Efficient delivery of siRNA into cytokine-stimulated insulinoma cells silences Fas expression and inhibits Fas-mediated apoptosis

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Abstract Fas/FasL interactions have been proposed as a potentially important mechanism mediating β-cell death in type 1 diabetes. Recent investigations suggest RNA interference, afforded by small interfering RNAs (siRNA), can provide specific and robust gene silencing in mammalian cells. The current study attempted to investigate the effects of silencing Fas expression with siRNA on Fas-mediated apoptosis in mouse insulinoma cells following cytokine incubation. Our results indicate that siRNA is capable of rapid inhibition of cytokine-induced Fas mRNA production and cell surface Fas protein. A complete suppression of the total Fas protein was only observed after prolonged incubation with siRNA, suggesting a slow turn-over of Fas protein. Moreover, siRNA significantly inhibited Fas-mediated β-cell apoptosis assessed by Caspase-3 and terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling assays, the extent of which positively correlated with the level of cell surface Fas. These observations provide additional evidence supporting a role for the Fas-mediated pathway in β-cell destruction, and suggest that siRNA targeting Fas may be of therapeutic value in preventing type 1 diabetes and improving islet cell viability in transplantation.

1. Introduction

Pancreatic β cells are destroyed by autoimmune responses in type 1 diabetes, leading to insulin deprivation and hyperglycemia [1]. Both CD4^{+} and CD8^{+} T cells are thought to be important effectors in β cell death. Multiple mechanisms contribute to this destructive process [2], among which CD4^{+} T cells appear to damage β cells through at least two independent pathways mediated by Fas and nitric oxide (NO) [3]. Proinflammatory cytokines secreted by infiltrating lymphocytes can upregulate Fas expression on β cells, thus activating Fas signaling [4,5]. Cytokines can also stimulate inducible nitric oxide synthase (iNOS) and subsequent NO formation, leading to impairment in insulin secretion and DNA fragmentation [3].

Fas/FasL interaction has been suggested to play an important role in the natural development and recurrence of autoimmune diabetes in murine models [4–10]. The involvement of Fas/FasL system may also hold true in the development of human type 1 diabetes, as suggested by histological investigation of pancreas specimens from new onset patients [11]. However, our understanding of Fas functions in autoimmune diabetes is yet to be complete [12]. Despite a potentially important role of Fas in β cell death, inhibition of this pathway has not been fully explored as a diabetes treatment [10,13]. Anti-FasL antibody has been tested in non-obese diabetic (NOD) mice, but only modest success was observed [10].

A growing body of the literature has provided convincing evidence that RNA interference (RNAi), via small interfering RNA (siRNA), can silence gene expression in a specific and potent manner in mammalian cells [14,15]. Rapid progress in siRNA delivery and function in vivo has further stimulated interests in exploring this approach as a therapeutic platform for human pathology [16–19]. As such, we believe siRNA may represent a viable means for efficient silencing of pro-apoptotic genes including Fas and iNOS and may thereby protect β cells from cytokine-induced apoptosis. In the current study, we chose Fas as the first target to assess the potentiality of using siRNA to inhibit β cell death. To achieve this goal, we developed a Fas-targeting siRNA and evaluated its capability in silencing Fas expression and inhibiting Fas-mediated β cell damage in response to proinflammatory cytokines. We first tested this possibility in transformed β cell lines.

2. Materials and methods

2.1. Fas siRNA

The 21-nt siRNA duplex was provided in the 2′-deprotected and desalted form of 2′-O-ACE-RNA (Dharmacon). Cy3-labeled siGLO RISC-free siRNA (Dharmacon) was used to visualize transfection efficiency. Three siRNA sequences were designed based on the murine Fas mRNA sequence (GenBank Accession No. NM_007987) following the Rational siRNA Design algorithm [20]. Fas1 siRNA, beginning at nt 415, 5′-GUG CAA GUG CAA ACC AGA C dTdT-3′ (sense), and 5′-GUC UGG UUU GCA CUU GCA C dTdT-3′ (antisense). Fas2

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siRNA, beginning at nt 924, 5′-AAG CCG AAU GUC GCA GAA C dTdT-3′ (sense), and 5′-GUU CUG CGA CAU UCG GCU U dTdT-3′ (antisense). Fas3 siRNA (control siRNA), beginning at nt 613, 5′-GUA CCG GAA AAG AAA GUG C dTdT-3′ (sense), 5′-GCA CUU UCU UUU CCG GUA C dTdT-3′ (antisense).

