Hepatocyte Growth Factor Preserves Beta Cell Mass and Mitigates Hyperglycemia in Streptozotocin-induced Diabetic Mice*

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Type I diabetes is an autoimmune disease that results in destructive depletion of the insulin-producing beta cells in the islets of Langerhans in pancreas. With the knowledge that hepatocyte growth factor (HGF) is a potent survival factor for a wide variety of cells, we hypothesized that supplementation of HGF may provide a novel strategy for protecting pancreatic beta cells from destructive death and for preserving insulin production. In this study, we demonstrate that expression of the exogenous HGF gene preserved insulin excretion and mitigated hyperglycemia of diabetic mice induced by streptozotocin. Blood glucose levels were significantly reduced in mice receiving a single intravenous injection of naked HGF gene at various time points after streptozotocin administration. Consistently, HGF concomitantly increased serum insulin levels in diabetic mice. Immunohistochemical staining revealed a marked preservation of insulin-producing beta cells by HGF in the pancreatic islets of the diabetic mice. This beneficial effect of HGF was apparently mediated by both protection of beta cells from death and promotion of their proliferation. Delivery of HGF gene in vivo induced pro-survival Akt kinase activation and Bcl-xL expression in the pancreatic islets of diabetic mice. These findings suggest that supplementation of HGF to prevent beta cells from destructive depletion and to promote their proliferation might be an effective strategy for ameliorating type I diabetes.

Diabetes is a devastating illness with significant morbidity and mortality, and its incidence has increased steadily worldwide (1). Type I diabetes is an autoimmune disease that results in extensive destruction of the insulin-producing beta cells in the islets of Langerhans in pancreas. Current clinical therapy for type I diabetes is to replace insulin through multiple injections, or replace the destroyed beta cell mass via islet or whole pancreas transplantation (2–4). Strategies in development include the establishment of human cell lines that can produce, process, store, and release insulin (5); direct intra-pancreatic injection of adenoviral vector containing human insulin gene (6, 7); and the induction of endocrine differentiation of stem cells or/and beta cell precursors (8–11). However, these approaches have their inherent limitations. For instance, injections at best only produce fluctuated, dysregulated levels of circulating insulin. Likewise, the shortage of human islets precludes the clinical use of islet transplantation on a large patient population. In addition, it remains a question whether the transplanted beta cells can survive for a long duration, in light of the continuous presence of the autoimmune triggers responsible for initial destruction of beta cells. In this context, we hypothesized that protecting pancreatic beta cells from destructive death may represent a new, novel strategy for preserving insulin production and for prevention and treatment of type I diabetes.

Hepatocyte growth factor (HGF) is a mesenchyme-derived, multifunctional protein that plays a critical role in cell survival, proliferation, migration, and differentiation (12–14). Earlier studies demonstrated that HGF and its specific receptor, a receptor tyrosine kinase encoded by c-met protooncogene, are highly expressed during pancreas development. HGF is also shown to function as an insulinotropic factor and promotes beta cell proliferation and regeneration (15–17). Transgenic mice overexpressing HGF in the islet beta cell resist the diabeticogenic effects of the beta cell toxin streptozotocin (STZ) (18, 19). Furthermore, intraperitoneal injection of HGF protein exhibits a favorable effect for amelioration of hyperglycemia in diabetic mice receiving a marginal mass of islet grafts (20). Collectively, these observations led us to speculate that HGF might play a critical role in promoting insulin-producing beta cell survival after injurious stimuli; if so, delivery of HGF may provide an effective means for preserving insulin excretion and for mitigating hyperglycemia in diabetes in vivo.

Earlier studies show that exogenous HGF protein has a very short half-life in blood circulation, presumably owing to its rapid clearance by the liver in vivo (21, 22). Thus, it is tremendously difficult to sustain circulating levels of exogenous HGF protein through direct injection, even at very short intervals. This problem may be potentially solved, at least partially, by developing an HGF gene transfer strategy that allows persistent expression in vivo. To this end, we recently described an efficient approach to introduce exogenous HGF gene in vivo by systemic administration of a naked plasmid vector. This hydrodynamics-based in vivo gene transfer strategy results in a high level of exogenous HGF protein in the circulation. Hence, this approach could provide an efficient way to examine the poten-
tial of exogenous HGF on preservation of beta cells in the pancreatic islets of diabetic animals.

