Insulin-Like Growth Factor (IGF)-I/IGF-Binding Protein-3 Complex: Therapeutic Efficacy and Mechanism of Protection against Type 1 Diabetes

WEI CHEN, Konstantin V. Salojin, Qing-Sheng Mi, Marsha Grattan, T. Craig Meagher, Peter Zucker, and Terry L. DeLovitch

Autoimmunity/Diabetes Group (W.C., K.V.S., Q.-S.M., M.G., T.C.M., P.Z., T.L.D.), Robarts Research Institute, London, Ontario N6G 2V4, Canada; and the Department of Microbiology and Immunology (T.C.M., T.L.D.), University of Western Ontario, London, Ontario N6A 5C1, Canada

IGF-I regulates islet β-cell growth, survival, and metabolism and protects against type 1 diabetes (T1D). However, the therapeutic efficacy of free IGF-I may be limited by its biological half-life in vitro. We investigated whether prolongation of its half-life as an IGF-I/IGFBP-3 complex affords increased protection against T1D and whether this occurs by influencing T cell function and/or islet β-cell growth and survival. Administration of IGF-I either alone or as an IGF-I/IGFBP-3 complex reduced the severity of insulitis and delayed the onset of T1D in nonobese diabetic mice, but IGF-I/IGFBP-3 was significantly more effective. Protection from T1D elicited by IGF-I/IGFBP-3 was mediated by up-regulated CCL4 and down-regulated CCL3 gene expression in pancreatic draining lymph nodes, activation of the phosphatidylinositol 3-kinase and Akt/protein kinase B signaling pathway of β-cells, reduced β-cell apoptosis, and stimulation of β-cell replication. Reduced β-cell apoptosis resulted from elevated Bel-2 and Bel-XL activity and diminished caspase-9 activity, indicating a novel role for a mitochondrial-dependent pathway of β-cell death. Thus, IGF-I/IGFBP-3 affords more efficient protection from insulitis, β-cell destruction, and T1D than IGF-I, and this complex may represent an efficacious therapeutic treatment for the prevention of T1D. (Endocrinology 145: 627–638, 2004)

Type 1 diabetes (T1D) is an autoimmune disorder resulting from the destruction of insulin-producing β-cells of pancreatic islets (1). The onset of spontaneous T1D in humans and nonobese diabetic (NOD) mice is preceded by a progressive leukocyte infiltration into islets (insulitis), which persists for a relatively long period of time before eventual massive islet β-cell destruction. Compelling evidence suggests that apoptosis is the principle mode of β-cell death during the development of T1D (2–4). Cytotoxic cytokines secreted by infiltrating leukocytes, such as TNF-α, interferon-γ (IFN-γ), and IL-1β, are major mediators of β-cell apoptosis via multiple signaling pathways (5–12). Traditionally, attempts to prevent β-cell destruction and T1D have involved immunological strategies and β-cell autoantigens to induce tolerance; however, it has become apparent that a number of approaches aimed at preserving β-cell functions may also have therapeutic relevance in preventing the pathogenesis of T1D. Currently, an approach receiving much attention is the blockade of apoptosis signaling pathways that occurs by influencing T cell function and/or islet β-cell growth and survival. Administration of IGF-I either alone or as an IGF-I/IGFBP-3 complex reduced the severity of insulitis and delayed the onset of T1D in nonobese diabetic mice, but IGF-I/IGFBP-3 was significantly more effective. Protection from T1D elicited by IGF-I/IGFBP-3 was mediated by up-regulated CCL4 and down-regulated CCL3 gene expression in pancreatic draining lymph nodes, activation of the phosphatidylinositol 3-kinase and Akt/protein kinase B signaling pathway of β-cells, reduced β-cell apoptosis, and stimulation of β-cell replication. Reduced β-cell apoptosis resulted from elevated Bel-2 and Bel-XL activity and diminished caspase-9 activity, indicating a novel role for a mitochondrial-dependent pathway of β-cell death. Thus, IGF-I/IGFBP-3 affords more efficient protection from insulitis, β-cell destruction, and T1D than IGF-I, and this complex may represent an efficacious therapeutic treatment for the prevention of T1D. (Endocrinology 145: 627–638, 2004)

Abbreviations: BGL, Blood glucose level; BrdU, 5-bromo-2-deoxyuridine; CY, cyclophosphamide; IFN, interferon; IGFBP, IGF binding protein; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NOD, nonobese diabetic; PI3-K, phosphatidylinositol 3-kinase; PLN, pancreatic lymph node; SOCS, suppressor of cytokine signaling; T1D, type 1 diabetes; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling.

Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.
glycemia in animal models of T1D (24). Studies of IGF-I/IGFBP-3 in an experimental model of multiple sclerosis and human T1D (25, 26) demonstrated that administration of IGF-I/IGFBP-3 is not only safer but also has a higher therapeutic index than free IGF-I. Thus, IGF-I/IGFBP-3 may provide an improved mode of delivery of IGF-I that will facilitate in vivo functional studies and potential clinical uses.

In the present study, we compared the effects of IGF-I and IGF-I/IGFBP-3 treatments on the incidence of T1D in NOD mice. In particular, we explored the optimal dose and treatment regimen of IGF-I/IGFBP-3 for preventing T1D. Due to the expression of IGF-I receptors on many cell types, including lymphocyte subpopulations and islet β-cells, we evaluated the effect of administration of IGF-I/IGFBP-3 on both systemic immune responses and islet β-cells in vivo. Finally, we investigated signaling pathway intermediates and signaling pathways involved in protective mechanisms mediated by IGF-I by in vitro functional analyses of freshly isolated primary islet β-cells and β-cell lines. Recent clinical studies of IGF-I and IGF-I/IGFBP-3 have proven their beneficial effects on many diseases, including type 1 and type 2 diabetes (26, 27). Our findings provide new insight into the mechanisms of β-cell apoptosis via the mitochondrial caspase-9-dependent pathway and the potential of IGF-I/IGFBP-3 as a prophylactic therapy in the prevention of autoimmune T1D in humans.

