Adenovirus-mediated Hepatocyte Growth Factor Expression in Mouse Islets Improves Pancreatic Islet Transplant Performance and Reduces Beta Cell Death*

Hepatocyte growth factor (HGF) increases beta cell proliferation and function in rat insulin promoter (RIP)-targeted transgenic mice. RIP-HGF mouse islets also function superiorly to normal islets in a transplant setting. Here, we aimed to determine whether viral gene transfer of the HGF gene into mouse islets \textit{ex vivo} could enhance the performance of normal islets in a streptozotocin-diabetic severe combined immunodeficient mouse model. In the present study, we therefore have explored the delivery of HGF transgenic mouse islets via an adenoviral delivery system. Specifically, we sought to (i) investigate the feasibility of adenoviral gene delivery of HGF in mouse islets \textit{ex vivo} and (ii) determine the effect of adenovirus-mediated HGF overexpression on the performance of mouse islets in a marginal mass model of islet transplant.

As anticipated, adenoviral delivery of HGF to mouse islets \textit{ex vivo} markedly improved islet transplant survival and performance. To our surprise, this protective effect was immediate and unlikely to be explained simply by improved beta cell proliferation. Instead, this rapid protective early effect was likely explained by a combination of enhanced beta cell function in the graft induced by HGF, as occurs \textit{in vivo} in the RIP-HGF mice, and/or increased graft beta cell survival, a novel beta cell action of HGF. The ability of HGF to enhance beta cell survival was directly documented using standard cell death assays and was confirmed in RIP-HGF mice and the beta cell line, INS-1. Mechanistically, the survival effect of HGF in beta cells appears to augment their function, or to be more resistant to cell death. Examples include insulin-like growth factors, glucagon-like peptide 1, exendin, parathyroid hormone-related protein, placental lactogen, and hepatocyte growth factor (HGF)\(^1\) (3–11).

Recently, we have developed a transgenic mouse model that overexpresses HGF in the pancreatic beta cell under the control of the rat insulin II promoter (RIP) (10, 11). These RIP-HGF transgenic mice display increased beta cell proliferation coupled with increased beta cell function. The latter resulting from an increase in the expression of insulin, glucokinase, and the glucose transporter, Glut-2. We have also shown that RIP-HGF transgenic mouse islets are significantly more effective than normal islets when transplanted into severe combined immunodeficient (SCID) diabetic mice (11). Thus, a marginal number of normal islets was insufficient to normalize blood glucose levels in SCID diabetic mice. In contrast, the same number of islets from RIP-HGF transgenic mice matched for size, protein, or DNA rapidly and completely normalized the blood glucose (11).

These findings suggest that gene transfer of the HGF cDNA into human islets could reduce the number of islets and donors required to correct diabetes in a human transplant setting. In the present study, we therefore have explored the delivery of HGF to mouse islets using an adenoviral delivery system. Specifically, we sought to (i) investigate the feasibility of adenoviral gene delivery of HGF in mouse islets \textit{ex vivo} and (ii) determine the effect of adenovirus-mediated HGF overexpression on the performance of mouse islets in a marginal mass model of islet transplant.

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Adolfo García-Ocana‡, Karen K. Takane, Vasumathi T. Reddy, Juan-Carlos Lopez-Talavera, Rupangi C. Vasavada, and Andrew F. Stewart

From the Division of Endocrinology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213

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† Recipient of a Junior Faculty Award by the American Diabetes Association. To whom correspondence should be addressed: BST E-1140, Division of Endocrinology, University of Pittsburgh School of Medicine, 3550 Terrace St., Pittsburgh, PA 15213, Tel.: 412-648-9770; Fax: 412-648-3290; E-mail: ocana@msx.dept-med.pitt.edu.

‡ Recipient of a Junior Faculty Award by the American Diabetes Association. To whom correspondence should be addressed: BST E-1140, Division of Endocrinology, University of Pittsburgh School of Medicine, 3550 Terrace St., Pittsburgh, PA 15213, Tel.: 412-648-9770; Fax: 412-648-3290; E-mail: ocana@msx.dept-med.pitt.edu.

1 The abbreviations used are: HGF, hepatocyte growth factor; RIP, rat insulin promoter; SCID, severe combined immunodeficient; PI3-kinase, phosphatidylinositol-3 kinase; m.o.i., multiplicity of infection; 5-X-gal, bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PBS, phosphate-buffered saline; STZ, streptozotocin; IE, islet equivalents; TUNEL, terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end-labeling; MTI, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Adv, adenovirus.

