Expression of the Transcription Factor STAT-1α in Insulinoma Cells Protects against Cytotoxic Effects of Multiple Cytokines*

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Destruction of pancreatic islet β-cells in type 1 diabetes appears to result from direct contact with infiltrating T-cells and macrophages and exposure to inflammatory cytokines such as interferon (IFN)-γ, interleukin (IL)-1β, and tumor necrosis factor TNF-α that such cells produce. We recently reported on a method for selection of insulinoma cells that are resistant to the cytokotoxic effects of inflammatory cytokines (INS-1<sub>res</sub>), involving their growth in progressively increasing concentrations of IL-1β plus IFN-γ, and selection of surviving cells. In the current study, we have investigated the molecular mechanism of cytokine resistance in INS-1<sub>res</sub> cells. By focusing on the known components of the IFN-γ receptor signaling pathway, we have discovered that expression levels of signal transducer and activator of transcription (STAT)-1α are closely correlated with the cytokine-resistant and -sensitive phenotypes. That STAT-1α is directly involved in development of cytokine resistance is demonstrated by an increase of viability from 10 ± 2% in control cells to 50 ± 6% in cells with adenovirus-mediated overexpression of STAT-1α (p < 0.001) after culture of both cell groups in the presence of 100 units/ml IFN-γ plus 10 ng/ml IL-1β for 48 h. The resistance to IL-1β plus IFN-γ in STAT-1α-expressing cells is due in part to interference with IL-1β-mediated stimulation of inducible nitric-oxide synthase expression and nitric acid production. Furthermore, overexpression of STAT-1α does not impair robust glucose-stimulated insulin secretion in the INS-1-derived cell line 832/13. We conclude that expression of STAT-1α may be a means of protecting insulin-producing cell lines from cytokine damage, which, in conjunction with appropriate cell-impermeant macroencapsulation devices, may allow such cells to be used for insulin replacement in type 1 diabetes.

Type 1 diabetes is caused by autoimmune destruction of pancreatic islet β-cells. Destruction of β-cells appears to result from direct contact with infiltrating T-cells and macrophages and exposure to inflammatory cytokines such as IFN-γ, IL-1β, and TNF-α that such cells produce (1–5). Insulin replacement by injection, the current treatment, fails to replicate the precise control of fuel homeostasis afforded by normal regulation of insulin secretion in response to glucose and other physiological cues. Islet transplantation has therefore been investigated as an alternative to insulin injection therapy for more than 3 decades (6, 7). Success in this area had been very limited until a recent trial in Edmonton, Alberta, Canada, in which a combination of mild immunosuppressive agents were used in conjunction with freshly isolated islet tissue to achieve insulin independence in seven successive patients studied for up to 14.9 months post-transplant (8).

Unfortunately, enthusiasm for this important finding is tempered by the fact that the number of human pancreata available for islet transplantation in the United States is on the order of several thousand per year (6, 7), which does not approach the number of patients that could benefit from this new form of therapy. To deal with this problem, we and others have been attempting to develop a replenishable source of cells that could serve as islet surrogates for cell-based insulin replacement in diabetes (9–13). One working concept is that immortalized cell lines engineered for robust glucose-stimulated insulin secretion can be transplanted in the context of a cell-impermeant macroencapsulation device, preventing contact of cellular elements of the immune system with the transplanted cells (11). However, because such devices are envisioned to allow rapid exit of insulin, as well as highly efficient equilibration of nutrients, oxygen, and waste products, it is anticipated that soluble mediators of immunological damage such as cytokines or reactive oxygen species will readily gain access to the cells within the device. Thus, development of methods to protect transplanted cells against diffusible, noncellular mediators of immunological destruction may be important for the eventual success of cell-based insulin replacement strategies.