Materials and Methods

Mice

NOD/Del and NOD.Scid mice were bred in a barrier facility at the Robarts Research Institute and were housed under specific pathogen-free conditions in the animal facility of University of Western Ontario for the duration of all experiments. In our colony of female NOD mice, the onset of insulitis and T1D occurs at 4–5 wk and 12 wk of age, respectively. The cumulative incidence of T1D is 75–85% by 35 wk of age. NOD.Scid mice were generously provided by Dr. L. Shultz (The Jackson Laboratory, Bar Harbor, ME). All procedures were performed under an approved protocol in compliance with guidelines set by the Animal Studies Committee of the University of Western Ontario.

Cell lines and reagents

The NIT-1 cell line was purchased from American Type Culture Collection (Manassas, VA), and cultured in Ham’s F-12K medium containing 10% fetal calf serum, 2 mm glutamine, and penicillin-streptomycin (Invitrogen, Inc., Burlington, Canada). Recombinant human IGF-I and IGF-I/IGFBP-3 complex (SomatoKine) used in this study were kindly provided by Genentech Inc. (San Francisco, CA) and Insmed Inc. (Glen Allen, VA), respectively. Recombinant human IGF-II was purchased from GroPep Inc. (Adelaide, Australia). Recombinant mouse TNF-α and IFN-γ were purchased from BD Bioscience (Mississauga, Canada). Cyclophosphamide (CY), the phosphatidylinositol 3-kinase (PI3-K) inhibitor LY294002 [2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride], and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO).

Islet isolation and culture

Islets were isolated from 4- to 6-wk-old male NOD mice as previously described (28). Distended pancreata were excised from anesthetized mice after intrabiliary duct collagenase digestion. Tissue was incubated in a 50-mL conical tube at 37 C for 10 min with agitation and digestion stopped with ice-cold Hanks’ basic salt solution. Islet tissue was filtered through a steel mesh (860 μm) and resuspended in dextran solution after washing twice with cold Hanks’ basic salt solution. The islet preparation was recovered after three-layer discontinuous dextran gradient centrifugation, and individual islets were hand picked to a purity of 95%. Purified islets were precultured in Ham’s F-12K medium at 37 C in 5% CO₂ overnight before exposure to cytokines.

Treatment of mice with IGF-I/IGFBP-3 complex and free IGFs

Littermate-matched female NOD mice (2–8 wk old) were randomly divided into treatment groups that included recipients of either IGF-I, IGF-II (diluted in PBS + 1% NOD mouse serum), or IGF-I/IGFBP-3 as well as a control group that received vehicle (PBS + 1% NOD mouse serum). Treatment reagents were injected sc for 4 wk at the various doses, and the regimens are indicated in the figure legends.

Histological analysis of insulitis

The incidence and severity of insulitis and insulin immunostaining were analyzed using formalin-fixed (10%) and paraffin-embedded pancreatic tissues. After hematoxylin and eosin staining, insulitis scores were graded based on the degree of lymphocytic infiltration as follows: G0, normal; G1, perinsulitis (mononuclear cells surrounding islets and ducts but no infiltration of the islet architecture); G2, moderate insulitis (mononuclear cells infiltrating less than 50% of the islet architecture); and G3, severe insulitis (more than 50% of the islet tissue infiltrated by lymphocytes, accompanied by a reduction in insulin staining). Three randomly obtained and nonadjacent sections of each pancreas from cored slides were scored independently by two blind observers. Insulitis index (I) was calculated using the following formula: $I = \frac{1}{3} [(\text{number of islets with score of } G0 \times 0) + (\text{number of islets with score of } G1 \times 1) + (\text{number of islets with score of } G2 \times 2) + (\text{number of islets with score of } G3 \times 3)] \times 3 \times (\text{total number of islets}).$

Assessment of diabetes

Mice were monitored for the development of T1D by measuring blood glucose levels (BGLs) either daily or weekly, dependent on the experimental design. Mice with a reading of BGL > 11.1 mmol/liter (200 mg/dl) on two consecutive occasions were diagnosed as being positive for T1D.

Detection of apoptosis in situ

For measurement of apoptosis, the in situ cell death detection kit (Roche Diagnostics, Laval, Canada) was used as suggested by the manufacturer. Briefly, a reaction of terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) was carried out on paraffin-embedded islet tissue sections. The sections were further stained with antinsulin antibody. The number of TUNEL/insulin double-positive cells per section were counted and are shown as a number of apoptotic nuclei per 100 islets. A minimum of 20 islets per mouse were analyzed, and six mice per group were used.

Assessment of β-cell death by an MTT assay

Pancreatic islets (50/well) or NIT-1 cells (2 × 10⁵/well) were precultured overnight in 96-well plates and then exposed to TNF-α + IFN-γ with or without IGF-I for the indicated times. IGF-I was added to cultures 1 h before exposure to cytokines, after which medium was removed and MTT (0.5 mg/ml) added during an incubation for 2 h at 37 C in 5% CO₂. The cells were then centrifuged, supernatants were removed, and acidic isopropanol (0.04N HCI) was added to dissolve MTT crystals. Absorbance of converted dye was measured at a wavelength of 575 nm with background subtraction at 655 nm.

Cytokine and chemokine assays

Pancreatic tissues and spleens were isolated and snap frozen in liquid nitrogen. Tissue samples were homogenized, sonicated in protease inhibitor buffered cocktail (Roche Diagnostics), and then filtered. Filtrates were analyzed for cytokine and chemokine levels by ELISA (BD Biosciences) as described (29), and the results were normalized relative to the amount of total pancreatic tissue protein.
Assay of caspase-3 activity

Caspase-3 activation/cleavage was determined by flow cytometry after intracellular staining of islet cells with a phycoerythrin-conjugated monoclonal antibody that recognizes only the processed/cleaved caspase-3 subunit (BD Biosciences), according to the protocol supplied by manufacturer.

