Biochemical and Biophysical Research Communications **290,** 443–451 (2002)
doi:10.1006/bbrc.2001.6215, available online at http://www.idealibrary.com on IDEAL®

Cell Surface Trafficking of Fas in NIT-1 Cells and Dissection of Surface and Total Fas Expression

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Received December 5, 2001

The appearance of Fas receptor at the surface of pancreatic β-cells affected by progressive insulitis strongly suggests that Fas-mediated β-cell apoptosis plays an important role in the pathogenesis of type 1 diabetes. In support of this concept, the present study has shown that islet cells from NOD mice and the β-cell line NIT-1 respond to the proinflammatory cytokines IL-1β and IFN-γ with Fas surface expression in a dose- and time-dependent manner. Moreover, the prevention of cytokine-induced surface Fas expression by actinomycin D, cycloheximide, and brefeldin A demonstrated that trafficking of Fas to the β-cell surface requires RNA and protein synthesis and, in addition is critically dependent on intracellular protein transport. Compared with total cellular Fas protein, the amount of Fas at the cell surface was relatively small and indicated that Fas is preferentially expressed in cytoplasmic compartments of NIT-1 cells. It is concluded that inflammatory insults specifically induce translocation of Fas to the β-cell surface and that interference with cell surface Fas expression is a new strategy to improve β-cell survival in inflamed islets. © 2002 Elsevier Science

Key Words: **Fas receptor; β-cell, apoptose; autoimmune β-cell destruction; NIT-1 cells; diabetes.**

Fas, also known as CD95 or APO-1, is a cell surface receptor of the tumor necrosis factor (TNF) receptor superfamily that transduces apoptotic signals critical for immune homeostasis and tolerance (1–3). The Fas protein is a 317-amino-acid-long membrane glycoprotein and consists of an extracellular domain, a transmembrane domain, and an intracellular domain representing important functional regions of the Fas receptor (3–5). Binding of Fas to its ligand FasL predominantly expressed on activated T cells (2) is mediated by cysteine-rich domains (CRD) 2 and 3 (5–7) and triggers a cascade of cytoplasmic events which result in cellular degradation and cell death (8, 9).

In the mouse abundant expression of Fas was found in thymus, liver, kidney, lung, ovary, heart and in activated lymphocytes (10). Whereas Fas surface expression is absent in resting insulin-producing β-cells (11), pancreatic β-cells express Fas at the cell surface in autoimmune type 1 diabetes (IDDM) when the islets are infiltrated by mononuclear cells (11). These immunopathological processes, also termed insulitis, lead to the destruction of pancreatic β-cells and, finally, to insulin deficiency (12).

The importance of the Fas/FasL-pathway for β-cell apoptosis was highlighted by histological investigations on pancreatic biopsies taken from subjects with newly diagnosed type 1 diabetes. Thus, Fas^+ and apoptotic β-cells surrounded by FasL-positive T-cells have been demonstrated in infiltrated islets of newly diagnosed diabetic children and NOD mice (11, 13, 14). *In vitro* experiments have revealed that proinflammatory cytokines such as IL-1β, IFN-γ and TNF-α produced by islet-infiltrating mononuclear cells (15) induce both Fas m-RNA synthesis (16–19) and Fas protein expression at the surface of β-cells in rodent and human islets (14, 16, 17, 20). Consistent with these findings, β-cell destruction was prevented when the Fas/FasL pathway was blocked by administration of anti-Fas antibody. Similarly, NOD mice (lpr/lpr) with a genetic defect in the Fas gene did not develop diabetes nor were islets derived from these animals attacked by the im-

Abbreviations used: ACT-D, actinomycin D; BFA, brefeldin A; CHX, cycloheximide; CRD, cysteine-rich domains; DAPI, diamidinophenylindole; sFAS, soluble Fas; FCS, fetal calf serum; FasL, Fas ligand; FITC, fluorescein isothiocyanate; IL-1β, interleukin-1 β; IFN-γ, interferon gamma; LMA, light microscopic appearance; NO, nitric oxide; PE, phycoerythrin; PI, propidium iodide; TNF, tumor necrosis factor.

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0006-291X/02 $35.00

mune system (21, 22). However, these findings have been discussed controversially. On the one hand, a pathogenic role for Fas/FasL interactions in autoimmune diabetes has been postulated, but, on the other hand, the absence of diabetes development in Fas-deficient NOD lpr/lpr mice has been associated with immune defects in the lpr mouse other than Fas deletion (20, 22–24).

Recent studies circumvented this problem by using newly generated NOD mouse strains which either failed to develop diabetes (NOD-*gld*/+ mice) or showed both a delayed onset and a reduced incidence of autoimmune diabetes (NOD-*lpr*/*lpr*-*scid*/*scid*) after adoptive transfer of diabetogenic NOD spleen cells (25). Notably, in spontaneously diabetic NOD mice syngenic islets transplanted beneath the kidney capsule were protected from autoimmune β-cell destruction by a FasL neutralizing antibody. Likewise, the invasion of leukocytes and the level of apoptosis of β-cells were markedly reduced in these grafts (26).

In view of the above reports (21–33), autoimmune β-cell destruction is a very complex process and the pathogenic role of Fas-mediated β-cell death in relation to other apoptotic pathways is still unknown and needs further elucidation. Therefore, it was the aim of this study to examine the relationship between surface and total Fas expression in cytokine-exposed, transformed β-cells and to clarify whether Fas is expressed predominantly at the surface or in the cytoplasm of β-cells. Furthermore, the translocation of Fas to the β-cell surface and its prevention by inhibition of protein transport as well as its dependency on RNA and protein synthesis was addressed.

