inhibitor aminoguanidin. *Diabetologia* **44** A142.
- Lally FJ, Ratcliff H & Bone AJ 2001 Apoptosis and disease progression in the spontaneously diabetic BB/S rat. *Diabetologia* **44** 320–324.
- Liu D, Pavlovic D, Chen MC, Flodstrom M, Sandler S & Eizirik DL 2000 Cytokines induce apoptosis in beta-cells isolated from mice lacking the inducible isoform of nitric oxide synthase (iNOS^{-/-}). *Diabetes* **49** 1116–1122.
- Mandrup-Poulsen T 1996 The role of interleukin-1 in the pathogenesis of IDDM. *Diabetologia* **39** 1005–1029.
- Mandrup-Poulsen T, Zumsteg U, Reimers J, Pociot F, Morch L, Helqvist S, Dinarello CA & Nerup J 1993 Involvement of interleukin 1 and interleukin 1 antagonist in pancreatic beta-cell destruction in insulin-dependent diabetes mellitus. *Cytokine* **5** 185–191.
- Mauricio D & Mandrup-Poulsen T 1998 Apoptosis and the pathogenesis of IDDM: a question of life and death. *Diabetes* **47** 1537–1543.
- Micheau O, Solary E, Hammann A & Dimanche-Boitrel MT 1999 Fas ligand-independent, FADD-mediated activation of the Fas death pathway by anticancer drugs. *Journal of Biological Chemistry* **274** 7987–7992.
- Moriwaki M, Itoh N, Miyagawa J, Yamamoto K, Imagawa A, Yamagata K, Iwahashi H, Nakajima H, Namba M, Nagata S, Hanafusa T & Matsuzawa Y 1999 Fas and Fas ligand expression in inflamed islets in pancreas sections of patients with recent-onset Type I diabetes mellitus. *Diabetologia* **42** 1332–1340.
- Nomura K, Imai H, Koumura T, Arai M & Nakagawa Y 1999 Mitochondrial phospholipid hydroperoxide glutathione peroxidase suppresses apoptosis mediated by a mitochondrial death pathway. *Journal of Biological Chemistry* **274** 29294–29302.
- O'Brien BA, Harmon BV, Cameron DP & Allan DJ 1997 Apoptosis is the mode of beta-cell death responsible for the development of IDDM in the nonobese diabetic (NOD) mouse. *Diabetes* **46** 750–757.
- Peter ME, Hellbardt S, Schwartz-Albiez R, Westendorp M, Walczak H, Moldenhauer G, Grell M & Krammer PH 1995 Cell surface sialylation plays a role in modulating sensitivity toward APO-1-mediated apoptotic cell death. *Cell Death and Differentiation* **2** 163–171.
- Rabinovitch A 1998 An update on cytokines in the pathogenesis of insulin-dependent diabetes mellitus. *Diabetes and Metabolism Reviews* **14** 129–151.
- Rane SG & Reddy EP 2000 Cell cycle control of pancreatic beta cell proliferation. *Frontiers in Bioscience* **5** D1–D19.
- Rossini AA, Handler ES, Mordes JP & Greiner DL 1995 Human autoimmune diabetes mellitus: lessons from BB rats and NOD mice – caveat emptor. *Clinical Immunology and Immunopathology* **74** 2–9.
- Sandler S, Bendtzen K, Eizirik DL, Sjöholm A & Welsh N 1989 Decreased cell replication and polyamine content in insulin-producing cells after exposure to human interleukin 1 beta. *Immunology Letters* **22** 267–272.
- Sjöholm A 1992 Differential effects of cytokines on long-term mitogenic and secretory responses of fetal rat pancreatic beta-cells. *American Journal of Physiology* **263** C114–C120.
- Stassi G, Todaro M, Richiusa P, Giordano M, Mattina A, Sbriglia MS, Lo-Monte A, Buscemi G, Galluzzo A & Giordano C 1995 Expression of apoptosis-inducing CD95 (Fas/Apo-1) on human beta-cells sorted by flow-cytometry and cultured in vitro. *Transplantation Proceedings* **27** 3271–3275.
- Stassi G, De Maria R, Trucco G, Rudert W, Testi R, Galluzzo A, Giordano C & Trucco M 1997 Nitric oxide primes pancreatic beta cells for Fas-mediated destruction in insulin-dependent diabetes mellitus. *Journal of Experimental Medicine* **186** 1193–1200.
- Suarez-Pinzon W, Sorensen O, Bleackley RC, Elliott JF, Rajotte RV & Rabinovitch A 1999 Beta-cell destruction in NOD mice correlates with Fas (CD95) expression on beta-cells and proinflammatory cytokine expression in islets. *Diabetes* **48** 21–28.
- Thomas HE, Parker JL, Schreiber RD & Kay TW 1998 IFN-gamma action on pancreatic beta cells causes class I MHC upregulation but not diabetes. *Journal of Clinical Investigation* **102** 1249–1257.
- Thomas HE, Darwiche R, Corbett JA & Kay TW 1999 Evidence that beta cell death in the nonobese diabetic mouse is Fas independent. *Journal of Immunology* **163** 1562–1569.
- Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA & Nagata S 1992 Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* **356** 314–317.
- Witt S, Ziegler B & Ziegler M 1989 Detection of islet cell specificity of monoclonal islet cell surface antibodies by means of double-staining immunofluorescence. *Acta Histochemica* **86** 111–115.
- Yamada K, Takane-Gyotoku N, Yuan X, Ichikawa F, Inada C & Nonaka K 1996 Mouse islet cell lysis mediated by interleukin-1-induced Fas. *Diabetologia* **39** 1306–1312.
- Zumsteg U, Frigerio S & Hollander GA 2000 Nitric oxide production and Fas surface expression mediate two independent pathways of cytokine-induced murine beta-cell damage. *Diabetes* **49** 39–47.

Received in final form 4 November 2002

Accepted 11 November 2002