Recently, we described a method for selection of cell lines with resistance to the cytokotoxic effects of exposure to a mixture of IL-1β and IFN-γ, known mediators of β-cell destruction in type 1 diabetes (14). This involved growth of INS-1 insulinoma cells in progressively elevated concentrations of the two cytokines over an 8-week period and selection of surviving cells. The resultant cell line, termed INS-1<sub>res</sub>, was 80% viable after 5 days of exposure to the combination of 10 ng/ml IL-1β and 100 units/ml IFN-γ, while less than 1% of parental INS-1 cells survived when treated in the same fashion. Interestingly, resistance to IL-1β alone persisted for up to 6 months after removal of cytokines, while resistance to IFN-γ or IL-1β plus IFN-γ was partially lost upon removal of cytokines and restored upon their return to the culture medium (14).

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§ The abbreviations used are: IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; IFN-R, IFN receptor; STAT, signal transducer and activator of transcription; pfu, plaque-forming units; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; iNOS, inducible nitric-oxide synthase.

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The current study was undertaken to define the factor or factors responsible for resistance to IFN-γ or IFN-γ plus IL-1β in selected insulinoma cell lines. By focusing on the known components of the IFN-γ receptor (IFN-R) signaling pathway, we have discovered that expression levels of signal transducer and activator of transcription (STAT)-1α are closely correlated with the cytokine-resistant and -sensitive phenotypes. Furthermore, we demonstrate that adenovirus-mediated overexpression of STAT-1α in cytokine-sensitive INS-1 cell lines confers resistance to the combination of IL-1β plus IFN-γ. Our findings suggest that expression of STAT-1α in insulin-secreting cell lines may be a means for enhancing their survival in a transplant setting.

**MATERIALS AND METHODS**

**Cells and Reagents—**The insulinoma cell line INS-1 (15) and clonal derivatives thereof (14, 16) were cultured in RPMI 1640 medium containing 10% fetal calf serum, 10 mM Hepes, 2 mM t-glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco) at 37 °C and 5% CO₂. Recombinant rat IL-1β was obtained from Endogen (Cambridge, MA). Recombinant rat γ-IFN was obtained from Life Technologies, Inc. Anti-STAT-1 and anti-phospho-STAT-1 antibodies were purchased from New England Biolabs, Inc (Beverly, MA), and anti-iNOS antibody was purchased from Transduction Laboratories (Lexington, KY).

**Cloning and Overexpression of Rat STAT-1α—**Cytokine-resistant 833/117 cells (14) were kept in medium containing IL-1β plus IFN-γ for 24 weeks prior to RNA isolation using TRIzol (Life Technologies). cDNA was reverse-transcribed by the Advantage reverse transcriptase-polymerase chain reaction kit (CLONTECH Inc., Palo Alto, CA). Primers were designed for the published mouse STAT-1 cDNA sequence (GenBank accession number U06924) and included BamHI restriction enzyme recognition sequences: 5′-GGATCCAGATGTCCAGTGTTCCG (sense) encoding nucleotides –4 to 16, and 5′-GGATCCTCGCCAGAGAAAAATGCTTG (antisense), encompassing nucleotides 2256–2275. Polymerase chain reaction products were cloned into pcRII from the TA cloning kit (Invitrogen, Carlsbad, CA). The amplified and subcloned rat STAT-1α cDNA was sequenced with a DNA Sequencer 377 (PE Biosystems, Foster City, CA), and the sequence was submitted to GenBank (accession number AF205804). A 2.2-kilobase BamHI fragment of the STAT-1α cDNA containing the protein coding sequence was cloned into the adenovirus vector pACCMV.pLpA (17), and a recombinant virus (AdCMV-STAT-1α) was prepared by previously described methods (18). Control experiments were conducted with a recombinant virus containing the bacterial β-galactosidase gene (AdCMV-βGal), prepared and used as described previously (19, 20).

**Measurement of Cytokine-mediated Cytotoxicity—**Cells were inoculated well into 48-well tissue culture plates (Corning Glass, Corning, NY). After 24 h, cells were treated with 1.0 × 10⁸ plaque-forming units (pfu) of AdCMV-STAT-1α or AdCMV-β-galactosidase recombinant viruses. After 12 h of viral treatment, culture medium was changed, and cells were incubated in the absence of virus for an additional 24 h prior to the addition of cytokines. After 48 h of cytokine treatment, media samples were collected for nitrite assay and replaced with 250 μl/well of medium with 75 μg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 1.5 h at 37 °C and 5% CO₂. The resulting formazan crystals were solubilized in 250 μl of 0.04 N HCl in isopropyl alcohol. The optical density of the solubilized formazan was read at 570 and 650 nm using a SpectraMax 340 (Molecular Devices, Inc., Sunnyvale, CA) plate reader. The reduction in optical density caused by cytokine treatment was used as a measurement of cell viability, normalized to cells incubated in control medium, which were considered 100% viable.