MATERIALS AND METHODS

Reagents. All reagents originated from Sigma (Deisenhofen, Germany) unless otherwise specified. Recombinant murine IL-1β and IFN-γ were obtained from Dunn (Asbach, Germany). Reagents for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) were exclusively supplied by Bio-Rad (Munich, Germany). Fas-antibodies Jo2 and M-20 and the M-20 Fas peptide for blocking experiments were purchased from BD-PharMingen (Heidelberg, Germany) and Santa Cruz Biotechnology (Heidelberg, Germany), respectively. Secondary antibodies used for flow cytometry were obtained from Dianova (Hamburg, Germany) and BD-PharMingen (Heidelberg, Germany). Alkaline-phosphatase labeled antibodies for Western blotting were purchased from Dianova (Hamburg, Germany).

NIT-1. The pancreatic β-cell line NIT-1 (27) was purchased from ATCC (ATCC No. CRL-2055; Rockville, MD) and cultured according to the recommendations of ATCC in F-12 Ham's medium (F12K) containing 10 mmol/L D-glucose supplemented with sodium bicarbonate and 10% heat-inactivated fetal calf serum (Invitrogen life Technologies, Karlsruhe, Germany) in 25-cm^2 tissue culture flasks (Greiner, Frickenhausen, Germany). NIT-1 cells were maintained at 37°C in a 5% CO_2/95% air mixture and were passaged at weekly intervals by trypsination (Invitrogen life technologies, Karlsruhe, Germany).

Aliquots of 2×10^5 NIT-1 cells were transferred into the wells of 24-well tissue culture plates (NUNC, Wiesbaden, Germany). After 48 h, medium was changed and the cells were exposed to IL-1β and IFN-γ or were kept untreated as controls. In experiments which included brefeldin A (BFA, 10 μg/ml), cycloheximide (CHX, 5 μg/ml) and actinomycin D (ACT-D, 10 μg/ml), NIT-1 cells were cocultured with cytokines and inhibitors for 6 h. For induction of apoptosis, FasL (Alexis, Grünberg, Germany) was added (100 ng/ml) to IL-1β and IFN-γ treated NIT-1 cells as well as to untreated controls. Immediately after cell culture, NIT-1 cells were inspected for their light microscopic appearance (LMA) to evaluate the integrity of the cell membranes, confluence of the monolayer and the portion of dead cells. The LMA index was graded from 1–3 (3 standing for very good, 2 for medium and 1 for bad LMA). Finally, cells were harvested by trypsin treatment and their viability was determined by propidium iodide (PI, 50 μg/ml) exclusion and flow cytometric analysis.

Islet isolation and culture. NOD mice were purchased from Møllegard-Breeding-Centre (M&B, A/S, Denmark) and bread at the animal facilities of the Institute of Diabetes "Gerhardt Katsch" Karlsburg. Islets were isolated according to the method of Thomas *et al.* (28). Briefly, pancreata were digested by cannulation of the bile duct and infusion of ice-cold collagenase P (1.5 mg/ml; Roche, Mannheim, Germany) solution *in situ*. After surgical removal of the pancreas, the entire gland was incubated at 37°C for 20 min. Islets were separated from the exocrine tissue by shaking for 1min. The islets were washed twice and purified using a Dextran gradient(29). The islets were collected from the upper interphase of the Dextran gradient, hand picked and cultured (37°C, 5% CO_2) in RPMI 1640 supplemented with 10% FCS and antibiotics.

Fas staining. Surface and total Fas expression in NIT-1 cells was investigated using standard protocols for flow cytometry. Briefly, surface Fas expression was analyzed on viable NIT-1 cells using the hamster anti-Fas antibody Jo2 (2 μg/ml) and hamster immunoglobulins (2 μg/ml) as negative control (BD Pharmingen, Heidelberg, Germany), respectively. The primary antibody was detected by biotinylated anti-hamster IgG (Dianova, Hamburg, Germany) and Streptavidin-PE (BD Pharmingen, Heidelberg, Germany). Viable NOD mouse thymocytes of which 89.2 ± 1.7% are Fas^+ ($n = 8$) were used as positive control.

To analyze total expression of Fas, NIT-1 cells were fixed and permeabilized using Permeafix (Ortho Diagnostics Systems, Neckargemünd, Germany) for 45 min at room temperature in the dark. After washing with PBS (BioWhittaker, Verviers, Belgium) containing 5% FCS and EDTA (0.2 mmol/L), NIT-1 cells were incubated with the polyclonal rabbit anti-Fas antibody M-20 (2 μg/ml) raised against a peptide mapping at the carboxy terminus of the mouse Fas molecule for 45 min followed by FITC-labeled anti-rabbit IgG. Rabbit IgG (Santa Cruz Biotechnology, Heidelberg, Germany) was used as negative control. Permeabilized NOD mouse thymocytes abundantly expressing Fas were used as positive control (82.7 ± 3.1% Fas^+; $n = 4$). The samples were analyzed on an EPICS/XL flow cytometer (Coulter, Krefeld, Germany) equipped with an air-cooled 488-nm argon laser. System II Software, version 3.0 (Coulter, Krefeld, Germany) was run for data acquisition and for analysis of the percentage and mean fluorescence intensity of Fas^+ cells.