2.2. Cell culture, transfection and cytokine treatment

Murine insulinoma NIT-1 cells and βTC-3 cells were used in the study. NIT-1 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (37 °C, 5% CO_2). βTC-3 cells were cultured in DMEM supplemented with 13.5% horse serum, 2.2% fetal bovine serum, 25 mM HEPES, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (37 °C, 5% CO_2). At 30–60% confluency, cells were transfected with 40 nM Fas siRNA complexed with Lipofectamine 2000 (Invitrogen) for 3 days (3-day experiment) or 9 days (first transfection on day 0 and second on day 4, 9-day experiment) prior to cytokine treatment with 100 U/ml mIL-1β and 1000 U/ml mIFN-γ (Sigma) for 16 h. During the 9-day experiment, cells were split 1:2 to avoid over-confluence, if necessary. For induction of apoptosis, cells were incubated with anti-mouse Fas Jo2 antibody (5 μg/ml, Pharmingen) and protein G (2 μg/ml) for 24 h following cytokine exposure. Floating cells were collected and counted. Adherent cells were harvested by trypsin-EDTA for RNA extraction, flow cytometry, and immunocytochemistry.

2.3. Semiquantitative and real-time RT-PCR analysis

Total RNA was extracted using Trizol (Invitrogen) and treated with RNase-free DNase I (Invitrogen). Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed with Access-Quick RT-PCR system (Promega) for 30 cycles. The primers for Fas mRNA were 5″-TGGCTCACAGTTAAGAGTTCATACTCAAGG-TAC-3′ (forward) and 5′-ATTGGTA TGGTTTCACGACTGGAGG-TTCTA-3′ (reverse). The primers for GAPDH mRNA were 5′-TGC-TGAGTATGTCGTGGAGTCTA-3′ (forward), and 5′-AGTGGGA-GTT GCTGTTGAAGTCG-3′ (reverse). Real-time RT-PCR was performed with TaqMan One-Step RT-PCR Master Mix Reagent kit (Applied Biosystems Cat# 4309169) and detected with ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A 25 μl reaction mixture contained 100 ng of total RNA, 12.5 μl 2× Taqman PCR Mix, 0.625 μl 40× reverse transcriptase, 300 nM forward and reverse primers (5′-GCGATTCTCCTGGCTGT-GAA-3′ forward, 5′-TGGCTCAAGGGTTCCATGTT-3′ reverse), and 150 nM TaqMan probe (5′-CTGTGTTCGCTGCGCCTCGTG-3′). Reactions were incubated at 48 °C for 2 min, 95 °C for 10 min, and then amplified for 40 cycles (95 °C for 15 s followed by 60 °C for 1 min in each cycle). Ribosomal RNA levels provide an endogenous control for PCR quantification studies. The TaqMan Ribosomal RNA control Reagents (Applied Biosystems Cat# 4308329) were used for the target quantification normalized to an endogenous control 18s rRNA. As the amplification efficiency of Fas mRNA in βTC-3 cells was approximately equal to that of 18s rRNA, the relative quantification of Fas mRNA in βTC-3 cells was expressed using the $2^{-\Delta\Delta Ct}$ (fold difference) method [21] upon normalization to 18s rRNA and relative to a calibrator (untreated control). Ct is the cycle number at which the amount of amplified gene of interest reaches a fixed threshold, ΔCt = Ct of target gene (Fas) – Ct of endogenous control (18s), and $\Delta\Delta Ct$ = ΔCt of samples – ΔCt of calibrator. As the amplification efficiency of Fas mRNA in NIT-1 cells was different from that of 18s rRNA, standard curves were included in each run. The amount of Fas mRNA and 18s rRNA was calculated from the standard curves, and the relative quantification of Fas mRNA in NIT-1 cells was expressed as (the amount of Fas mRNA divided by the amount of 18s rRNA).