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peared to involve the PI3-kinase/Akt pathway and likely other pathways. Collectively, our prior studies with RIP-HGF mice together with the results reported in the present manuscript using adenoviral HGF gene therapy suggest that HGF overexpression in the islet markedly improves islet graft performance through a combination of effects including improved beta cell survival, enhanced glucose sensing and insulin secretion, and accelerated beta cell proliferation. Although the precise quantitative definition of these effects at specific time points in the post-transplantation period will require additional study, each would appear to be important. These results suggest that ex vivo gene transfer of HGF into human islets may be particularly useful in improving islet transplant performance and reducing the number of islets required for humans with Type 1 diabetes.

MATERIALS AND METHODS

Generation of Recombinant Adenovirus Vectors—Adenovirus was prepared according to the methods of Becker et al. (12) using Ad.5 constructs generously provided by Dr. Christopher Newgard (Duke University, Durham, NC). Complementary DNAs encoding β-galactosidase (Invitrogen) and murine HGF (10) were ligated into the cytomegalovirus promoter-containing, adenovirus-based plasmid, pPACKMVP.LPA (12). They were then co-transfected with a second plasmid (pJM17) (12), rupturing the E1 region and DNA from a replication-defective adenovirus, into HEK-293 cells (ATCC, Manassas, VA) using Superfect (Qiagen Inc., Valencia, CA), according to the manufacturer’s instructions. After recombinant and lysis of the cells, the medium was collected, and the remaining cells were lysed by freezing and thawing. The cell debris was pelleted, and the viral supernatant was saved for subsequent experiments. Adenovirus DNA was sequenced to confirm the correct orientation and sequence of β-galactosidase and mouse HGF cDNAs. Adenovirus was purified on a CsCl/Tris gradient, separated into aliquots, and stored at −20 °C until use. m.o.i. was determined by A260 and plaque assays (12).

Islet Isolation and Gene Transfer—Murine islets from adult CD-1 mice were isolated as previously described (10). Briefly, the pancreas was injected through the pancreatic duct with Hanks’ buffered saline solution containing collagenase, incubated, and then filtered through a 500-μm wire mesh. The digested pancreas was rinsed with Hanks’ buffered saline solution, and islets were separated by density gradient in Histopaque (Sigma). After several washes with Hanks’ buffered saline solution, islets were hand-picked under a microscope.

Mouse pancreatic islets were washed with RPMI 1640 medium and exposed to 100 μl of adenovirus for 1 h at 37 °C. Islets were then washed and incubated in 1 ml of RPMI 1640 medium containing 10% fetal bovine serum, 5 mm glucose, 100 units/ml penicillin, and 100 μg/ml streptomycin. Twenty-four hours after the infection, islets were harvested and used in the experiments described below. After 24 h in infection, with 250 m.o.i.AdvLacZ under these conditions, 29 ± 6% (n = 4) of islet cells were stained for X-gal following the standard methods for staining islets infected with AdvLacZ (13). This infection rate is comparable with that reported previously with rat and human islets (13, 14).

RNA Isolation, Reverse Transcription, and Relative Semiquantitative PCR Analysis of HGF mRNA Expression—Total DNA-free RNA was isolated from uninfected or adenovirus-transduced islets by using the DNA-free RNA isolation kit (Ambion, Austin, TX, according to the manufacturer’s instructions. Reverse transcription was performed as previously described (11). PCR was performed with 5 μl of cDNA in a final volume of 25 μl containing 1X Taq buffer (Promega, Madison, WI), 2.5 mm MgCl2 (Promega), 200 μM dNTPs (Promega), 0.5 μM of [α-32P]deoxyctydine triphosphate (3000 Ci/mmol, Amersham Biosciences), 400 nM HGF primer pair (11), 4 nM actin primer pair (Ambion), 16 nM actin competitors (Ambion) that allow actin RNA to be used as an internal control, and 1.25 units of Taq DNA polymerase (Promega). Tubes were placed in a thermal cycler, and the following program was applied: 3 min at 94 °C followed by 25 cycles at 94 °C for 30 s, 74 °C for 1 min (Promega), 200 μM dNTPs (Promega), 0.5 μM of [α-32P]deoxyctydine triphosphate (3000 Ci/mmol, Amersham Biosciences), 400 nM HGF primer pair (11), 4 nM actin primer pair (Ambion), 16 nM actin competitors (Ambion) that allow actin RNA to be used as an internal control, and 1.25 units of Taq DNA polymerase (Promega). Tubes were placed in a thermal cycler, and the following program was applied: 3 min at 94 °C followed by 25 cycles at 94 °C for 30 s, 72 °C for 1 min. The PCR products (actin, 293 bp, and HGF, 146 bp) were separated on a 6% polyacrylamide gel in Tris borate-EDTA buffer, and the gel was dried and exposed to x-ray film.