Analysis of β-cell replication and islet size

For measurement of β-cell replication, mice were injected ip with 5-bromo-2-deoxyuridine (BrdU) (Sigma), 50 mg/kg body weight at the end of treatment. Six hours later, the pancreas was extracted and embedded in paraffin. Sections were stained for insulin and BrdU (mouse anti-BrdU monoclonal antibody, BD Biosciences). The number of insulin-stained BrdU-positive nuclei per total nuclei was determined. At least 1000 islet β-cell nuclei were counted in each pancreatic section, and six mice per group were analyzed.

Islet size and area (µm²) was determined by calculation of sections using a digital camera mounted with a light microscope and analysis of each islet using Northern Eclipse Imagine analyzer. At least five sections (>20 islets) were measured per mouse.

cDNA microarray and RT-PCR analysis

Total RNA was extracted from tissue samples with the RNeasy Midi kit (QIAGEN, Mississauga, Canada). The quality of RNA was determined using Agilent Technologies 2100 Bioanalyzer (Caliper Technologies Corp., Mountain View, CA). Aliquots of RNA (3–4 µg) were used to analyze the apoptosis gene expression profile in islets by GEArray technology (Mouse Apoptosis GEArray Q series, catalog No. MM-002N-4, SuperArray Inc., Bethesda, MD). Relative amounts of mRNA transcripts were quantified using a Molecular Imager System and Molecular Analyst Imaging software (Bio-Rad, Hercules, CA). Gene transcript levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase. A relative signal difference of ≥2-fold in signal intensity was considered significant. Cytokine and chemokine gene expression was analyzed by RT-PCR, as described (29). To analyze CCL3 (MIP-1α) and CCL4 (MIP-1β) chemokine gene expression, the following primers were used: MIP-1α, 5'-TTCCTCGTACAGGCTTCTGCT-3' and 5'-GTCCCTCGATGGCTTCTGCTGCA-3'; and MIP-1β, 5'-TGAAGCCTTGGGCCCCGAGTTCGCTGAG-3'.

Immunoblotting

Islets or cells were lysed in ice-cold modified RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA) supplemented with a mixture of protease and phosphatase inhibitors (100 µM ρ-nitrophenyl guanidonibezoate, 1 mM phe- nylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 5 µg/ml pepstatin, 2 mM Na3VO4, and 10 mM NaF) (all obtained from Sigma). Protein concentrations in each sample were determined with a protein assay kit (R&D Systems, Mississauga, Canada). Equal amounts of sample protein were solubilized in 2× Laemml sample buffer, resolved by SDS-PAGE 8–16% gradient gel (Novex, San Diego, CA) under reducing conditions, transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH), and immunoblotted with antibodies. To avoid sample loading errors, β-actin expression was determined in the blots to adjust and normalize the amount of sample loaded. Anti-Akt (goat polyclonal), anti-Bad (rabbit polyclonal), anti-Bax (rabbit polyclonal), anti-Bcl-2 (mouse monoclonal IgG1), and anti-Bcl-XL (rabbit polyclonal) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-Akt, anti-caspase-9 and anti-caspase-12 polyclonal antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Signal intensities were quantified using a Molecular Imager System and Molecular Analyst Imaging software (Bio-Rad) and are presented as OD/mm².

Statistical analysis

Statistical analyses were performed using the Student's t test, Fisher’s exact test, or the Mann-Whitney sum of ranks test depending on the experimental design. Differences were considered statistically significant when P < 0.05.

Results

IGF-I/IGFBP-3 treatment reduces the severity of insulitis

To test whether administration of IGF-I/IGFBP-3 regulates the extent of lymphocyte infiltration into pancreatic islets, IGF-I/IGFBP-3 (1 mg/kg twice per day) was administered as described for IGF-I (21). To rule out possible effects of IGF-I on glucose metabolism, the BGLs on fasting and 1-h post injection of IGF-I/IGFBP-3 were determined after three daily injections. The fasting BGLs were represented as the mean value ± s.d and were found to be 5.5 ± 0.5 vs. 5.7 ± 0.5 mmol/liter between treated and control groups, respectively. One hour after injection, the BGLs of treated and control mice were 6.3 ± 0.7 and 6.7 ± 1.0 mmol/liter, respectively. No clinical signs of hypoglycemia were observed in the treated mice during the treatment period, indicating that administration of IGF-I/IGFBP-3 does not significantly influence glucose metabolism. Next, we compared the efficacy of IGF-I/IGFBP-3 vs. IGF-I or IGF-II treatment in reducing the severity of insulitis. Groups of 4- to 5-wk-old female NOD mice were injected sc with either IGF-I/IGFBP-3 (2 mg/kg per day) or the equivalent amount of IGF-I or IGF-II (0.4 mg/kg) twice a day for 4 wk. Recipients of IGF-I/IGFBP-3 showed a significantly higher percentage of intact islets (G0 = 55%) with less severe infiltration (G3 = 14%, I = 0.291; Fig. 1A) compared with vehicle-treated control mice (G0 = 30%, G3 = 24%, I = 0.461; P < 0.001). Mice treated with IGF-I alone showed a similarly lower insulin score compared with control mice (Fig. 1B; I = 0.221 vs. I = 0.432, respectively; P < 0.01). Moreover, the insulitis scores observed upon IGF-I/IGFBP-3 or IGF-I treatment did not differ significantly from each other. However, mice receiving IGF-II exhibited similar insulitis scores to those of control mice (data not shown). These data suggest that treatment with IGF-I/IGFBP-3 and IGF-I, but not IGF-II, efficiently blocks islet inflammation to an equivalent extent during the development of TID in NOD mice.