In the present study, we report the effects of exogenous HGF on the development of diabetes in a mouse model induced by STZ. Our results suggest that exogenous HGF promotes beta cell survival and proliferation and, thereby, mitigates hyperglycemia in STZ-induced diabetic mice.

EXPERIMENTAL PROCEDURES

Animals—Male CD-1 mice weighing 18–22 g were purchased from Harlan Sprague-Dawley (Indianapolis, IN). They were housed in the animal facilities of the University of Pittsburgh Medical Center with free access to food and water. Animals were treated humanely in accordance with National Institutes of Health guidelines and by use of approved procedures of the Institutional Animal Use and Care Committee at the University of Pittsburgh.

Plasmid Injection—The recombinant human HGF expression plasmid (pCMV-HGF) that contains full-length human HGF cDNA driven under a human cytomegalovirus (CMV) promoter was cloned as described previously (23). The empty expression plasmid vector pCDNA3 was purchased from Invitrogen (Carlsbad, CA). Plasmid DNA was administrated into mice by a hydrodynamic-based gene transfer technique via rapid injection of a large volume of DNA solution through the tail vein, as previously described (24–26). Briefly, 20 µg of plasmid DNA was dissolved in 1.6 ml of saline at a concentration of 12.5 µg/ml, and the solution was injected into the circulation within 5–10 s. Mice were injected with pCMV-HGF plasmid at 16 h prior to administration of STZ (Sigma, St. Louis, MO). Control mice were injected with 20 µg of empty vector pCDNA3 plasmid in an identical manner. STZ was given by a single intravenous injection at the dose of 100 mg/kg body weight (BW), unless indicated otherwise.

Groups of mice (n = 6) were sacrificed at 7 days after administration of STZ, respectively, and the pancreas was removed. For some experiments, additional groups of mice were sacrificed at 6, 12, 24, and 48 h after administration of STZ, respectively, and the pancreas was removed. For some experiments, additional groups of mice were sacrificed at 6, 12, 24, and 48 h after administration of STZ, respectively. One part of the pancreas was fixed in 10% buffered formalin for histological studies after paraffin embedding. Another part was immediately frozen in optimal cutting temperature compound for cryosectioning.

Blood Glucose Measurement—Blood was collected at 1, 3, and 7 days after administration of STZ by retro-orbital bleeding. Blood glucose levels were determined by using a colorimetric glucose assay kit, according to the procedures specified by the manufacturer (Sigma).

Determination of Insulin Level—Mouse serum insulin levels were determined by using an ultrasensitive insulin enzyme-linked immunosorbent assay (ELISA) kit (Crystal Chem Inc., Chicago, IL). Briefly, serum samples were collected at 1, 3, and 7 days after administration of STZ. Five-microliter samples or different concentrations of mouse insulin standard solutions and 95 µl of sample diluents were added to the 96-well microtiter plates pre-coated with the guinea pig anti-rat insulin antibody. After incubating for 2 h at 4 °C and followed by extensive washing, a 100-µl aliquot of horseradish peroxidase-conjugated anti-rat insulin antibody was added, and the plates were incubated for 30 min at room temperature. Following washing, they were then incubated with 100 µl of enzyme substrate solution. The plates were allowed to stand for 40 min at room temperature, and the reaction was stopped by addition of enzyme reaction stopping solution. The absorbance was read at 405 nm by an EL800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT). The concentration of serum insulin was expressed as nanograms per milliliter. For determination of the insulin content in pancreatic islets, isolated islets were resuspended in acid/ethanol (0.18 M HCl in 70% ethanol), sonicated, and extracted at −20 °C as described previously (27). After neutralization, the insulin content in the islet extracts was determined by using the same ELISA kit. The data were expressed as nanograms per microgram of total protein.