**Measurement of Nitrite Formation—**NO production was estimated by measurement of nitrite levels, as described previously (14, 21), and data were normalized to protein content per well, measured by the Bradford assay (Bio-Rad).

**Insulin Secretion Experiments—**Cells were cultured in 24-well plates. Assays were initiated by removal of culture medium, washing of cells with 1 ml of PBS (Life Technologies) and incubation in 1 ml of secretion assay buffer containing 3 mM glucose for 1 h, followed by incubation in secretion assay buffer containing 3 or 15 mM glucose for 2 h at 37 °C. Secretion assay buffer contains 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.16 mM MgSO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, 20 mM HEPES, and 0.25% fatty acid-free bovine albumin (Sigma), pH 7.4.

After centrifugation of media samples at 3000 rpm for 6 min to remove floating cells, the supernatants were subjected to insulin radioimmunoassay (DPC Coat-A-Count). The cells remaining in the wells were then washed once with PBS and lysed with 60 μl of PBS containing 0.1% Triton X-100 for measurement of total protein (Bio-Rad).

**Immunoblot Analysis—**Cells were lysed with PBS and lysed with buffer containing 1% Triton X-100, 50 mM Hepes, 150 mM NaCl, 0.2 mg/ml phenylmethylsulfonyl fluoride, 100 mM NaF, 2 mM sodium vanadate, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin, pH 7.2. Lysates were vortexed, kept on ice for 20 min, and centrifuged at 14,000 rpm in a refrigerated microcentrifuge. Supernatants were collected, and protein concentrations were determined. Samples containing 25 or 50 μg of protein were suspended in 5× sample buffer (300 mM Tris, 10% SDS, 0.5% b-mercaptoethanol, 500 mM dithiothreitol, pH 6.8), heated for 5 min in boiling water, and electrophoresed using 10% precast Tris-glycine gels (Bio-Rad). Protein was transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) and blocked with 5% dry milk in TBST (10 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 8.0). Blots were incubated with anti-STAT-1, anti-phospho-STAT-1, or anti-iNOS antibodies according to protocols supplied by the manufacturers. Protein bands were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Pharmacia Biotech) and enhanced chemiluminescence (Amersham Pharmacia Biotech). Immunoblots were scanned with a UMAX UC 9540 scanner, and band intensities were measured by NIH Image 1.59.

**RNA Isolation and Blot Hybridization Analysis—**Total RNA was isolated by extraction with the TRizol reagent (Life Technologies) according to the manufacturer’s protocol. 10 μg of total RNA was resolved on a 1.3% formaldehyde/agarose gel, and samples were transferred to nylon membrane and hybridized in Rapid-Hyb buffer (Amersham Life Sciences) with 32P-labeled STAT-1α cDNA probe, prepared with the Redi-priming labeling kit (Amersham Life Sciences). After hybridization and washing, nylon membranes were exposed to film to create autoradiographs.

**Statistical Methods—**Statistical analysis of the data was performed using the two-tailed Student’s t test, assuming unequal variances.

**RESULTS**

**Expression of STAT-1α Correlates with Cytokine Resistance—**We have previously isolated cytokine-resistant insulinoma cell lines (INS-1α) by culture of the cells in the presence of increasing concentrations of IL-1β plus IFN-γ for a period of 8 weeks (14). INS-1αα cells exhibited impaired translocation of the NF-κB transcription factor in response to IL-1β, which may explain their attendant block of NO production and iNOS expression. However, iNOS expression and NO production did not account for resistance to IFN-γ or IFN-γ plus IL-1β, as evidenced by the fact that LNF6-monomethyl-1-arginine, an iNOS inhibitor, only partially blocked killing of INS-1 cells induced by IL-1β plus IFN-γ (14). To gain understanding of the mechanism by which the selection process confers resistance to IFN-γ or IFN-γ plus IL-1β, we have analyzed known components of the IFN-γ signal transduction system in cytokine-resistant and -sensitive cell lines.