2.4. Flow cytometric analysis

For detection of cell surface Fas, 1×10^6 live cells were incubated with 1:100 Alexa 488-conjugated hamster anti-mouse Fas antibody (Jo2, Pharmingen) or 1:100 Alexa 488-conjugated hamster IgG2 (Pharmingen) as negative control for 30 min at 4 °C. For detection of total Fas, cells were fixed with 1% paraformaldehyde and permeabilized with 0.1% Triton X-100 prior to incubation with 1:100 rabbit anti-mouse Fas antibody (X-20, Santa Cruz) or rabbit IgG as negative control, followed by incubation with 1:3000 Qdot 705-conjugated Goat anti-rabbit IgG (Quantum Dot). For detection of active Caspase-3, cells were fixed, permeabilized and incubated with 1:100 PE-conjugated rabbit anti-Caspase-3 recognizing the active form of the enzyme (Pharmingen), or rabbit Ig detected by biotinylated anti-rabbit Ig and streptavidin-PE as negative control. Cells were analyzed with LSR II (BD Pharmingen, San Diego, CA, USA) acquiring 100,000 events per sample. Unstained cells and isotype controls were run concurrently.

2.5. Immunocytochemistry and TUNEL assay

Cells were cytospun on Superfrost Plus slides (Fisher), air dried for 30 min and stored at −80 °C until staining. For Fas detection, cells were fixed with −20 °C acetone, blocked with 5% goat serum, incubated with 1:20 rabbit anti-mouse Fas antibody (M-20, Santa Cruz), followed by biotinylated goat anti-rabbit IgG, and detected with ABC Alkaline Phosphatase Kit and Vector Blue chromogen (Vectors). Cells were counterstained by Nuclear Fast Red. The terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay was performed with Apoptosis Detection Kit (Upstate). Apoptotic cells with FITC-dUTP-labeled DNA (green fluorescence) were visualized by fluorescent microscopy (Olympus IX-71, Melville, New York, USA) and images were taken at the same exposure time. Cell nuclei were stained with DAPI (blue fluorescence). Percentage of Fas$^+$ cells and apoptotic cells were counted with Microsuite Imaging Software (SIS, Lakewood, Colorado, USA), in a blinded fashion, from six 10× fields per slide and two slides per condition.

2.6. Statistical analysis

All experiments were repeated two or three times with duplicate samples each time. Results were presented as means ± S.E.M. Statistical analysis was performed using one-way ANOVA test and statistical significance is defined as $P < 0.05$.

3. Results and discussion

In the current study, we attempted to evaluate the ability of siRNA targeting Fas in inhibiting Fas-mediated pancreatic β-cell apoptosis in face of proinflammatory cytokines. Two mouse insulinoma cell lines, NIT-1 (derived from NOD mice) and βTC-3 (derived from C57BL/6 mice) cells, were investigated. The purpose of this choice was to test whether Fas siRNA would work in two distinct β cell lines varied in mouse origin and morphological characteristics.

In order to achieve sufficient siRNA transfection, four transfection reagents were tested, including Lipofectamine 2000 (Invitrogen), Transmessenger (Qiagen), siPORT Amine and siPORT Lipid (Ambion). Only Lipofectamine 2000 facilitated successful delivery (>90%) of Cy3-labeled siRNA into both NIT-1 and βTC-3 cells with 85–95% viability (Fig. 1). No Cy3 fluorescence was detected in control cells that were untreated, or treated in the absence of Lipofectamine 2000 or siRNA. A rapid uptake of siRNA was observed within 30 min post transfection (data not shown), and the presence of Cy3 fluorescence persisted for at least four days, albeit at a reduced level and in a lower percentage of cells, suggesting slow degradation and dilution of synthetic siRNA molecules.

In order to investigate the effects of siRNA on Fas-mediated, cytokine-induced apoptosis, cytokine concentrations required titration to minimize the impact of NO pathway. It has been shown that Fas and NO productions in β cells are dose-dependent on cytokines [22–24]. Cell surface Fas levels appeared to continuously increase within a wide range of IL-1β and IFN-γ concentrations [22], while cytokine-stimulated nitrite release reached a plateau at 10–25 U/ml of IL-1β [23,24]. Our preliminary experiments that examined various IL-1β and IFN-γ concentrations on cell surface Fas and nitrite production in