Immunofluorescence Staining—Indirect immunofluorescence staining was performed using an established procedure (14, 28). Briefly, pancreatic cryosections were prepared and mounted on poly-L-lysine-coated slides and fixed in 3% paraformaldehyde in PBS for 30 min, followed by incubation with 0.2% Triton X-100 for 15 min. The slides were stained with the specific primary antibodies using the Vector M.O.M. immunodetection kit according to the protocols specified by the manufacturer (Vector Laboratories, Burlingame, CA). The primary antibodies used were monoclonal mouse insulin antibody (Clone 2D11-H5, Ventana Medical Systems Inc.), anti-glucagon (A0565, alpha cell marker), anti-somatostatin (A0566, delta cell marker), and anti-pancreatic polypeptide (A0619, PP cell marker) antibodies (Dako, Carpinteria, CA). To visualize the primary antibodies, tissues were stained with either Cy3- or fluorescein isothiocyanate-conjugated secondary antibodies (Jackson Immunoresearch Laboratories). The slides were then stained with 4',6-diamidino-2-phenylindole, HCl (DAPI) to visualize the nuclei. Stained slides were mounted with anti-fade mounting medium (Vector Laboratories) and viewed with a Nikon Eclipse E600 Epi-fluorescence microscope equipped with a Nikon DXM 200 digital camera (Melville, NY). For quantification, the percentages of a particular cell type in the pancreatic islets were calculated, which were determined on at least five islets per animal, six animals per group.

Immunohistochemical Staining—Pancreatic sections from the mice were prepared at 4 µm thick by a routine procedure. Sections were deparaffinized and rehydrated through a graded series of alcohol to distilled water. The sections were then incubated for 15 min into 3% H2O2 in methanol at room temperature. Double immunohistochemical staining was performed using the M.O.M. immunodetection kit according to the specifications of the manufacturer (Vector Laboratories). The slides were first stained with monoclonal mouse insulin antibody (Clone 2D11-H5, Ventana Medical Systems Inc.) or a mixture of polyclonal rabbit anti-glucagon, anti-somatostatin, and anti-pancreatic polypeptide antibodies, followed by incubating with their respective secondary antibodies. For visualizing proliferating cell nuclear antigen (PCNA), the slides were re-incubated with anti-PCNA antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). As a negative control, the primary antibody was replaced with nonimmune mouse IgG. Apoptotic cells were identified via the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining, according to the procedure described previously (26, 29). A fluorescein-based TUNEL staining protocol was employed using an Apoptosis Detection System (Promega, Madison, WI). Cryosections of the pancreas were washed with PBS, fixed with 3% paraformaldehyde, and then treated with 0.2% Triton X-100 for 10 min. After pre-equilibration in 100 µl of buffer containing 200 mM potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 0.2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, and 2.5 mM cobalt chloride, strands of DNA were end-labeled by incubation at 37 °C for 1 h in 50 µl fluorescein-12-dUTP, 100 µM dATP, 10 µM Tris-HCl, pH 7.6, 1 mM EDTA, and TdT. The reaction was stopped by adding 2× sodium chloride/sodium citrate hybridization buffer for 15 min. To localize the pancreatic islets, immunofluorescence staining was performed on cryosections with anti-insulin antibody, as described elsewhere (14). For visualizing cell nuclei, the slides were also stained with DAPI. After washing, the slides were mounted with Vectashield anti-fade mounting media (Vector Laboratories) and observed on Nikon Eclipse E600 Epi-fluorescent microscope. The apoptotic index in the islets was defined as a percentage of apoptotic cells in total islet cell population, which were determined on at least five islets per animal, six animals per group.

Isolation of Pancreatic Islets—Mouse pancreatic islets were isolated by collagenase digestion followed by separation on Ficoll-Conray gradients, according to the procedure described previously (19). Briefly, the pancreas was injected through the pancreatic duct with 3 ml of 1.7 mg/ml collagenase (Sigma) in Hanks’ buffered saline solution (HBSS), removed, incubated at 37 °C for 20 min, and then passed through a 500-µm wire nylon mesh. The digested pancreas was rinsed with HBSS, and the islets were separated by density gradient in the Ficoll-Conray distribution with HBSS. The islet population was immunohistochemically stained manually using a Pasteur pipette with the aid of a dissecting microscope and pooled for protein extraction.