Hypodiploid nuclei. Hypodiploid nuclei were determined by flow cytometry according to the protocol of Thomas *et al.* (20). Briefly, both adherent and nonadherent cells were harvested, centrifuged at 200*g* for 10 min, and washed twice with phosphate-buffered saline. The cells were gently resuspended in a hypotonic PI solution (50 μg/ml) containing 0.1% sodium citrate and 0.1% Triton X-100 and afterwards incubated at 4°C overnight in the dark. To permit doublet discrimination, the FL3 peak was assigned to AUXiliary and impulse height versus impulse area was measured. Fluorescence of PI was acquired in the log mode after exclusion of doublets and degraded DNA from cell debris. Hypodiploid nuclei were identified by the analysis of the sub-G1 area.

Western blotting. Following the exposure to cytokines, NIT-1 cells and NOD mouse islets were lysed on ice in RIPA buffer (PBS containing 1% Igepal, 0.5% deoxycholic acid and 0.1% SDS) supple-

TABLE 1

Induction of Apoptosis in IL-1β + IFN-γ-Treated NIT-1 Cells (100 + 1000 U/ml) by Coculture with FasL for 24 h

	Viability (%)	LMA index	Hypodiploidy (%)	Chromatin condensation (%)	Nitrite oxide (μM)
Control	83.2 ± 1.3	2.7 ± 0.1	6.4 ± 1.4	2.5 ± 0.6	1.7 ± 0.1
Control + FasL	80.7 ± 1.9	2.4 ± 0.1*	11.8 ± 1.8*	4.4 ± 0.9	2.0 ± 0.2
IL-1β + IFN-γ	72.2 ± 2.9°°	1.8 ± 0.1°°	16.4 ± 2.7°°	7.9 ± 1.7°°	4.0 ± 0.5°°
IL-1β + IFN-γ + FasL	57.6 ± 4.7**°°	1.4 ± 0.1*°°	30.5 ± 3.3**°°	22.3 ± 3.6**°°	5.1 ± 0.7°°

Note. Data are given as means ± SE from at least 8 individual experiments (*$P < 0.05$, **$P < 0.01$ vs treatment without FasL; °$P < 0.05$, °°$P < 0.01$ vs untreated control).

mented with freshly prepared protease inhibitor cocktail according to the supplier's protocol (Roche, Mannheim, Germany). After 30 min lysis, samples were sonicated for three impulses, incubated again for 30 min on ice and centrifuged at 10,000*g*. Protein concentrations in supernatants were determined by the Lowry technique and equal amounts of proteins (30 μg total protein/slot) were separated by SDS–polyacrylamide (12%) gel electrophoresis. The proteins were blotted onto polyvinylidene difluoride membranes (PVDF) using a tank blotting system (Bio-Rad, Munich, Germany). The membranes were subsequently blocked in 100 mM Tris-buffered saline (pH 7.5) containing 0.9% NaCl and 3% BSA. Afterward the membranes were incubated with M-20 Fas antibody (0.4 μg/ml) followed by alkaline phosphatase-conjugated antibodies to rabbit immunoglobulins. Rabbit IgG was used as negative control. The specificity of M-20 binding for Fas was tested in competition studies using the corresponding Fas control peptide at a 5-fold excess. The immunoreaction was visualized by the SigmaFast BCIP/NBT substrate. The blots were scanned and analyzed by measuring the average counts of pixels within the object using the Molecular Imager Software (Bio-Rad, Munich, Germany).

Nitric oxide synthesis. The NO production was measured as nitrite accumulation in conditioned media as determined by the Griess reaction. In brief, 150 μl of cell-free medium was mixed with an equal volume of the Griess reagent (one part 0.1% naphthylethylene diamine dihydrochloride and one part 1% sulfanilamide 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 absorbence was measured at 540 nm on an immunoreader (ELx800UV; BioTek Instruments, Winooski, U.S.A.).

Chromatin condensation. NIT-1 cells were fixed at 4°C in 4% phosphate-buffered paraformaldehyde for 30 min. After washing, cells were permeabilized (BD FACS Permeabilizing Solution I, BD-Pharmingen, Heidelberg, Germany) as recommended by the supplier, washed again and stained with DAPI (50 ng/ml) at 37°C for 20 min. Analysis was performed using an fluorescence microscope (Axioscope, Zeiss, Jena, Germany).

Statistical analysis. Results are expressed as means ± SE. Statistical significance was assessed using unpaired Student's t-test or analysis of variance (ANOVA). If ANOVA revealed significant differences, multiple comparison to the control group was performed by the Dunnett's test. Differences were considered to be statistically significant at a level of $P < 0.05$.