Immunoblot Analysis of HGF Protein Expression—Islet extracts were made in freshly prepared ice-cold lysis buffer (phosphate-buffered saline [PBS] with 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM sodium dodecyl sulfate, 1 mM sodium orthovanadate, 100 μg/ml phenylmethylsulfonyl fluoride, and 5 μg/ml aprotinin), incubated for 30 min on ice, and sonicated, the supernatant containing the cell lysate was separated, and protein concentrations were measured using the MicroBCA assay (Pierce). Fifteen micrograms of protein from uninfected or adenovirus-infected islets were resuspended in loading buffer containing β-mercaptoethanol, boiled for 5 min, and analyzed using a 10% SDS-polyacrylamide gel. Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) using standard techniques. After blocking for 1 h at room temperature with 5% nonfat milk in PBS containing 0.1% Tween 20, blots were incubated overnight at 4 °C with the following primary antibodies: anti-HGF antibody (1:200 dilution, Santa Cruz Biotechnology, Inc.) or against actin (rabbit polyclonal antibody at 1:100 dilution, Sigma). After several washes with PBS containing 0.1% Tween 20, blots were incubated with the corresponding peroxidase-conjugated secondary antibodies for 1 h at room temperature. Chemiluminescence was detected using the ECL system (Amersham Biosciences).

Glucose-stimulated Insulin Secretion in Isolated Islets—Insulin release from uninfected and adenovirus-transduced islets was measured in triplicate for each glucose concentration tested as previously described (11), with some modifications. Briefly, islets obtained 24 h after infection with AdvLacZ or AdvHGF and uninfected islets were preincubated for 45 min with Krebs-Ringer bicarbonate buffer supplemented with 10 mM HEPES, 1% bovine serum albumin, and 5.5 mM glucose for 1 h at 37 °C in a 5% CO2 incubator. After washing the islets once with the same solution, groups of 10 islets of similar size for each condition were incubated in 1 ml of fresh Krebs-Ringer bicarbonate buffer plus 1% bovine serum albumin and 5.5 or 22.2 mM glucose at 37 °C in the 5% CO2 incubator. After a 30-min incubation, buffer was removed and frozen at −20 °C until insulin measurement by radioimmunoassay (Linco Research, St. Louis, MO). Islets were then washed 3 times with PBS and digested overnight in 1 ml of 0.1 n NaOH at 37 °C. After neutralization with HCl, protein was measured by the Bradford method. Results are expressed as a percentage of insulin concentration obtained with uninfected islets incubated at 5.5 mM glucose.

Islet Transplantation—Murine CD-1 islets isolated and transduced with AdvLacZ or AdvHGF as described above were transplanted under the kidney capsule of streptozotocin (STZ)-induced diabetic, SCID mice (BALB/cByJ) (The Jackson Laboratory, Bar Harbor, ME) as previously described (11). Briefly, SCID mice were rendered diabetic by injecting 250 mg/kg body weight of STZ intraperitoneally. Diabetes was confirmed by the presence of hyperglycemia (>300 mg/dl), polyuria, and weight loss. Random non-fasted blood glucose was measured from the snipped tail by a Precision Q.I.D. portable glucometer (Medisense, Bedford, MA). After three consecutive days of hyperglycemia, SCID diabetic mice were transplanted with either uninfected or AdvLacZ- or AdvHGF-transduced islets (12). Briefly, islets were harvested from diabetes-resistant kidneys from 6- to 8-week-old C57BL/6 mice and transplanted into the kidney capsule of STZ-induced, SCID mice. After transplantation, blood samples were obtained from the snipped tail and analyzed for glucose levels using the portable glucometer.