Western Blot Analysis—The isolated pancreatic islets as described above were homogenized in radioimmunoprecipitation assay lysis buffer (1% Nonidet P-40, 0.1% SDS, 100 µg/ml phenylmethylsulfonyl fluoride, 1% sodium deoxycholate, 0.5% sodium dodecyl sulfate, 1 mg/ml aprotinin, 2 µg/ml antipain, and 2 µg/ml leupeptin in PBS) on ice, and the supernatants were collected after centrifugation at 13,000 × g for 4 °C at 20 min. Protein concentration was determined using a bicinchoninic acid protein assay kit (Sigma), and islet lysates were mixed with an equal amount 2× SDS loading buffer (100 µl Tris-HCl, pH 6.8, 2% glycerol, 2% SDS, 0.001% bromphenol blue) at 100 °C. The samples were heated at 100 °C for 5–10 min before loading and separated on precast 10% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA). The proteins were electro-transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ) in transfer buffer containing 48 mM Tris-
HGF Gene Therapy for Diabetes

Fig. 1. Blood glucose and serum insulin levels in streptozotocin-induced diabetic mice after a single, intravenous injection of HGF plasmid. Mice were injected intravenously with either naked HGF plasmid pCMV-HGF or empty vector pcDNA3 at 16 h prior to administration of STZ, respectively. A and B, kinetics of blood glucose and serum insulin levels in STZ-induced diabetic mice. Blood glucose (A) and serum insulin (B) levels were determined at different time points as indicated after intravenous administration of STZ at 100 mg/kg body weight. C and D, blood glucose and insulin levels in mice at 7 days after injection of different doses of STZ. Groups of mice were administrated intravenously with 50, 100, and 150 mg/kg BW of STZ. At 7 days after STZ, blood glucose (C) and insulin (D) levels were determined. Data are presented as mean ± S.E. from six animals per group (n = 6). *, p < 0.05; **, p < 0.01.

RESULTS

HGF Mitigates Hyperglycemia and Preserves Insulin Production in Streptozotocin-induced Diabetic Mice—Mice were administrated intravenously with HGF plasmid at 16 h prior to injection of a specific diabetogenic agent, STZ. As previously reported (25), following a single, intravenous injection of naked plasmid encoding human HGF cDNA driven by a cytomegalovirus promoter (pCMV-HGF), serum HGF levels increased as early as 4 h, reaching a peak at 12 h. Quantitative determination by a specific ELISA for human HGF protein revealed ~8 ng/ml exogenous HGF in the circulation at the peak level (data not shown). Although serum HGF levels tended to decline, significant HGF protein (more than 1 ng/ml) was detectable in the circulation at 7 days after a single injection.

Blood glucose levels at different time points in mice after STZ injection are presented in Fig. 1A. We found that HGF significantly mitigated the STZ-induced hyperglycemia in diabetic mice. In mice injected with empty pcDNA3 vector, blood glucose levels started to elevate as early as 1 day and continued to increase to more than 300 mg/dl at 7 days after intravenous administration of STZ at 100 mg/kg BW. The dynamics of hyperglycemia observed in this study manifested the onset and development of diabetes in mice, presumably due to the destruction and depletion of beta cells in the islet of Langerhans in pancreas, as typically seen in this model. However, in mice given pCMV-HGF plasmid, blood glucose levels at various time points were significantly reduced, compared with the pcDNA3 control group (Fig. 1A). This reduction of blood glucose levels by

HCl, 39 mM glycine, 0.037% SDS, and 20% methanol at 4 °C for 1 h. Non-specific binding to the membrane was blocked for 1 h at room temperature with 5% Carnation nonfat milk in TBS buffer (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20). The membranes were then incubated for 16 h at 4 °C with various primary antibodies in blocking buffer containing 5% milk. The antibodies against phosphospecific Akt kinase and total Akt were obtained from Cell Signaling (Beverly, MA). The antibodies against Bcl-xL and actin were purchased from Santa Cruz Biotechnology, Inc. Following extensive washing for three times, the membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad) for 1 h at room temperature in 5% nonfat milk. The signals were visualized by the enhanced chemiluminescence system (ECL, Amersham Biosciences).