RESULTS

Surface Fas Expression

NIT-1 cells were exposed to the cytokine combination IL-1β + IFN-γ (50 + 500, 100 + 1000, 200 + 2000 U/ml) for 6, 12, 24, and 48 h. The biological activity of IL-1β and IFN-γ was demonstrated by the significantly ($P < 0.01$) increased release of NO from NIT-1 cells after cytokine exposure (Table 1). Fas surface expression was induced on NIT-1 cells within 6 h of cytokine exposure (Fig. 1A) and the percentage of Fas$^+$ NIT-1 cells increased up to 12 h of cytokine exposure. Exposure to cytokines for more than 24 h resulted in stable surface Fas expression as reflected by plateau percentage levels of Fas$^+$ NIT-1 cells (Figs. 1A and 1B). Induction of surface Fas expression was confirmed on islet

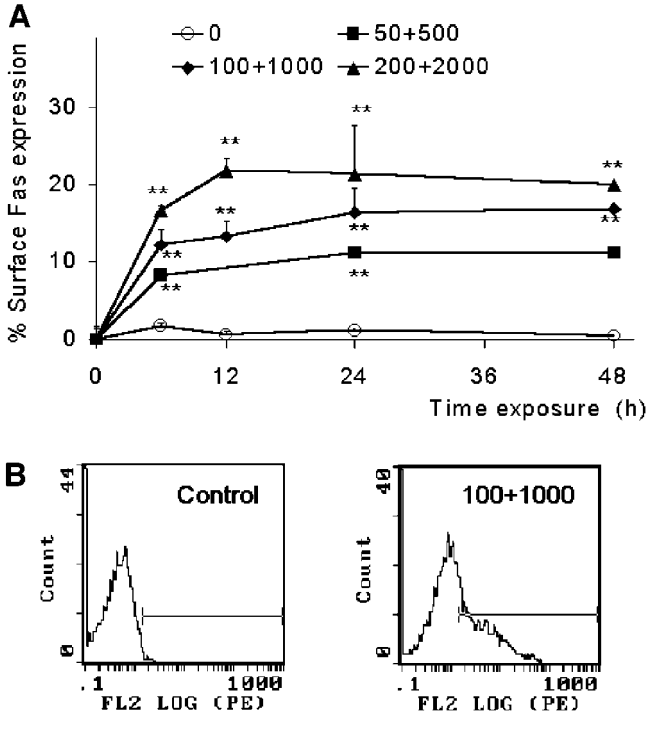


FIG. 1. (A) Time course of cytokine-induced surface Fas expression on NIT-1 cells exposed to IL-1β + IFN-γ (50 + 5000, 100 + 100; 200 + 1000 U/ml) for 6, 12, 24, 36, and 48 h. Data (mean ± SE) from at least six individual experiments are shown (*$P < 0.05$; **$P < 0.01$ vs untreated controls). (B) Representative flow cytometric histograms of surface Fas expression on NIT-1 cells cocultured for 24 h with IL-1β + IFN-γ (100 + 1000 U/ml) or kept untreated as controls.

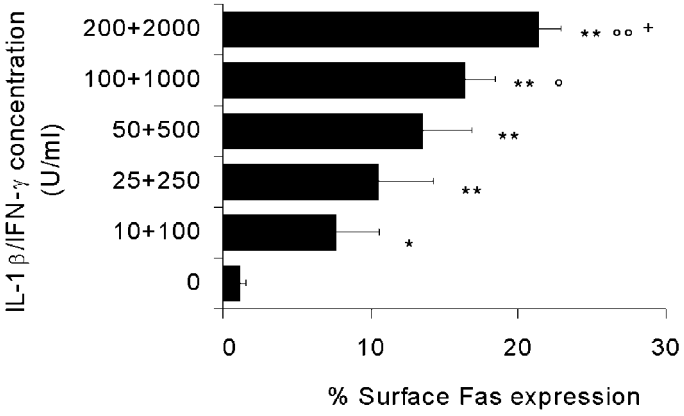


FIG. 2. Surface Fas expression on NIT-1 cells exposed for 24 h to different doses of IL-1β and IFN-γ ranging from 10 + 100 to 200 + 1000 U/ml. Data are given as means ± SE from at least four individual experiments (*$P < 0.05$; **$P < 0.01$ vs untreated controls; °$P < 0.05$,°°$P < 0.01$ vs treatment with 10 + 100 U/ml IL-1β + IFN-γ; $^{+}P < 0.05$ vs treatment with 25 + 250 U/ml IL-1β + IFN-γ).

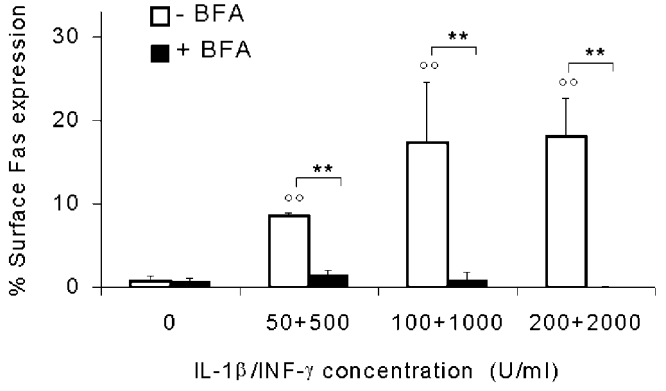


FIG. 3. Effect of brefeldin A on cytokine-induced surface Fas expression. NIT-1 cells were exposed to IL-1β and IFN-γ in the presence of BFA for 6 h and then analyzed be flow cytometry (**$P <$ 0.01 vs IL-1β + IFN-γ exposure in presence of BFA; °°$P < 0.01$ vs untreated control).

cells from NOD mice ($P < 0.01$) exposed to 100 + 1000 U/ml IL-1β+IFN-γ (56.3 ± 4.9% vs 9.4 ± 2.4% in untreated controls; $n = 4$).