The IFN-γ receptor transduces signals via its associated Jak kinase activity, which in turn binds to and phosphorylates STAT-1 transcription factors (22–24). We therefore treated clonal cell lines derived from INS-1αα cells (lines 833/15 and 833/117) or parental INS-1 cells (line 834/40) acutely with IFN-γ or IL-1β plus IFN-γ for a period of 20 min and then assayed phosphorylation and expression of STAT-1α by immunoblot analysis. Stimulation of STAT-1α phosphorylation in response to acute cytokine treatment was similar in all groups of cells, as shown in Fig. 1. Nevertheless, there was a clear correlation between susceptibility to cytokine damage and STAT-1α protein levels. Thus, 833/15 and 833/117 cells that are grown in the continual presence of cytokines are IFN-γ- and IFN-γ plus IL-1β-resistant (14), and contain 6.9 and 7.0 times as much STAT-1α protein, respectively, as the same cell lines that had been grown in the absence of cytokines for 4 weeks or more (rendering them sensitive to IFN-γ and
IL-1 plus IFN-γ-mediated killing). Essentially identical results were obtained in a second independent experiment (data not shown). STAT-1α levels were also low in 834/40 cells, which had not been through the selection procedure and are thus highly susceptible to cytokine damage. These data suggest that STAT-1α expression may directly contribute to cytokine resistance induced by the selection protocol.

**STAT-1α Expression in Resistant Cells Decreases upon Withdrawal of Cytokines from the Culture Medium**—To learn more about the regulation of STAT-1α expression by cytokines in the culture medium, we measured STAT-1α protein levels as a function of time after cytokine withdrawal. As shown in Fig. 2, culture of the INS-1res-derived cell lines 833/15 and 833/117 in the continual presence of cytokines resulted in very high STAT-1α protein levels. 2 days after cytokine removal, STAT-1α expression was unchanged in 833/15 cells and only mildly decreased in 833/117 cells. However, by 6 days after cytokine removal, STAT-1α protein levels were decreased by 48 and 67% in the two lines, respectively, relative to starting levels. The time course and magnitude of the decrease in STAT-1α expression was confirmed in a second independent experiment identical in design to the representative experiment shown in Fig. 2 (data not shown). Thus, constant IFN-γ treatment is required to maintain the high STAT-1α protein levels associated with cytokine resistance in INS-1res cells.

**IL-1β Inhibits IFN-γ-induced STAT-1α Expression in Cytokine-sensitive INS-1 Cells**—We initially assumed that STAT-1α protein levels were being regulated mainly by IFN-γ in the tissue culture medium, as has been reported in other systems (25, 26). Indeed, as shown in Fig. 3, treatment with IFN-γ alone stimulates STAT-1α expression to a similar degree in cytokine-resistant and sensitive clones. However, the coaddition of IL-1β and IFN-γ to a cytokine-sensitive cell line (line 834/40) for 12 or 24 h resulted in accumulation of only 8.1 and 20% as much STAT-1α protein as observed with IFN-γ alone, while in cytokine-resistant cells (line 833/117), no inhibitory effect of co-stimulation with IL-1β was observed. This result was confirmed in three other independent experiments, similar in design to the representative experiment shown in Fig. 3. Thus, in unselected cells, IL-1β inhibits and delays IFN-γ-induced expression of STAT-1α. In contrast, selected cells lose IL-1β-mediated suppression of IFN-γ-mediated up-regulation of STAT-1α expression. The implication is that cytokine resistance in selected INS-1 cells may be a sequential process, in which development of IL-1β resistance alleviates inhibition of IFN-γ-mediated up-regulation of STAT-1α, allowing STAT-1α to accumulate to levels that protect against the cytotoxic effects of IFN-γ alone or the combination of IFN-γ plus IL-1β.