Immunoprecipitation—Immunoprecipitation was carried out essentially according to the procedures described previously (30). Briefly, pancreatic islets isolated from various groups were homogenized in radioimmune precipitation assay lysis buffer (1% Nonidet P-40, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml antipain, and 2 μg/ml leupeptin in PBS) on ice, and the supernatants were collected after centrifugation at 13,000 × g at 4 °C for 20 min. After protein concentration was determined, 200 μg of the lysates was pre-cleared by adding 0.25 μg of normal rabbit IgG and 20 μl of protein A/G Plus-agarose (Santa Cruz Biotechnologies) into 1 ml of whole islet lysates. After incubation for 1 h at 4 °C, supernatants were collected by centrifugation at 1,000 × g for 5 min at 4 °C. Lysates were immunoprecipitated overnight at 4 °C with 1 μg of anti-mouse c-Met (Santa Cruz Biotechnologies) and anti-phosphotyrosine-PY20 (BD PharMingen/Transduction Laboratories, San Diego, CA), respectively, followed by precipitation with 20 μl of protein A/G Plus-agarose for 3 h at 4 °C. After four washes with radioimmune precipitation assay buffer, the immunoprecipitates were boiled for 5 min in SDS sample buffer. The resulting precipitated complexes were separated on SDS-polyacrylamide gels and blotted with various antibodies as described above.

Statistical Analysis—Animals were randomly assigned to control and treatment groups. Data were expressed as mean ± S.E. Data were analyzed using SigmaStat software (Jandel Scientific, San Rafael, CA). Comparison between groups was made using one-way analysis of variance followed by the Student-Newman-Keuls test. A p value of less than 0.05 was considered significant.
HGF was reproducible in the diabetic mice induced by different doses of STZ (Fig. 1C), indicating that delivery of HGF gene substantially mitigates the hyperglycemia in diabetic animals.

We next examined the serum insulin levels in the STZ-induced diabetic mice. As shown in Fig. 1B, during the course of the development of diabetes induced by STZ, serum insulin levels progressively declined and reached to ~20% of normal level at 7 days after injection of STZ at 100 mg/kg BW. The reduction of serum insulin levels was closely correlated with an increase in blood glucose levels (Fig. 1A). Single injection of HGF gene significantly improved serum insulin levels at various time points in the diabetic mice induced by STZ. At 7 days, serum insulin levels in mice receiving HGF plasmid injection were more than 3-fold of that receiving empty vector (Fig. 1B). Consistently, HGF also increased serum insulin levels in the diabetic mice induced by various doses of STZ (Fig. 1D).

HGF Preserves Insulin-producing Beta Cells in Mouse Pancreas—The beneficial effects of exogenous HGF described above prompted us to further investigate the impacts of exogenous HGF on the survival and preservation of insulin-producing beta cells in the islets of pancreas in diabetic mice. To distinguish the beta cells from non-beta cells in the islets, we employed a double immunofluorescence staining technique. Beta cells were stained with anti-insulin antibody (red), whereas non-beta islet cells were recognized by a mixture of antibodies against glucagon (alpha cell marker), somatostatin (delta cell marker), and pancreatic polypeptide (PP cell marker) (green). Fig. 2 shows representative micrographs of the pancreatic islets of diabetic mice at different time points after STZ administration. In mice injected with STZ at 100 mg/kg BW, the numbers of insulin-positive beta cells in the pancreatic islets dropped very rapidly. At 48 h after STZ, very few, if any, islet cells with positive staining with insulin were observed in mice receiving empty pcDNA3 vector injection (Fig. 2C). Of note, there was a significant number of cells that were not stained with either insulin (red) or non-beta islet cell markers (green) within the islets after STZ. Such cells are presumably the degranulated beta cells caused by severe hyperglycemia. It is of interest to note that, owing to marked depletion of beta cells after STZ, non-beta islet cells (green) apparently aggregated in the center of the islets where beta cells normally were positioned (Fig. 2, C and D).

Delivery of HGF gene significantly mitigated the depletion of the beta cells in diabetic mice. Substantial numbers of insulin-positive beta cells was still present at 7 days after STZ in mice receiving pCMV-HGF plasmid injection (Fig. 2H). Fig. 2I shows the percentage of beta cells in the pancreatic islets at different time points after STZ between pcDNA3 and pCMV-HGF groups. At 7 days after injection of STZ at 100 mg/kg BW, the percentage of beta cells in the pancreatic islets dropped below 20%; most of them, if not all, were insulin-negative, degranulated beta cells. However, in mice receiving pCMV-HGF injection, about 60% of the total cell population within the islets was beta cells, and most of them displayed insulin positivity (Fig. 2, H and I). Consistent with these observations, we found a marked difference in the insulin content in the isolated islets at 7 days after STZ between pcDNA3 and pCMV-HGF groups (Fig. 2J), as determined by a quantitative measurement using specific ELISA.