IGF-I/IGFBP-3 treatment prevents TID in NOD mice

To determine whether administration of IGF-I/IGFBP-3 protects NOD mice from TID and to identify an optimal regimen of IGF-I/IGFBP-3 treatment, female NOD mice (4–5 wk old) were treated sc with various doses of IGF-I/IGFBP-3 for 4 wk and then monitored for their incidence of TID. Treatment with IGF-I/IGFBP-3 at a dose of 2 mg/kg/d, but neither 0.2 nor 1 mg/kg/d, significantly reduced the incidence of TID by 50% compared with the control group (P < 0.05) (Fig. 2A). An increase in dose of IGF-I/IGFBP-3 to 4 mg/kg/d showed no further beneficial effect (25% incidence vs. 50% incidence in control mice at 24 wk of age; n = 8–10 mice per group). Surprisingly, 80% of mice that received IGF-I/IGFBP-3 at 1 mg/kg every other day displayed the lowest incidence of TID (20%) up to 35 wk of age. The latter treatment protocol may represent an optimum therapeutic regimen, because use of this protocol beginning at either 2 or 8–10 wk of age did not further reduce the incidence of TID in NOD mice (our unpublished observations).
To explore whether a similar protocol of IGF-I treatment prevents the onset of T1D, we monitored the incidence of T1D in NOD mice either treated from 4 wk of age with IGF-I (0.4 mg/kg/d, twice a day) or vehicle or left untreated. Disease onset was delayed for only approximately 4 wk in IGF-I-treated compared with vehicle-treated or untreated mice, and the incidence of T1D was similar in all three treatment groups at 32 wk of age ($P < 0.05$) (Fig. 2B). Thus, IGF-I/IGFBP-3 appears to afford more efficient protection from T1D than free IGF-I.

Further evidence in support of the use of IGF-I/IGFBP-3 to protect against T1D was obtained from analyses of CY-induced T1D in NOD mice. Mice treated with IGF-I/IGFBP-3 at 2 mg/kg/d showed a 2-wk delay in disease onset compared with vehicle-treated control mice, but a high cumulative incidence of T1D (55 and 75%, respectively) was observed in both groups (Fig. 2C). In contrast, administration of IGF-I/IGFBP-3 at 1 mg/kg/d every other day reduced the incidence of T1D to 30% ($P < 0.01$), demonstrating that IGF-I/IGFBP-3 treatment can also prevent T1D in an accelerated and more aggressive model of the disease.

**IGF-I/IGFBP-3 treatment may protect from T1D by providing an antiinflammatory environment in the pancreas rather than activating regulatory T cells**

We have shown that IL-4-induced polarization of T helper-1 to T helper-2 cell responses is associated with the pro-
tection from T1D in NOD mice (30, 31). In addition, we reported that increased expression of the CCL3 chemokine in the pancreas is associated with progression to T1D, whereas increased CCL4 expression in the pancreas leads to protection from T1D in NOD mice (32). Because activated T cells express IGF-I receptors (33), it is possible that protection from T1D by IGF-I/IGFBP-3 is mediated by the modulation of activated T cell responses to islet β-cells. To test this possibility, we initially examined the effects of IGF-I/IGFBP-3 treatment on the expression of IFN-γ, IL-4, CCL3, and CCL4 in the spleen and pancreas of female NOD mice. After a 4-wk treatment with IGF-I/IGFBP-3, decreased CCL3 and increased CCL4 mRNA expression was observed in the pancreatic lymph nodes (PLNs) but not in the islets (Fig. 3A) or spleen (data not shown) of treated mice relative to that detected in vehicle-treated control mice. A modest reduction (15% decrease vs. control; \( P < 0.05 \)) in CCL3 but not CCL4 protein expression was noted in the pancreas (Fig. 3B). Similarly, IGF-I/IGFBP-3 treatment significantly reduced the level of IFN-γ protein expression in the pancreas (Fig. 3C) but did not affect the level of IL-4 expression (Fig. 3D). These findings are consistent with the notion that IGF-I/IGFBP-3 treatment may elicit a T helper-2 cell-like regulatory T cell environment in the pancreas that preserves β-cell survival and function. However, our adoptive T cell transfer studies demonstrated that splenic T cells from IGF-I/IGFBP-3-treated mice do not transfer protection from T1D in NOD-Scid recipients (Fig. 3E), suggesting that regulatory T cells are not induced by our IGF-I/IGFBP-3 treatment protocol. Thus, the protection from T1D conferred by IGF-I/IGFBP-3 may be partially related to the induction of an antiinflammatory milieu rather than activation of regulatory T cells.

Fig. 3. IGF-I/IGFBP-3 treatment protects from T1D by stimulating an anti-inflammatory environment in the pancreas of NOD mice. A and B, RT-PCR analysis of CCL3 and CCL4 gene expression in NOD PLNs and islets. Total tissue RNA was pooled from six mice per group after IGF-I/IGFBP-3 treatment (2 mg/kg/d). PCRs were run on an ethidium bromide-stained 1.5% agarose gel and amplification products visualized and quantified using a molecular imager as described in Materials and Methods. Data are represented as mean ± SD of intensities of RT-PCR signals for chemokine mRNA, as determined by densitometry of cDNA followed by normalization to the glyceraldehyde-3-phosphate dehydrogenase signals in individual samples of three experiments. *, \( P < 0.05 \); **, \( P < 0.01 \) compared with controls. C–E, Chemokine and cytokine levels in the pancreas after treatment. Intrapancreatic concentrations of CCL3 and CCL4 (C), IFN-γ (D), and IL-4 (E) were determined by ELISA. Data represent the mean concentrations ± SD in six mice per group. *, \( P < 0.05 \) vs. controls. F, Cumulative incidence of T1D in NOD-Scid recipient mice. Female NOD-Scid recipients were injected ip with diabetogenic spleen T cells \( (5 \times 10^8) \) and an equal number of spleen T cells from either IGF-I/IGFBP-3- or vehicle-treated female NOD mice. Results of one of two reproducible experiments are shown. A Student's t test was used to analyze statistical differences between the above data sets.
implying that IGF-I/IGFBP-3 may have more direct effects on the survival and function of islet β-cells than T cells.