**Adenovirus-mediated Expression of STAT-1α in Cytokine-sensitive Cells Confers Protection against Cytokine Damage**—To investigate whether STAT-1α expression in cytokine-sensitive INS-1 cell lines can confer protection against cytokine damage, it was first necessary to clone the rat STAT-1α cDNA. This was achieved by reverse transcriptase-polymerase chain reaction-mediated preparation of cDNA from the INS-1 cell line 833/117 after treatment with IFN-γ for 48 h to induce STAT-1α expression. The sequence of the rat STAT-1α cDNA was determined and deposited in GenBank™ (accession number AF205604), and a fragment containing the entire protein coding region was used to prepare a recombinant adenovirus (AdCMV-STAT-1α). Treatment of INS-1 cells with a range of AdCMV-STAT-1α titers from 2.5 × 10⁸ to 1 × 10⁹ pfu/ml resulted in titer-dependent increases in STAT-1α protein overexpression, as evaluated by immunoblot analysis (Fig. 4A).

Based on these results, the cytokine-sensitive cell line 834/40 was treated with 1 × 10⁰ pfu/ml of AdCMV-STAT-1α or, as a control, the same amount of AdCMV-βGal virus. These cell groups, as well as a group of cells not treated with adenovirus, were cultured in normal medium for 24 h and then treated with 10 ng/ml IL-1β, 100 units/ml IFN-γ or the combination of IL-1β plus IFN-γ. After 48 h of cytokine treatment, viability was measured by the MTT assay. As shown in Fig. 4B, adenovirus-mediated overexpression of STAT-1α in 834/40 cells increased the viabilities of the IL-1β-, IFN-γ-, and IL-1β plus IFN-γ-treated groups from 41.7 ± 2.0, 56.5 ± 3.2, and 19.0 ± 0.9%, respectively (control group with no viral treatment), or 60.9 ± 7.8, 44.0 ± 4.9, and 10.1 ± 2.4% (control group treated with AdCMV-βGal) to 81.1 ± 3.8, 72.9 ± 7.9, and 50.1 ± 5.6% (AdCMV-STAT-1α-treated group), respectively. These data show that STAT-1α overexpression is sufficient to provide sub-
INS-1-derived (834/40) and INS-1res-derived (833/117) cells were cultured in normal medium lacking cytokines for a period of 6 months. Cells were then plated in 12-well plates and treated with IFN-γ (100 units/ml) or IFN-γ (100 units/ml) plus IL-1β (10 ng/ml). At the indicated time, cells were washed with PBS and lysed in 200 μl of lysis buffer. 25 μg of total protein was separated by 10% SDS-PAGE and subjected to immunoblot analysis with rabbit anti-STAT-1 antibody. In the cytokine-sensitive line, induction of STAT-1 expression was observed in response to IFN-γ treatment. Our results also suggest that the synergistic effects of IL-1β plus IFN-γ signaling are lost in the IL-1β-resistant cell line. Data shown are representative of four independent experiments.

**FIG. 3.** IL-1β impairs IFN-γ-mediated induction of STAT-1α expression in cytokine-sensitive but not -resistant INS-1-derived cell lines. INS-1-derived (834/40) and INS-1res-derived (833/117) cells were cultured in normal medium lacking cytokines for a period of 6 months. Cells were then plated in 12-well plates and treated with IFN-γ (100 units/ml) or IFN-γ (100 units/ml) plus IL-1β (10 ng/ml). At the indicated time, cells were washed with PBS and lysed in 200 μl of lysis buffer. 25 μg of total protein was separated by 10% SDS-PAGE and subjected to immunoblot analysis with rabbit anti-STAT-1 antibody. In the cytokine-sensitive line, induction of STAT-1 expression was observed in response to IFN-γ treatment. Our results also suggest that the synergistic effects of IL-1β plus IFN-γ signaling are lost in the IL-1β-resistant cell line. Data shown are representative of four independent experiments.