HGF Protects Beta Cells from Death in Diabetic Mice Induced by STZ—We further investigated the cellular events that led to an increase in insulin-positive beta cells in STZ-induced diabetic mice by HGF. Because STZ is a diabetogenic agent that specifically induces beta cell depletion, we first examined the effects of HGF on beta cell death in the pancreatic islets of diabetic mice in vivo. Fig. 3 shows the effects of HGF gene transfer on apoptotic cell death in the pancreatic islets of diabetic mice induced by STZ, as demonstrated by TUNEL stain-
Fig. 3. HGF prevents cell death in mouse pancreas after administration of STZ. Cell apoptotic death in the islet of pancreas was determined by TUNEL staining at different time points as indicated after administration of STZ. A–F, representative micrographs show apoptotic cells identified by TUNEL staining (C and F) in the pancreatic islets at 12 h after administration of STZ at 150 mg/kg BW in mice receiving pcDNA3 (A–C) or pCMV-HGF (D–F). Cryosections were also stained with insulin antibody for localization of the islets in pancreatic sections (B and E). DAPI was used to stain the nuclei to facilitate easy counting of cell numbers (A and D). Scale bar, 30 μm. G, time course of the apoptotic index in the pancreatic islets after STZ administration at 150 mg/kg BW. At least five islets were counted per animal. Data are presented as mean ± S.E. of six mice (n = 6). *p < 0.05 versus normal control; **p < 0.01 versus pcDNA3.

HGF Promotes Beta Cell Proliferation in Vivo—Because alterations in either cell death or cell growth could potentially play a role in the preservation of beta cells, we next investigated the effects of HGF on beta cell proliferation in the pancreatic islets of diabetic mice in vivo. As shown in Fig. 4, HGF significantly promoted cell proliferation in the pancreatic islets in the early time points after injection of STZ. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA), an indicative marker for G1 to S phase transition, revealed a significant increase in the percentages of positive cells in the pancreatic islets of diabetic mice pre-administered with HGF plasmid (Fig. 4). This induction of PCNA expression in the pancreatic islets was prominent at 6 and 12 h after STZ (Fig. 4E); much less increase in PCNA was observed beyond 48 h after the onset of diabetes. Double-immunohistochemical staining showed that most PCNA-positive cells in the islets did not express non-beta cell markers (Fig. 4, A and B). Instead, co-localization of PCNA and insulin was clearly evident in the islets of pancreas in mice receiving HGF plasmid (Fig. 4D), indicating that HGF may preferentially promote beta cell proliferation. These results suggest that HGF preservation of the beta cell mass in the pancreatic islets of diabetic mice may also be mediated by enhancing beta cell proliferation.

HGF Promotes Beta Cell Survival by Activating Cell-survival Signaling—To elucidate the potential mechanism underlying HGF promotion of beta cell survival and amelioration of hyperglycemia in diabetic mice, we investigated the signal transduction pathways leading to cell survival by using isolated pancreatic islets from diabetic animals. As shown in Fig. 5, HGF receptor activation was confirmed by detecting the tyrosine phosphorylation of c-Met protein in the isolated islets. Immunoprecipitation studies revealed that more than 2-fold induction of tyrosine-phosphorylated c-Met was observed in the pancreatic islets from the mice receiving pCMV-HGF injection, compared with that in mice receiving pcDNA3 or in normal control mice (Fig. 5B), suggesting that the c-Met receptor in the pancreatic islets is activated after intravenous injection of naked HGF plasmid vector.

We examined the activation of protein kinase B/Akt kinase, a signaling protein that plays a central role in dictating cell survival or death (31), in the isolated pancreatic islets from the mice receiving either pcDNA3 or pCMV-HGF plasmid. As shown in Fig. 6, STZ at 100 mg/kg BW reduced the levels of phosphorylated Akt at 6 h, but not at 12 h, in the pancreatic islets. However, administration of HGF plasmid markedly induced phosphorylated Akt levels in the islets in a time-dependent manner. More than 4-fold induction of phosphorylated Akt was found in the isolated islets at 6 h after STZ administration. This induction of phosphorylated Akt in the pancreatic islets was transient; the phosphorylated Akt level only slightly increased and tended to return toward baseline at 12 h after the initial injury.