The correlation of surface Fas expression and cytokine-dosage is shown in Fig. 2. Beginning at concentrations of 25 + 250 U/ml, the combination IL-1β and IFN-γ induced significantly increased percentages of Fas^{+} NIT-1 cells versus untreated controls. Furthermore, 100 + 1000 and 200 + 1000 U/ml of IL-1β and IFN-γ induced significantly ($P < 0.01$) higher levels of Fas expression in NIT-1 cells than the low dose of 10 + 100 U/ml did. Similarly, exposure of NOD-mouse islets to enhanced doses of IL-1β and IFN-γ resulted in steadily increasing surface Fas expression (25 + 250 U/ml: 37.5 ± 3.8%, 50 + 500 U/ml: 42.5 ± 4.0%, 100 + 1000 U/ml: 56.3 ± 4.9%; $P < 0.05$ for 25/250 vs 100 + 1000 U/ml IL-1β + IFN-γ). The functional integrity of Fas was shown by exposure of NIT-1 cells to IL-1β and IFN-γ (100 + 1000 U/ml) for 24 h in the presence of FasL. This treatment resulted in a significant impairment ($P < 0.01$) of cell viability, elevated percentages of hypodiploid nuclei and worsening of the LMA index. In addition, FasL enhanced the appearance of chromatin condensation (Table 1).

Fas Surface Expression Depends on Protein Transport Mechanisms and Requires Protein and RNA Synthesis

Disruption of intracellular protein transport processes by BFA abolished cytokine-induced trafficking of Fas to the surface of NIT-1 cells (Figs. 3 and 4; $n = 5$). This effect could not be overcome by increasing the IL-1β + IFN-γ concentrations ($n = 3$) because Fas surface expression was not restored under these conditions. Surface Fas expression was totally suppressed by ACT-D and a 85% inhibition was seen in the presence of CHX (16.1 ± 1.89% vs 2.44 ± 0.7% Fas^{+} NIT-1 cells; Fig. 4). To verify that BFA treatment wasn't noxious to NIT-1 cells, cell viability and the cell condition as well as the percentage of hypodiploid nuclei were determined. Exposure of NIT-1 cells to BFA as well as ACT-D and CHX neither induced cell death nor resulted this treatment in DNA fragmentation (Table 2).

Total Fas Expression

Total Fas expression was analyzed by flow-cytometry in permeabilized NIT-1 cells after exposure to IL-1β and IFN-γ (50 + 500, 100 + 1000, 200 + 1000 U/ml) for 12, 24, and 48 h using the Fas antibody M-20 and revealed that the level of total Fas expression was independent of the IL-1β and IFN-γ concentration tested (Fig. 5). Notably, a high percentage of untreated

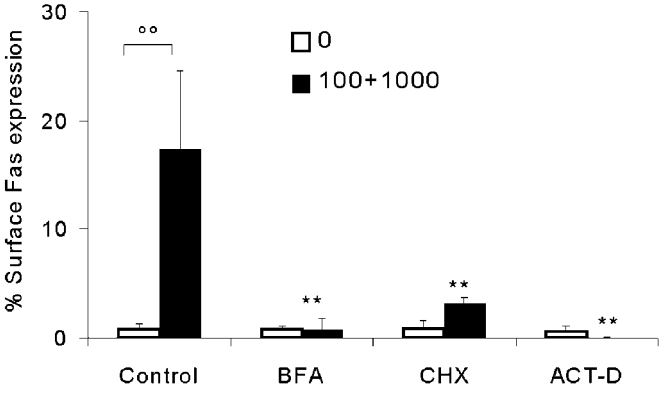


FIG. 4. Effect of brefeldin A, cycloheximide, and actinomycin D on cytokine-induced surface Fas expression. NIT-1 cells were exposed to IL-1β and IFN-γ in the presence of BFA, CHX or ACT-D for 6 h and then analyzed by flow cytometry (**$P < 0.01$ vs IL-1β + IFN-γ exposure without inhibitors; °°$P < 0.01$ vs untreated control).

TABLE 2

Effects of BFA, CHX, or ACT-D on Viability (%) and Hypodiploidy (%) of IL-1β + IFN-γ-Treated NIT-1 Cells (100 + 1000 U/ml) and Untreated Controls after 6 h Culture

	Coculture							
	None		BFA		CHX		ACT-D	
	Control	IL-1β + IFN-γ	Control	IL-1β + IFN-γ	Control	IL-1β + IFN-γ	Control	IL-1β + IFN-γ
Viability	90.5 ± 2.2	88.8 ± 7.7	90.1 ± 1.8	90.2 ± 4.9	89.5 ± 1.7	89.6 ± 1.5	81.2 ± 2.7	79.6 ± 2.8
Hypodiploidy	4.5 ± 1.4	6.9 ± 5.9	5.0 ± 4.1	4.1 ± 4.2	4.6 ± 1.2	5.2 ± 1.5	2.3 ± 0.3	2.5 ± 0.1

Note. Data are given as means ± SE from at least five independent experiments.

NIT-1 cells was also found to be Fas positive. Binding of M-20 to NIT-1 cells was completely inhibited after competition using the immunogen peptide as detected by flow-cytometry.