**FIG. 4.** Adenovirus-mediated expression of STAT-1α in a cytokine-sensitive INS-1-derived cell line confers broad resistance to cytokine-induced cytotoxicity. A, INS-1 cells were treated with the indicated concentrations of AdCMV-STAT-1α for 12 h and then incubated in fresh medium for 24 h prior to cell harvesting and immunoblot analysis with an anti-STAT-1α antibody. Control cells were incubated for the same period but without virus treatment. B, the cytokine-sensitive cell line 834/40 was treated with 1 × 10⁹ pfu/ml of AdCMV-STAT-1α, the same amount of AdCMV-βGAL virus, or no virus. These cell groups were cultured in normal medium for 24 h and then treated with 10 ng/ml IL-1β, 100 units/ml IFN-γ or both cytokines for 48 h. Cell viability was then determined with the MTT assay, as described under “Materials and Methods.” Data represent the mean ± S.E. for four independent experiments. The symbols refer to comparisons of viability of STAT-1α-overexpressing cells with other groups as follows. @, p < 0.001, versus untreated control and p < 0.03 versus AdCMV-βGAL-treated control; #, p = 0.05 versus untreated control and p < 0.007 versus AdCMV-βGAL-treated control. *p < 0.002 versus either control group.

Statistical analysis included the use of Student’s t test, one-way ANOVA, or Dunnett’s test for multiple comparisons as indicated. Differences were considered significant at p < 0.05.

**Table 1.**

| Total STAT-1 | - | - | - | - | - | - | - | - | - | - | - | - |
|-------------|---|---|---|---|---|---|---|---|---|---|---|---|
| IL-1β (10ng/ml) | - | - | - | - | + | + | + | + | + | + | + | + |
| γ-IFN (100U/ml) | - | - | - | - | - | + | + | + | + | + | + | + |
| Cells | 834/40 | 833/117 | 834/40 | 833/117 | 834/40 | 833/117 | 834/40 | 833/117 | 834/40 | 833/117 | 834/40 | 833/117 |
| Time (H) | 0 | 12 | 24 | 48 | 0 | 12 | 24 | 48 | 0 | 12 | 24 | 48 |

This table shows the expression levels of STAT-1α in different cell lines and treatment conditions. The table represents the mean ± S.E. for four independent experiments. The symbols refer to comparisons of viability of STAT-1α-overexpressing cells with other groups as follows. @, p < 0.001, versus untreated control and p < 0.03 versus AdCMV-βGAL-treated control; #, p = 0.05 versus untreated control and p < 0.007 versus AdCMV-βGAL-treated control. *p < 0.002 versus either control group.
shown in Fig. 6. Taken together, these data suggest that protection against cytokine-mediated cytotoxicity in STAT-1α-overexpressing cells is in part attributable to reduced NO production and iNOS expression. It is interesting to note that the impairment of cytokine-induced NO production in STAT-1α-overexpressing cells is partial, in contrast to our findings in cells taken through the cytokine selection process, in which NO production was completely blocked (14). These results imply that factors other than STAT-1α expression, possibly including impairment of IL-1β-induced NF-κB translocation (14), contribute to the more complete blockade of NO production noted in the INS-1res cell lines.

**Adenovirus-mediated Overexpression of STAT-1α Does Not Impair Robust Glucose-stimulated Insulin Secretion in INS-1-derived Cell Lines**—If STAT-1α expression is to serve as a viable strategy for protecting insulin-producing cells against cytokine-mediated damage, it is important to demonstrate that expression of this gene does not impair glucose-stimulated insulin secretion. We have recently demonstrated that the rat insulinoma cell line INS-1 (15) is composed of a mixture of glucose-responsive and -unresponsive cells (14, 16). We therefore tested the effect of STAT-1α expression in a robustly glucose-responsive INS-1-derived cell line, 832/13, which was obtained from parental, unselected INS-1 cells by stable transfection with a plasmid containing the neomycin resistance gene and harvesting of independent colonies (16). As shown in Fig. 7, incubation of 832/13 cells with 15 mM glucose stimulated insulin secretion by 12–14-fold relative to cells incubated at 3 mM glucose, regardless of whether they had first been treated with AdCMV-STAT-1α or AdCMV-βGAL or left untreated. We conclude that STAT-1α overexpression has no effect on insulin secretion in a robustly glucose-responsive INS-1-derived cell line.