**IGF-I/IGFBP-3 reduces β-cell apoptosis in NOD mice**

To assess whether IGF-I/IGFBP-3 regulates β-cell survival in treated mice, an in situ TUNEL assay was used to evaluate the levels of β-cell apoptosis in pancreatic tissue sections obtained from NOD mice treated with IGF-I/IGFBP-3 or vehicle control. Islets from mice that received IGF-I/IGFBP-3 displayed a 2.5-fold reduction ($P < 0.02$) in the number of intra-islet apoptotic nuclei relative to control mice (Fig. 4A). Positive staining for insulin identified the apoptotic nuclei to be localized in β-cells. To further determine whether IGF-I/IGFBP-3 rescues β-cells from apoptosis, primary islets were cultured in vitro with apoptosis-inducing cytokines and the effects of IGF-I on β-cell death were monitored. Our preliminary studies indicated that IGF-I, in contrast to IGF-I/IGFBP-3, does not function efficiently in vivo as a complex when cultured with either islets or β-cells. This result suggested that there is a lack of function of specific proteases that release free IGF-I from the complex; such proteases are abundant in serum and blood vessels (34). In addition, we found that IGFBP-3 alone or in combination with IGF-I did not alter the bioactivity of islet β-cells. Thus, free IGF-I was used for the following in vitro studies to explore whether the protection from T1D is mediated by an IGF-I/IGFBP-3 complex. TNF-α and IFN-γ were chosen for study, as these cytokines mediate insulitis and elicit β-cell apoptosis (6, 35–37). In vitro exposure of primary islet β-cells to TNF-α + IFN-γ in the absence of IL-1β for 48 h efficiently induced apoptosis in approximately 50% of the cells (Fig. 4B), as estimated by an MTT assay (38). Addition of IGF-I to these cultures reduced the percentage of apoptotic islet cells to approximately 25%, indicating their greater resistance to cytokine-induced cell death ($P < 0.01$). Next, we examined whether cytokine-induced cell death occurs through activation of caspase-3, a mediator and early indicator of apoptosis (33). After exposure to TNF-α + IFN-γ for 24 h, 35% of islet cells stained positive for caspase-3 compared with 10% for control samples. Upon addition of IGF-I, the number of caspase-3-positive islet cells was reduced to approximately 20%. These data further support the idea that β-cell death induced by TNF-α + IFN-γ occurs via apoptosis and that IGF-I blocks this apoptosis and results in β-cell survival. Thus, administration of IGF-I/IGFBP-3 may afford protection from T1D by preventing the apoptosis of β-cells induced by cytokines expressed during disease development.

**Regulation of Bcl-2 family members and caspase-9 activity by IGF-I contributes to inhibition of β-cell apoptosis**

Bcl-2 protein family members possesses both pro- and antiapoptotic functions in a mitochondrial-mediated pathway of β-cell apoptosis (13, 39, 40). To elucidate the mechanism(s) of how IGF-I inhibits β-cell apoptosis induced by cytokines, we examined the expression of Bcl-2 family members and caspase-9 activity in the NIT-1 β-cell line and primary islets after exposure to TNF-α + IFN-γ ± IGF-I in vitro. NIT-1 cells exposed to TNF-α + IFN-γ express a 2-fold increase in proapoptotic Bax as revealed in immunoblots (Fig. 5A, lane 3), suggesting that Bcl-2 family members regulate islet β-cell homeostasis. Addition of IGF-I (100 ng/ml) significantly reduced the level of Bax expression (8-fold) and increased the level of Bcl-XL expression (2-fold) relative to that observed for NIT-1 cells exposed to TNF-α + IFN-γ alone (Fig. 5A, lane 4). This stimulation of Bcl-XL expression...
and significant reduction of Bax expression was not observed when IGF-I was added at 1000 ng/ml (Fig. 5A, lane 5). Similar to Bax, Bad expression was normal and efficiently phosphorylated in NIT-1 cells cultured for 24 h in medium alone (Fig. 5B, lane 1). Exposure to IGF-I alone reduced the expression of Bad and maintained its level of phosphorylation (Fig. 5B, lane 2). In contrast, a normal level of Bad expression was maintained (relative to medium alone), but Bad phosphorylation was significantly diminished in cells exposed to TNF-α + IFN-γ (Fig. 5B, lane 3). Addition of IGF-I to cultures containing TNF-α + IFN-γ significantly reduced the level of Bad expression and augmented the level of Bad phosphorylation (Fig. 5B, lane 4).

We next examined whether similar mechanisms mediate the apoptosis of primary islet β-cells. NOD mouse islet cells were cultured for 48 h under the same conditions used for NIT-1 cells. IGF-I-stimulated islet cells expressed 4-fold more Bcl-2 and 4-fold more Bcl-XL (Fig. 5C, lane 2) compared with control islet cells (Fig. 5C, lane 1). Similar increases in Bcl-2 and Bcl-XL expression were detected for islet cells stimulated...
in the presence of TNF-α + IFN-γ (Fig. 5C, lane 3), suggesting that antiapoptotic molecules may be expressed as a compensatory measure in islet cells stressed under these conditions. It is noteworthy that IGF-I stimulation further enhanced both Bcl-2 (2-fold) and Bcl-XL (2.3-fold) expression in islet cells exposed to only TNF-α + IFN-γ (Fig. 5C, lane 4). These results emphasize the potential importance of the mitochondrial-dependent pathway of caspase activation in TNF-α + IFN-γ-induced apoptosis of β-cells and suggest that IGF-I may promote islet β-cell survival by regulating the levels of Bcl-2 family members and down-regulating mitochondrial-dependent apoptosis induced by cytokines.