To further specify these findings, Fas expression in NIT-1 cells was analyzed by Western blotting after exposure to IL-1β and IFN-γ (100 + 1000 U/ml) for 6, 12, and 24 h. Fas expression was evident in untreated as well as cytokine-treated NIT-1 cells (Fig. 6). Densitometric analysis of Western blots demonstrated that NIT-1 cells expressed 110.2%, 103.9% and 82.8% (n = 3) of total Fas-expression detected in controls (considered as 100%) after 6, 12, and 24 h exposure to IL-1β and IFN-γ (100 + 1000 U/ml), respectively. The immunoreactivity of M-20 in Western blotting was completely blocked by pretreatment with the Fas peptide that was used for raising the Fas antibody.

DISCUSSION

The morphological hallmark of autoimmune β-cell destruction is the lymphocytic infiltration of pancreatic

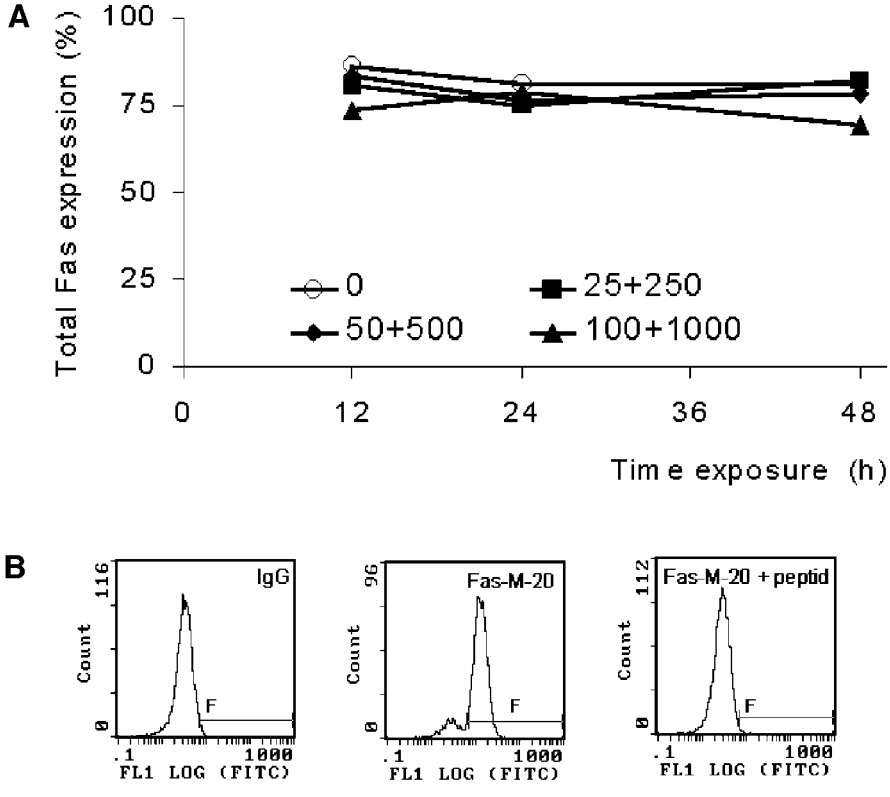
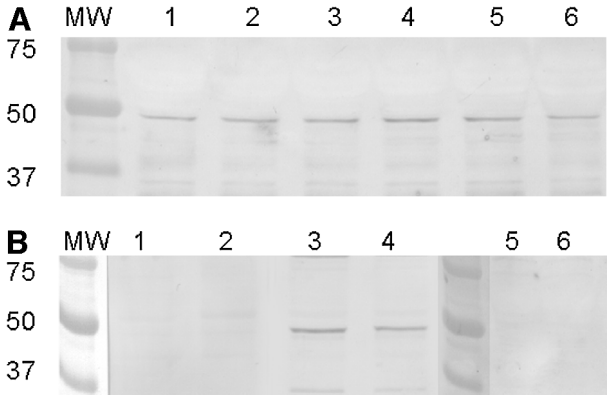


FIG. 5. (A) Flow-cytometric examination of total Fas expression using the Fas antibody M-20. NIT-1 cells were incubated with IL-1β and IFN-γ as indicated and permeabilized before Fas staining. Data are given as means ± SE from at least four individual experiments. (B) Representative histograms of total Fas staining using M-20 matched to an isotype control were obtained from untreated NIT-1 cells. Binding of M-20 was completely inhibited by the peptide used for raising the antibody.



Lane:	1	2	3	4	5	6
Time (h):	6		12		24	
Treatment:	Control	IL1β + IFN-γ	Control	IL1β + IFN-γ	Control	IL1β + IFN-γ
M-20	+	+	+	+	+	+

Lane:	1	2	3	4	5	6
Time (h):	24				12	
Treatment:	Control	IL1β + IFN-γ	Control	IL1β + IFN-γ	Control	IL1β + IFN-γ
M-20	+	+	+	+	−	−
Peptide	+	+	−	−	−	−
Rabbit IgG	−	−	−	−	+	+

FIG. 6. (A) Western blots of NIT-1 cells treated with IL-1β and IFN-γ (100 + 1000 U/ml; lanes 2, 4, and 6,) or kept untreated as controls (lanes 1, 3, and 5). A representative experiment of NIT-1 cells lysed after 6 h (lanes 1 and 2), 12 h (lanes 3 and 4) and 24 h (lanes 5 and 6) exposure time is shown. Blots were immunostained with Fas-antibody M-20 (lanes 1–6). The molecular weight markers are demonstrated in lanes MW (kDa). (B) Inhibition of M-20 binding to Fas by the Fas peptide used for raising the antibody. NIT-1 cells were treated with IL-1β and IFN-γ (100 + 1000 U/ml; lanes 2, 4, and 6,) or kept untreated as controls (lanes 1, 3, and 5). Binding of M-20 was abolished after competition with the peptide (lanes 1, 2). The reactivity of rabbit immunoglobulins used as negative control is demonstrated in lanes 5 and 6–10). The molecular weight markers are demonstrated in lane MW (kDa).