Fig. 7 shows the expression levels of pro-survival protein Bcl-xL in the pancreatic islets of the diabetic mice. Administration of the HGF gene markedly induced Bcl-xL overexpression in the pancreatic islets. More than 6-fold induction of Bcl-xL protein was observed at 6 h after administration of STZ at 100 mg/kg BW in the islets from the mice receiving pCMV-HGF plasmid (Fig. 7). Of note, severe diabetes induced by a
higher dose of STZ (such as 150 mg/kg BW) was associated with a repressed endogenous Bcl-xL protein expression, and HGF not only reversed this repression of Bcl-xL but also induced its expression in such conditions (data not shown). Thus, exogenous HGF dramatically activates and induces pro-survival signaling that results in Akt phosphorylation and Bcl-xL overexpression in the pancreatic islets of diabetic mice.

**DISCUSSION**

Although the pathogenesis underlying the onset and development of type I diabetes remains elusive, it is generally considered as an autoimmune disease that results in extensive destruction and depletion of insulin-producing beta cells in the islets of Langerhans in pancreas by cytotoxic T cells (32, 33). In this regard, protection of beta cells and/or promotion of their proliferation by supplementing growth factor could be a novel strategy to enhance beta cell resistance to apoptotic and necrotic damage inflicted by the immune system and various injuries (10, 34). In this study, we have demonstrated that systemic delivery of naked HGF plasmid protected beta cells from death and promoted their proliferation in a mouse model of diabetes induced by beta cell toxin STZ. The preservation of beta cells was accompanied by amelioration of hyperglycemia and increased circulating insulin. These findings suggest that protection of beta cells from destructive depletion and promotion of their proliferation by HGF might be an effective approach for ameliorating type I diabetes.

HGF preservation of beta cell mass in the pancreatic islets after injury is likely mediated by a dual mechanism: a decrease in beta cell loss and an increase in beta cell proliferation. However, it remains elusive as to a quantitative contribution of these mechanisms in mediating the beneficial effects of HGF on preservation of beta cell mass. In light of the fact that the primary pathogenesis of diabetes in STZ model is beta cell death, it is plausible to speculate that HGF preservation of the insulin production may be largely mediated by protection of the beta cells against apoptotic cell death after injurious stimulus. This cytoprotective role of HGF for pancreatic beta cells in vivo is consistent with a previous report in which transgenic mice overexpressing the HGF gene under rat insulin promoter resist the diabetogenic effects of STZ (18). It has also been shown that repeated injections of HGF protein ameliorate hyperglycemia in STZ-induced diabetic mice receiving a marginal mass of intrahepatic islet grafts (20). Although the mechanism underlying the beneficial effects of HGF was not determined in that study, it is possible that HGF elicits its actions through a protective effect on grafted islet cells from apoptosis in diabetic mice after transplantation (20), because earlier studies show that islet cell injury in the islet grafts after transplantation is mediated through the mechanisms of apoptosis (35, 36). However, cell proliferation may also play a significant role in mediating the beneficial effects of HGF, because there was an induction of the expression of PCNA (Fig. 4), a marker for G1 to S phase transition, in the insulin-positive beta cells after delivery of HGF gene. Regardless of the mechanisms involved, the fact that HGF inhibits beta cell apoptosis and promotes...
their proliferation in vivo underscores the remarkable ability of HGF to preserve beta cell mass of the pancreatic islets after destructive challenges.