Caspase-9 and -12 have been proposed to influence islet β-cell apoptosis (41–43). Here, we analyzed whether IGF-I has an effect on these early apoptosis mediators whose accumulation is triggered by mitochondrial damage or stressed endoplasmic reticulum. After exposure to TNF-α + IFN-γ for 6 h, caspase-9 was significantly cleaved in NIT-1 cells (Fig. 5D, lane 3), suggesting that activation of caspase-9 participates in cytokine-induced β-cell death. Addition of IGF-I efficiently blocked the cleavage of caspase-9 activated by TNF-α + IFN-γ. On the other hand, caspase-12 was not detectable in NIT-1 cells stimulated under the above-described conditions (our unpublished data), indicating that caspase-12 does not participate in TNF-α + IFN-γ-induced β-cell apoptosis. Our additional studies demonstrated that β-cell death induced by TNF-α + IFN-γ cannot be fully blocked by inhibitors of caspase-3, -8, and -9 (our unpublished data), suggesting that initiator caspsases, such as caspase-2, may also regulate cytokine-induced β-cell apoptosis (44).

**IGF-I-mediated β-cell protection against apoptosis depends on activation of the PI3-K/Akt-1 signaling pathway**

The PI3-K/Akt-1 signaling pathway is a key pathway for β-cell metabolism, proliferation and survival (45–47). To determine whether activation of this pathway contributes to IGF-I-mediated resistance to β-cell apoptosis induced by inflammatory cytokines, we first examined the role of PI3-K activity in NIT-1 cell apoptosis by evaluating the effects of PI3-K blockade on β-cell death. A high concentration (50 μM) of LY294002, an inhibitor of PI3-K, reduced NIT-1 cell viability by approximately 45% compared with that observed for cells cultured with medium alone, indicating that PI3-K controls the normal survival and/or proliferation of NIT-1 cells (Fig. 6A). As expected, TNF-α + IFN-γ also induced NIT-1 cell death to a similar extent. However, NIT-1 cells are much more susceptible to TNF-α + IFN-γ-induced apoptosis in the presence of 50 μM LY 294002, as cell viability was...
s significantly decreased from approximately 54 to 11%. IGF-I-mediated rescue from apoptosis was also partially inhibited by LY294002 in a dose-dependent manner (5–20 μM). Given that one of the most important downstream effectors of PI3-K is Akt-1, which exerts its antiapoptosis function via the phosphorylation-dependent inactivation of Bad (48), we assessed Akt-1 activity in NIT-1 cells after exposure to IGF-I alone or TNF-α + IFN-γ ± IGF-I. Whereas IGF-I activated the phosphorylation of Akt-1 in NIT-1 cells (Fig. 6B, lane 2), this phosphorylation was not changed upon exposure to TNF-α + IFN-γ compared with the control medium alone (Fig. 6B, lane 4). However, inclusion of IGF-I in the latter cultures partially enhanced the level of Akt-1 phosphorylation (Fig. 6B, lane 5) to that observed upon stimulation by cytotoxic cytokines (Fig. 6B, lane 4). The presence of LY294002 (20 μM) blocked the increase in Akt-1 phosphorylation induced by IGF-I (Fig. 6B, lane 6). Immunoblotting analyses of total Akt indicated that equivalent levels of Akt were present in NIT-1 cells under the various incubation conditions. Taken together, our data indicate that the stimulation of β-cell survival by IGF-I in a proinflammatory and proapoptotic milieu depends on the PI3-K/Akt-1 signaling pathway.

**Increased β-cell replication in IGF-I/IGFBP-3-treated mice**

Because of its mitogenic capacity, IGF-I can regulate β-cell proliferation in vitro and in vivo (49–51). It is possible that long-term treatment with IGF-I may prevent T1D in NOD mice by enhancing β-cell proliferation. To test this possibility, we assayed β-cell proliferation by BrdU incorporation in response to IGF-I and also measured the size of islets after IGF-I/IGFBP-3 treatment. β-cell proliferation was significantly enhanced in mice treated with IGF-I/IGFBP-3 compared with vehicle-treated mice (Fig. 7A). These newly proliferating β-cells were localized predominantly near the infiltrating zone of islets (our unpublished data). No increase in β-cell mass was observed in mice treated with IGF-I/IGFBP-3. In addition, the distribution of islet size was not altered in mice treated with IGF-I/IGFBP-3 compared with vehicle-treated control mice, although a high number of small-size islets (<100 μm²) were observed in IGF-I/IGFBP-3-treated mice (Fig. 7B). Thus, IGF-I may protect from the development of T1D by stimulating β-cell proliferation.

**IGF-I suppresses the expression of several genes that encode proteins in the apoptotic signaling pathways of islet cells**

To further understand how IGF-I prevents the apoptosis of islet β-cells, we used cDNA microarrays to analyze the expression profiles of several genes that encode proteins in NOD islet cell apoptotic signaling pathways triggered by TNF-α ± IFN-γ. After exposure to these cytokines for 24 h, 12 islet gene transcripts were detected on a cDNA template filter consisting of 96 apoptosis-related genes (Table 1). These transcripts include those of the DAP kinase (2), DFFA, DR3, DR6, Myd88, TNF-β, and Trail proapoptotic genes. Other proapoptotic genes, such as Flash and TNFRSF11A, exhibited lower levels of transcription. On the other hand, the expression of some antiapoptotic genes, including IAP-1 and NIP-3, was also elevated, which may indicate that islet cells up-regulate their self-antiapoptosis machinery to rescue themselves from cell death induced by cytokines. In comparison with gene profiles of islets exposed to cytokines alone, islets exposed to IGF-I did not yield the activation of some proapoptotic genes such as DAP kinase (2), DFFA, and Flash. Surprisingly, IGF-I significantly down-regulated the expression of a group of newly defined proapoptotic genes, such as DR3, DR6, Myd88, TNF-β, TNFRSF11A, and Trail. The expression of some proapoptotic genes was also suppressed in islets cultured with IGF-I due to hypoxia resulting from the islet isolation procedures (data not shown). These results implicate the complexity of genes involved in the regulation of β-cell apoptosis pathways activated by TNF-α + IFN-γ and reinforce the importance of IGF-I as a potent antiapoptotic agent for β-cells.