islets, termed insulitis (12). β-cells in inflamed islets are surrounded by activated immune cells such as macrophages, NK cells, B lymphocytes, $CD4^+$- and $CD8^+$ T cells producing various proinflammatory and type 1 cytokines (15). IL-1β, TNF-α and IFN-γ have been reported to be abundantly present during the insulitis process and to damage β-cells as indicated by impaired β-cell function, appearance of DNA fragmentation and activation of inducible nitric oxide synthetase generating β-cell toxic nitric oxide (15, 30–33). Likewise, cytokines prime β-cells for the Fas/FasL-pathway of apoptosis by inducing surface Fas expression (14, 16, 17, 20, 34). Up to now little is known regarding the processes involved in translocation of Fas to the β-cell surface. Addressing this issue, we asked in this study whether the translocation of Fas to the β-cell surface requires protein transport mechanisms and depends on RNA and protein synthesis. Additionally, we investigated Fas expression at the protein level by measuring both surface and total Fas appearance after stimulation of transformed and primary β-cells deriving from NOD mouse by IL-1β and IFN-γ.

First, we monitored cytokine-induced Fas expression of NIT-1 cells in a time- and dose-dependent fashion. Remarkably, NIT-1 cells responded to cytokine exposure by expression of the Fas receptor on their surfaces already within 6h and reached a plateau after 24 h. Comparable to NIT-1 cells, mouse islets have been reported to present Fas on their surfaces and to express Fas mRNA after 6 h IL-1β-treatment (16). Similarly, β-cells from BB rat and β-TC1 cells responded to IL-1β alone or in combination with IFN-γ with mRNA expression (16, 35). In accordance with our results, Zumsteg *et al.* (17) demonstrated an increase in Fas m-RNA expression in NIT-1 cells after 15 h exposure to IL-1β, IFN-γ and TNF-α that reached a plateau after 24 h along with detection of Fas protein on the surface of NIT-1 cells. Furthermore, surface Fas expression on human β-cells has been reported to be maximal after 24 h of IL-1β exposure (14, 34). Certainly, the dose of IL-1β and IFN-γ was directly related to the amount of Fas protein on NIT-1 and NOD islet cells surfaces. Major differences in the level of surface Fas expression were seen within a cytokine dose range from 10 + 100 to 200 + 1000 U/ml. Within this range 10- and 20-fold elevations of the cytokine dosage resulted in 2.3- and 2.8-fold increases in surface Fas expression on NIT-1 cells, respectively.

To investigate whether cytokine-induced Fas expression is dependent on intracellular transport and protein secretion mechanisms, we induced surface Fas expression by 6 h exposure to IL-1β and IFN-γ in the presence of BFA. BFA is a protein transport inhibitor that redistributes Golgi proteins to the endoplasmatic reticululum or near the microtubular organizing center. The rationale for this short-term exposure comes from following facts. First, we observed a significant increase of surface Fas expression already after 6 h and second extended exposure to BFA is considered to be cytotoxic due to the intracellular accumulation of proteins. We found that the cytokine-induced surface Fas expression in the β-cell line NIT-1 is completely inhibited by BFA. To our knowledge this is the first report clearly demonstrating the absolute requirement of protein transport mechanisms for cytokine-induced surface Fas expression in NIT-1 cells. Furthermore, our results are supported by reports on BFA-mediated blocking of Fas expression and apoptosis of hepatocytes, muscle cells and colorectal tumor cells (36–38). The suppression of translocation of Fas to the β-cell surface could be a new strategy to prevent Fas-induced β-cell apoptosis. The feasibility of this approach has been demonstrated recently in mice suffering from ex-

perimentally induced liver toxicity. In this model, prevention of Fas-induced apoptosis by colchicine resulted in a reduction of Fas receptor trafficking to the cell surface (39).

To assess whether the process of Fas trafficking to the cell surface is dependent on protein and mRNA synthesis, we induced surface Fas expression in the presence of cycloheximide and actinomycin D, inhibitors of protein- and RNA synthesis, respectively. Both inhibitors influenced the ability of NIT-1 cells to translocate Fas to the cell surface. While ACT-D completely abolished cell surface Fas expression, 15% of cytokine-induced Fas expression were unaffected by CHX treatment. Nevertheless, the majority of Fas receptors in NIT-1 cells requires protein synthesis for translocation to the cell surface. Similar to our present results, cytokine-induced surface Fas expression in thymocytes was shown to be inhibited by cycloheximide (40). However, Fas up-regulation in anti-CD3-activated thymocytes (40) and UV-B-enhanced Fas expression in several cell lines did not require new protein synthesis (41). In p53-induced surface Fas expression (36), translocation to the cell surface was also unaffected by CHX and ACT-D whereas surface expression of Fas was decreased by ACT-D in tumor cell lines (37, 42). Surface expression of DR4, another member of the TNF receptor family, also relies on new RNA synthesis (43). Thus, the requirement of protein and RNA-synthesis for the translocation of Fas to the cell surface appears to depend on the nature of the inducing stimulus of Fas surface expression and on the cell type under investigation (36). In β-cells, cytokine induced surface Fas expression appears to be a quick answer which depends on protein- and RNA synthesis as well as protein transport mechanisms.