**DISCUSSION**

In a previous study, we described a method for selecting insulinoma cell lines with resistance to the cytotoxic effects of IFN-γ plus IL-1β (INS-1res), involving culture of the cells in increasing concentrations of the cytokines over an 8-week period (14). A potential mechanistic explanation for the development of resistance to IL-1β was provided by our finding that activation of NF-κB translocation by the cytokine was impaired in INS-1res cells relative to cells that had not undergone selection. NF-κB is a known activator of iNOS expression, possibly explaining the blockade of IL-1β-induced NO production that was also observed in INS-1res cells (14). However, incubation of INS-1 cells with N⁴-monomethyl-L-arginine, an inhibitor of iNOS, only partially prevented the cytotoxic effects of the combined addition of IL-1β plus IFN-γ and had no effect on IFN-γ-mediated cytotoxicity. Furthermore, levels of IFN-R mRNA were not reduced in INS-1res cells relative to parental INS-1 cells. Thus, the mechanism by which INS-1res cells gained resistance to IFN-γ and the combination of IFN-γ plus IL-1β was not resolved.

In the current study, these unresolved issues have been investigated. By tracing the known signal transduction pathway for IFN-γ-mediated signaling in mammalian cells, we have discovered that there is a tight correlation between the level of expression of the transcription factor STAT-1α and resistance to cytokine damage. Thus, cells that have undergone the selection process and have been grown in the continual presence of IL-1β plus IFN-γ are resistant to the combined effects of the two cytokines (14). These cells have very high levels of STAT-1α expression. Withdrawal of the cytokines from the culture medium for periods as little as 6 days and as long as 32 weeks results in a loss of resistance to IFN-γ and a partial loss of resistance to IL-1β plus IFN-γ but full retention of resistance to IL-1β (14). These changes are correlated with a sharp drop in STAT-1α protein levels, such that expression becomes equal to that in cells that have not been through the selection procedure (see Figs. 1 and 2). Adenovirus-mediated expression of STAT-1α in naive INS-1 cells confers partial resistance to IL-1β, to IFN-γ, and most prominently, to the combined cytotoxic effects of IL-1β plus IFN-γ. We conclude...
that the induction of STAT-1α expression that occurs during selection by growth in the presence of cytokines is directly linked to the resistant phenotype of INS-1res cells.

Our data also provide fresh insight into cross-talk between separate cytokine signaling pathways in islet β-cells, as exemplified by prior studies in which IFN-γ was shown to prime rat islets for IL-1β-induced iNOS expression (27). An important finding of the current work is that IL-1β impairs IFN-γ-induced expression of STAT-1α in unselected INS-1 cells. This means that in cells that have been subjected to the selection procedure, the ability of IL-1β to suppress induction of STAT-1α expression by IFN-γ is partially blocked. Since we have shown that increased expression of STAT-1α is sufficient to induce resistance to IFN-γ and IL-1β plus IFN-γ-mediated cytotoxicity, it follows that ablation of IL-1β-mediated suppression of STAT-1α expression is an important event in the development of resistance to these cytokines.

Another interesting example of cross-talk is the apparent interaction of IFN-γ and IL-1β signaling pathways in control of NO production in INS-1 cells. In previous studies, we have shown that incubation of nonresistant INS-1 cells with IL-1β alone induces a large increase in iNOS expression and NO production, while IFN-γ has no effect on these variables (14). However, overexpression of STAT-1α, a transcription factor that clearly participates in IFN-R-mediated signaling, is able to partially block NO production and iNOS expression in response to IL-1β (Figs. 6 and 7). STAT-1α is not known to participate in IL-1β-mediated signaling. However, the promoter region of the iNOS gene does contain several copies of the consensus sequence that mediates IFN-γ regulation of gene expression through STAT-1α binding (GAS sites) (5). Thus, possible mechanisms by which STAT-1α overexpression interferes with iNOS expression and NO production could include a change in transcription factor binding to the iNOS promoter or interference of STAT-1α with more proximal steps in the IL-1R1/IFN-αb/iNOS signaling pathway. Further experimentation will be required to resolve these possibilities.