In harmony with the ability to promote beta cell survival, delivery of the HGF gene resulted in marked activation of cell survival signaling in the pancreatic islets in vivo. Activation of PKB/Akt kinase and induction of Bcl-xL in the isolated pancreatic islets as presented in this study (Figs. 6 and 7) provide mechanistic explanations for the impressive beta cell survival-promoting capacity of HGF. Consistent with this observation, a recent study (27) also demonstrates an Akt activation in the isolated islets after treatment with HGF adenoviral vector. Of note, both Akt activation and Bcl-xL induction occur maximally at 6 h after STZ administration, a time point that significantly precedes the peak of apoptosis in the pancreatic islets (12 h). Akt kinase has recently emerged as a key component of cell survival machinery that plays a central role in regulating cell apoptosis and survival (31, 37). Extensive studies demonstrate that Akt activation will lead to multiple signal transduction pathways that result in cell survival. We previously showed that, in renal tubular epithelial cells, HGF induces Akt phosphorylation as early as 5 min through a phosphoinositide 3-kinase-dependent pathway (38), which is followed by phosphorylation and resultant inactivation of Bad, a pro-death member of Bcl-2 family (31). Furthermore, HGF can override Bad-induced renal epithelial cell death by stimulating Bad phosphorylation at both Ser112 and Ser136 sites (38). Hence, the dramatic activation of Akt kinase by HGF in isolated pancreatic islets implies that the pathway involving sequential phosphorylation of phosphoinositide 3-kinase, Akt, and Bad could also be a major signal mechanism leading to beta cell survival. It should be noted that, in addition to inducing Bad phosphorylation and inactivation, Akt has been shown to phosphorylate the Forkhead family of transcription factors such as FKHRL1, leading to their inactivation and inability to transcriptionally activate the genes essential for cell death (39, 40). Therefore, multiple possibilities exist as to the pathways elicited by activated Akt in mediating beta cell survival. It remains a question as to whether one single pathway plays a predominant role, or multiple pathways are necessary for mediating HGF/Akt-induced beta cell survival in the pancreatic islets. Further studies are needed to clarify these issues and to delineate the signaling mechanism leading to beta cell survival.

Besides Akt activation, the protein abundance of Bcl-xL, a pro-survival member of Bcl-2 family proteins, is markedly induced in the isolated pancreatic islets in mice receiving HGF plasmid injection. Of interest, the endogenous Bcl-xL protein level falls in the islets after STZ in a dose-dependent fashion, suggesting that a decrease in pro-survival Bcl-xL protein may account for, at least in part, the increasing apoptosis induced by the beta cell toxin. It is intriguing to note that exogenous...
of using a gene therapy approach for delivering HGF, rather proliferation by providing HGF may be an effective strategy for beta cells from destructive cell death and promotion of their apoptosis presumably by a mitochondria-dependent pathway, the marked induction of Bcl-xL by HGF provides a unique niche in pancreatic islets in which mitochondria-dependent apoptosis is blunted. Along this line, a recent report suggested that endothelial cell apoptosis induced by high d-glucose is associated with the translocation of Bax protein from cytosol to the mitochondrial membrane, whereas HGF inhibited the Bax translocation by inducing Bcl-2 (42). Therefore, beta cells in the pancreatic islets may be particularly sensitive to HGF protection by inducing overexpression of pro-survival Bcl-xL, thereby leading to inhibition of Bax protein translocation to the mitochondria and blockade of mitochondria-dependent apoptosis.

It is of importance to indicate that the protective effects on the beta cells of pancreatic islets are mediated by HGF, because its specific receptor, c-Met, was phosphorylated at the tyrosine residue in the pancreatic islets (Fig. 5). The magnitude of c-Met receptor activation, as demonstrated by its tyrosine phosphorylation, was likely underestimated at the time point (6 h after STZ administration; 22 h after HGF plasmid injection) examined in this study, because c-Met receptor-triggered intracellular signal transduction events occur within minutes after HGF stimulation in vivo (38).

The present study validates a hypothesis that protection of beta cells from destructive cell death and promotion of their proliferation by providing HGF may be an effective strategy for ameliorating type I diabetes. One might question the rationale of using a gene therapy approach for delivering HGF, rather than simply administering HGF protein. There are many obstacles for direct injection of HGF protein in vivo. Among them, the principal one is the impossibility of maintaining a constantly high level of exogenous HGF in circulation even when using repeated injections at short intervals, because exogenous HGF is cleared from the circulation by the liver within a few minutes after intravenous injection (22, 43). In accordance with this, a previous study (20) shows that repeated injections of HGF protein alone without dextran sulfate could not ameliorate hyperglycemia in diabetic mice after islet transplantation. Thus, developing a gene delivery strategy allowing efficient expression of HGF in vivo may be a prerequisite for evaluating its therapeutic efficacy in whole animals. In this context, intravenous injection of naked HGF plasmid as described in this report provides a convenient, simple, and highly efficient way to introduce exogenous HGF expression (26, 44) and to demonstrate its potential for beta cell preservation after injury in vivo.

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REFERENCES

1. Harris, M. I. (1998) Diabetes Care 21, Suppl. 3, C11–C14
2. Shapiro, A. M., Ryan, E. A., and Lakey, J. R. (2001) Lancet 358, (suppl.) S21