To compare the time-course of cytokine-induced surface and total Fas expression, we permeabilized cytokine-treated as well as untreated NIT-1 cells before immunostaining and measured the level of total Fas expression by flow cytometry. To our surprise, Fas protein was detected in the majority of permeabilized untreated and cytokine-treated NIT-1 cells and cytokine exposure had no augmenting effect on total Fas expression in NIT-1 cells. These results inspired us to investigate Fas expression in NIT-1 cells by Western blotting. Our results obtained by Western blotting confirmed the flow cytometric data on total Fas expression and demonstrated the presence of Fas in lysates of cytokine-treated and untreated NIT-1 cells. Moreover, Fas protein was detected in NOD mouse islets independent of cytokine exposure (data not shown). Consistent with our present findings, Fas expression was also demonstrated by Ingelsson *et al.* (44) in NOD mouse islets by Western blotting. Indeed, Fas expression was preferentially detectable in islets of older mice, indicating an age-dependent increase of Fas expression in islets of NOD mice (44) probably related to progression of destructive insulitis. Taken together, comparison of total and surface Fas expression of cytokine-treated NIT-1 cells revealed a high percentage of NIT-1 cells being positive for total Fas expression and a lower percentage of NIT-1 cells being positive for surface Fas expression. What might be the reasons for these unexpected results? For detection of total Fas expression we used the polyclonal antibody M-20 directed against a linear Fas epitopes while the Jo2 antibody recognizes a conformational Fas epitope that is sensitive to SDS–PAGE, blotting and fixation. The specificity of the immune reactions detected by M-20 was proven by blocking experiments with the same peptide that have been employed for generation of the antibodies. On the proviso that the antibody is specific for Fas, we seem to look at different species of the Fas protein. The Fas receptor translocation to the cell surface of NIT-1 cells is rapidly induced by cytokines and requires new m-RNA synthesis (17) as supported by several studies on cytokine exposed rodent and human islets (16, 18). The level of total Fas expression was constant and independent of cytokine exposure. High levels of total Fas expression were also reported in muscle cells with transient surface Fas expression (36), indicating that at least in some cell types Fas may be stored in intracellular compartments. This hypothesis is supported indirectly by recent reports (35, 45). Analysis of cytokine-induced genes in pancreatic β-cells by high-density oligonucleotide arrays failed to detect changes in the Fas gene expression level of purified rat β-cells after 6 h cytokine exposure (35). In addition, after proteome analysis of IL-1β induced changes in protein expression in rat islets, effects on Fas have not been reported so far (45). Further insight into the relation between surface and total Fas protein expression was presented by Peter and colleagues in a study using cell lines with a high level of Fas expression (46). The authors characterized Fas as a stable long-living protein and observed that even cells with a high level of surface Fas expression synthesized Fas at a low rate (46). In addition, in comparison to total Fas protein, Fas expressed at the cell surface was smaller and low sialylated and these different biochemical features were related to the susceptibility for Fas-mediated apoptosis (46, 47). Thus it is conceivable that β-cells share similar characteristics of Fas protein expression.

To induce the Fas pathway of apoptosis in NIT-1 cells similar to the *in vivo* situation, we used a cross-linked soluble FasL reported to be as active as membrane-bound FasL in its interaction with the Fas receptor (48). Using this approach, we circumvented the limitations of recombinant FasL and agonistic Fas antibodies. Recombinant FasL has been shown to exert variable apoptosis-inducing effects while agonistic antibodies apparently may interact with Fas differently to FasL (5, 49). Under these conditions we were able to

demonstrate the functional integrity of surface Fas receptors expressed on the surface of NIT-1 cells by detecting typical signs of apoptosis such as appearance of condensed chromatin and hypodiploid nuclei. Thus, NIT-1 cells seem to represent an appropriate model for investigating Fas-induced apoptosis in pancreatic β-cells.

In conclusion, NIT-1 cells appear to express Fas constitutively in their cytoplasm whereas surface Fas expression apparently represents a specific functional cellular response to harmful proinflammatory insults. The induction of the Fas receptor at the surface of NIT-1 cells by proinflammatory cytokines within 6 h indicates that a relatively short period of time is sufficient to prime β-cells for Fas-mediated cell death. Also, the cytokine dosage can modulate the extent of Fas surface expression whereas total Fas expression is not augmented by cytokine exposure. Therefore, the amount of proinflammatory cytokines produced by islet-invading immune cells may determine the amount of Fas receptor translocated to the cell surface, and thus can influence the sensitivity of β-cells for Fas-induced apoptosis. Transport of cytokine-induced Fas to the cell surface requires active protein transport mechanisms and mRNA synthesis. A small portion of cytokine-induced Fas surface expression appears to be independent of protein synthesis. However, the vast majority of Fas surface expression is prevented by inhibition of protein synthesis, indicating that protein synthesis is a prerequisite for trafficking of Fas to the surface of the pancreatic β cell. The blockade of cytokine-induced surface Fas expression may be a new strategy to prevent Fas-induced β-cell apoptosis.

ACKNOWLEDGMENTS

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 and Dr. B. Hehmke for critical reading of the manuscript.

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