IFN-γ exerts its biological effects by binding to its cell surface receptor, which is a heterodimer composed of α and β subunits (22–24). Binding of IFN-γ causes receptor dimerization and association of soluble Jak kinases with distal regions of the β-chain of the IFN-R receptor. Jak kinase then phosphorylates a nearby tyrosine in the receptor β-chain, allowing it to bind to STAT-1α via its C-terminal Src homology 2 domain. Juxtaposition of STAT-1α and Jak kinase results in phosphorylation of receptor-associated STAT-1α, rendering it competent for homo- or heterodimerization (e.g. with other members of the STAT family of proteins), nuclear translocation, DNA binding, and activation of transcription. Within this framework, the site at which STAT-1α overexpression causes resistance to IFN-γ and IFN-γ plus IL-1β-mediated cytotoxicity remains to be elucidated. It has been appreciated for some time that levels of STAT-1α mRNA and protein are increased in response to IFN-γ treatment of a variety of different cell types (25, 26), but the significance of this increase has remained obscure. Particularly puzzling is the finding that the effects of IFN-γ on expression of its target genes wanes within 2–4 h of application of the cytokine, while STAT-1α expression continues to increase in this time frame and then remains at high levels for as long as IFN-γ is applied (22, 25, 26). The results presented here suggest that increases in expression of STAT-1α serve as a mechanism for blunting untoward effects of IFN-γ on cellular processes, possibly by direct impairment of IFN-γ signal transduction or by cross-talk with other cytokine signaling pathways (e.g. that for IL-1β). Interestingly, accumulation of STAT-1α protein in response to long term culture of cells in cytokines does not cause a proportional increase in phosphorylated STAT-1α (see Fig. 1), suggesting that the overexpressed protein is not efficiently phosphorylated, dimerized, or translocated to the nucleus. Possibilities for future investigation include interference of the overexpressed, unphosphorylated STAT-1α with normal homodimer formation or nuclear translocation or interference with other cellular processes via “promiscuous” Src homology 2 domain-mediated complex formation.

Our results suggest that expression of STAT-1α in cell lines designed for insulin replacement in type 1 diabetes may be a means of protecting them against cytokine-induced damage occurring in response to transplantation. An apparent advantage of this approach is that it confers partial protection against a mixture of two cytokines prominently associated with inflammation, IFN-γ and IL-1β. This is in contrast to the more specific protection conferred by other genetic engineering strategies, such as expression of Mn-superoxide dismutase, which provides protection against IL-1β and IL-1β plus TNF-α, but not against IFN-γ (21), or expression of dominant-negative FADD domain proteins, which partially block TNF-α signaling (28, 29). Expression of anti-apoptotic members of the bcl gene family have been reported to confer partial protection against cytokine mixtures (30, 31), but at high levels of expression, these proteins may also impair glucose metabolism and glucose-stimulated insulin secretion (32). In contrast, adenovirus-mediated expression of STAT-1α in the strongly responsive INS-1-derived cell line 832/13 (16) did not impair the 12–14-fold increase in insulin secretion induced by raising the glucose concentration from 3 to 15 mM (Fig. 7).

Nevertheless, further work will be required before we can conclude that STAT-1α expression is a practical or broadly applicable strategy for cell transplantation. One concern is that STAT proteins are involved in a wide variety of receptor pathways, including those of the gp130, growth hormone, and receptor tyrosine kinase families (22). Thus, long term expression of STAT-1α may alter signaling through pathways other than that mediated by IFN-γ and IFN-R, affecting the viability, growth, or function of cells in as yet undefined ways. Furthermore, the protection conferred by STAT-1α expression in naive INS-1-derived lines is incomplete, in contrast to the complete protection afforded by the selection procedure (14), suggesting that IL-1β resistance occurring through impairment of NF-κB signaling must be present as a complement to STAT-1α overexpression for complete protection to be gained. Studies involving stable transfection of INS-1res cell lines (which exhibit long term IL-1β resistance (14)) with STAT-1α and evaluation of the growth and function of these cells in macroencapsulation devices will be required to fully evaluate the potential of this new approach.

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