**Discussion**

In this study, we examined the effects of IGF-I/IGFBP-3 on both spontaneous and CY-induced T1D in NOD mice. We demonstrate for the first time that administration of IGF-I/IGFBP-3 to NOD mice starting at 4 wk of age not only diminished the severity of insulitis but also significantly re-
complex.

unlikely that IGFBP-3 functions independently of IGF-I in- clinical (hyperglycemia and reduced insulin sensitivity) obtained in previous animal (IGFBP-3 transgenic mice) and ptotic function of IGF-I. Our shown as the ratio of gene expression in islets exposed to cytokines to genes encoding proteins expressed in apoptosis pathways. Results are shown as the ratio of gene expression in islets exposed to cytokines to that in islets exposed to cytokines + IGF-I +, indicates that gene transcripts were detected only in islets exposed to cytokines.

duced the incidence of spontaneous and CY-induced T1D at 35 wk of age in a dose-dependent manner. Note that the content of IGF-I in the IGF-I/IGFBP-3 complex used here was one fifth (i.e. 0.2 mg/kg) of that used in a previous investigation (25). Furthermore, free IGF-I had only a transient effect on reducing insulitis but did not afford any significant protection against the development of T1D. Our observations demonstrate that the bioactivity of IGF-I present in the form of an IGF-I/IGFBP-3 complex is considerably enhanced in vivo compared with that of free IGF-I and provide further support for the potential therapeutic application of IGF-I/ IGFBP-3 treatment in the prevention of human T1D. Nonetheless, it is important to bear in mind that the therapeutic efficacy of IGF-I/IGFBP-3 treatment can also be influenced by the dose and tissue sensitivity of this complex as well as the disease status of prospective recipients. An example of this was reported in a study of experimental allergic encephalomyelitis, in which a high dose of the IGF-I/IGFBP-3 complex administered after disease onset increased the severity of disease (25).

In addition to its role in IGF-I transport, IGFBP-3 can also regulate cell survival and apoptosis (34). Upon treatment with IGF-I/IGFBP-3, the protective effect in T1D mediated by IGF-I may be influenced in part by IGFBP-3 bioactivity. Interestingly, at the outset of this study, the effect of IGFBP-3 on islet cell survival and apoptosis was examined in vitro. Our data suggest that IGFBP-3 alone or in combination with IGF-I (10–1000 ng/ml) neither induces islet cell proliferation nor enhances cell survival under conditions of cytokine-induced islet β-cell cytotoxicity (our unpublished observations). Rather, we found that IGFBP-3 blocks the antiapoptotic function of IGF-I. Our in vivo data support those obtained in previous animal (IGFBP-3 transgenic mice) and clinical (hyperglycemia and reduced insulin sensitivity) studies of the IGF/IGFBP-3 complex (26, 52, 53). Thus, it is unlikely that IGFBP-3 functions independently of IGF-I independent when present in the form of an IGF-I/IGFBP-3 complex.

To evaluate the effects of IGF-I on immune responses against islet cells, we determined the cytokine and chemokine profiles in the spleen and pancreas of IGF-I/IGFBP-3-treated mice and monitored their splenic regulatory T cell activity. These mice did not exhibit profound changes in their levels of IFN-γ, IL-4, CCL3, and CCL4 in the pancreas, although there was an observed trend toward reduced IFN-γ and CCL3 compared with vehicle-treated mice. However, reduced CCL3 and increased CCL4 mRNA expression was observed in the PLNs of IGF-I/IGFBP-3-treated mice. In the regulation of the CCL3 and CCL4 chemokines by IGF-I/IGFBP3, changes observed in the chemokine protein level in pancreatic tissues seem less significant than the changes detected in mRNA levels assayed by RT-PCR. This discrepancy may be due to several reasons. First, due to the relatively short half-life of chemokines at the site of inflammation, the high content of proteases, and excellent blood circulation in the pancreas, CCL3 and CCL4 may be quickly degraded or diluted during the euthanasia of mice and subsequent sample preparation. Thus, the difference in protein levels between the two groups may be artificially reduced. Second, at 8–10 wk of age, the distribution of chemokine-producing T cells and other leukocytes may differ between PLNs and islets. IGF-I may also have more functional activity in the PLNs relative to islets in treated mice. The latter point is supported by our RT-PCR data. However, when we measured chemokine protein levels in whole pancreatic tissue (includes a few small lymph nodes), the difference between the two groups of PLN samples was reduced possibly due to dilution by whole tissue-derived proteins. Considering the protein and mRNA levels of chemokines, severity of insulitis, and our laboratories’ previous reports on these chemokines during insulitis (32, 54, 55), we believe that the changes observed in CCL3 and CCL4 levels are associated with IGF-I-mediated protection from diabetes possibly through the inhibition of migration of diabetogenic T cells to islets. In fact, our ongoing study of CCL4 activity indicates that the ability of this chemokine to mediate protection from T1D involves the reduced recruitment of CDB4 + T cells to islets during the development of T1D (our unpublished observations).

Recently, β-cell apoptosis has been identified as a key mechanism of islet destruction in the pathogenesis of T1D. Here we examined the ability of IGF-I/IGFBP-3 treatment to counteract β-cell apoptosis in NOD mice. We found that mice treated with IGF-I/IGFBP-3 had fewer apoptotic islet β-cells than vehicle-treated mice. These data provide direct evidence that the administration of IGF-I in vivo in the form of an IGF-I/IGFBP-3 complex effectively enhances β-cell resistance to cytokine-induced cytotoxicity. Although local expression of IGF-I in β-cells prevents β-cell apoptosis induced by multi-low-dose streptozotocin treatment in transgenic C57BL/6-SJL mice and human islets (20, 50), our study is the first to demonstrate that IGF-I administered systemically inhibits β-cell apoptosis in NOD mice that spontaneously develop T1D. We further observed that this IGF-I-induced blockade of β-cell apoptosis is mediated by the up-regulation of antiapoptotic Bcl-2 and Bcl-Xl and down-regulation of Bad and Bax in β-cells. Moreover, we showed that IGF-I inhibits the caspase-9-mediated mitochondrial-dependent pathway of β-cell apoptosis. These observations not only emphasize the importance of the mitochondrial-dependent death path-

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cytokines:cytokins + IGF-I</th>
<th>Cytokins only</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. IAP1</td>
<td>2.8</td>
<td>+</td>
</tr>
<tr>
<td>2. DAP kinase (2)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3. DPFA</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>4. NIP-3</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>5. Flash</td>
<td>7. TNF-β</td>
<td></td>
</tr>
<tr>
<td>6. DR6</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>7. DR3(apo3)</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>8. Myd88</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>9. TNFRSF11A</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>10. Trail</td>
<td>2.9</td>
<td></td>
</tr>
</tbody>
</table>

Pancreatic islets (500 per sample) were treated with TNF-α and IFN-γ (each at 5 ng/ml) in the presence or absence of IGF-I (100 ng/ml) for 24 h. RNA was isolated and analyzed by cDNA array analyses of genes encoding proteins expressed in apoptosis pathways. Results are shown as the ratio of gene expression in islets exposed to cytokines to that in islets exposed to cytokines + IGF-I +, indicates that gene transcripts were detected only in islets exposed to cytokines.
way of β-cells activated by cytokines but also reveal possible antiapoptotic mechanisms mediated by IGF-I as a survival signal in preventing β-cell apoptosis.

The PI3-K and Akt-1 signaling pathway regulates β-cell growth and metabolism (19, 45, 46, 56, 57). Previously, we showed that IGF-I is not detectable in lymphocyte-infiltrated pancreatic islets (16), suggesting that cytokines may diminish IGF-I-activated survival signals and render islet β-cells more susceptible to cytokine-induced apoptosis. In support of this notion, we found that the LY294002 inhibitor of PI3-K abrogated the protective effect of IGF-I on NIT-1 cells. In addition, enhanced NIT cell death was detected in the presence of this inhibitor. Immunoblots of NIT-1 cell extracts showed that changes in Akt-1 activity parallel those in cell viability. Thus, our data further demonstrates that cytotoxic cytokines can inhibit survival signaling pathways in β-cells, and emphasizes the importance of the PI3-K/Akt-1 signaling cascade as a key pathway of IGF-I-mediated protection from apoptosis in β-cells. Although it remains to be determined how IGF-I counteracts cytokine-mediated death signaling in β-cells, members of the suppressor of cytokine signaling (SOCS) protein family may be involved as SOCS family members are known to regulate IGF-I signaling pathways (58, 59). Whether a SOCS protein(s) indeed blocks cytokine-induced β-cell apoptosis by activating IGF-I signaling pathways requires additional experimentation.

We observed that NOD mice receiving IGF-I/IGFBP-3 had augmented β-cell proliferation rather than increased β-cell mass and islet size. In transgenic mouse studies, overexpression of IGF-I and the constitutive expression of Akt-1 in β-cells results in increased β-cell mass, hyperplasia of islets, and resistance to streptozotocin-induced diabetes (50, 56). In these transgenic models, isletigenic pathways are not continuously suppressed by cytotoxic cytokines, and β-cells are destroyed relatively rapidly. These factors may offer the residual surviving islet β-cells an opportunity for regeneration. However, during the pathogenesis of T1D in NOD mice, islet β-cell death is a chronic event and the mitogenic activities initiated by the autocrine/paracrine form of IGF-I may be suppressed by cytokines secreted by islet-infiltrating lymphocytes. Moreover, considering the numerous stresses islets face during the process of inflammation, β-cell survival might be a physiologically more important process than proliferation. Therefore, it is conceivable that IGF-I/IGFBP-3 treatment may provide only a limited mitogenic signal for β-cell replication in NOD mice. Regardless, we believe that enhancement of β-cell replication partially contributes to reduced susceptibility of T1D in NOD mice after IGF-I/IGFBP-3 treatment.

Various death receptor activation pathways, including those mediated by Fas/FasL and TNF receptors, are stimulated in islet β-cells (13, 40, 60). However, it remains unknown how different death pathways interact with each other to regulate cell death and whether IGF-I is able to suppress these amplified death signals, because β-cells are exposed to multiple cytokines during the pathogenesis of T1D. Our results show that many TNF receptor superfamily genes (DR3, DR6, TNF-β, and Trail) are activated by TNF-α + IFN-γ stimulation in islets, suggesting an amplification of death signals resulting from the expression of more than one surface death receptor. Moreover, we found that IGF-I down-regulated several of these death receptor gene transcripts. These findings further illustrate that IGF-I efficiently prevents β-cell apoptosis, especially when induced by inflammatory stress.

In summary, the present study demonstrates the efficacy of IGF-I/IGFBP-3 treatment in protection from T1D in NOD mice. Because treatment of IGF-I/IGFBP-3 has proven to inhibit acute inflammation, protect against tissue injury, and offer improvement of metabolism in clinical studies (61), our study may provide a new approach for prevention of T1D in humans.

Acknowledgments

We thank all the members of our laboratory for their advice, critique, and encouragement.

Received September 23, 2003. Accepted November 3, 2003.

Address all correspondence and requests for reprints to: Dr. Terry L. Delovitch, Director, Autoimmunity/Type I Diabetes Group, Robarts Research Institute, 1400 Western Road, London, Ontario N6G 2V4, Canada. E-mail: del@robarts.ca.

This work was supported by grants (to T.L.D.) from the Canadian Institutes of Health Research (MT-5729) and the Juvenile Diabetes Research Foundation (JDRF). W.C. was supported as a postdoctoral fellow by the JDRF (152–03-1999). T.L.D. is the Sheldon H. Weinstein Scientist in Diabetes at the University of Western Ontario.

References

1. Delovitch TL, Singh B 1997 The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. Immunity 7: 727–738
22. Dinger DE, Cheatham TD, Crowe EC. 1995 Insulin-like growth factors (IGFs) and IGF-I treatment in the adolescent with insulin-dependent diabetes mellitus. Metabolism 44:119–123
34. Chen et al. • Protection from T1D by IGF-I/IGFBP-3 Complex