Determination of Insulin Content—Insulin was extracted from graft-containing kidneys as previously reported (10) with some modifications. Briefly, graft-containing kidneys were washed with ice-cold PBS, finely minced, and homogenized through a 22-gauge needle, washed with ice-cold acid/ethanol (0.18 Ti HCl in 70% ethanol), sonicated for 15 s, and extracted at 37 °C. Tubes were then centrifuged at 2500 rpm for 10 min at 4 °C, and the supernatant was stored at −20 °C. After neutralization, the insulin content in the extracts was measured by insulin radioimmunoassay (Linco).

Intraperitoneal Glucose Tolerance Test—Forty-nine days after islets were transplanted in STZ-induced SCID diabetic mice (see above), glucose tolerance was analyzed in 16-h-fasted mice by intraperitoneal injection of 2 g of glucose/kg of body weight (Promega). Blood samples were obtained from the snipped tail and analyzed for glucose levels using the portable glucometer.

Immunoblot Analysis of Beta Cell Death by Propidium Iodide Staining—Pancreata and graft-containing kidneys from four animals per group in each case were fixed in Bouin’s solution, embedded in paraffin, and sectioned. In the graft-containing kidneys, three serial sections separated by 25 μm each were immunostained after deparaffinization and
rehydration. Beta cell death was detected by co-staining for insulin and prolidase (Sigma) as previously reported (15). Immunoprecipitates were carried out with a guinea pig anti-porcine insulin antibody (Zymed Laboratories Inc., San Francisco, CA) as primary antibody followed by a fluorescein isothiocyanate-conjugated rabbit anti-guinea pig IgG secondary antibody (Zymed Laboratories Inc.). After several washes with PBS, samples were incubated for 10 min at 37 °C with 2 μg/ml proteinase K (Boehringer Mannheim) in PBS, washed several times with water, and cover-slipped using the Prolong antifade kit (Molecular Probes, Inc., Eugene, OR).

Terminal Deoxynucleotidyltransferase (TdT)-mediated dUTP Nick End-labeling (TUNEL) and Immunostaining in RIP-HGF Mice—Twelve hours after the treatment of four RIP-HGF transgenic mice and four normal littermates (3–6 months old) with a single intraperitoneal injection of streptozotocin (150 mg/kg of body weight), pancreata were removed, fixed in Bouin’s solution, embedded in paraffin, sectioned, and immunostained after deparaffinization and rehydration. Cell death was detected by enzymatic in situ labeling for DNA strand breaks using the TUNEL method using the in situ cell death detection kit (Roche Molecular Biochemicals) according to the manufacturer’s protocol. Staining was achieved using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Vector Laboratories Inc., Burlingame, CA) as substrate. Subsequent to TUNEL, sections were stained with the anti-insulin antibody described above. Visualization was achieved using antibody-coupled peroxidase and diaminobenzidine tetrahydrochloride substrate (Biogenex, San Ramon, CA).

Cell Viability in STZ-treated INS-1 Cells—Cell viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) assay and by cell counting. Viability tests were performed in rat insulinoma cells (INS-1) cells kindly provided by Dr. Doris Stoffers (University of Pennsylvania School of Medicine) and seeded at a density of 4 × 10^6 cells/well onto 96-well plates in RPMI 1640 medium containing 11 mM glucose supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml β-mercaptoethanol. Forty-eight hours later, cells were reseeded with fresh medium containing vehicle or different agents (HGF, STZ, and/or wortmannin). Cell viability was measured 24 h after the addition of the tested agents. Cell counting was performed in the presence of trypan blue to detect non-viable cells. MTT assay was performed by incubating the cells with 0.5 mg/ml MTT for 2 h at 37 °C in 5% CO₂ as previously described (16). Formazan produced in the cells was dissolved with isopropanol, and absorbance was read at 570 and 690 nm.

Phosphatidylinositol-3 Kinase (PI3-kinase) Assay—INS-1 cells and mouse islets were serum-depleted for 24 h before the addition of 25 ng/ml HGF. After a 10-min incubation, cells were rinsed once with ice-cold PBS and twice with ice-cold buffer A (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, and 100 μM Na₃VO₄). Cells were solubilized in buffer A containing 1% Nonidet P-40 and 10% glycerol, cell extracts were centrifuged at 13,000 × g for 10 min, and the protein content in the supernatant was measured by the Micro BCA method (Pierce). PI3-kinase activity was measured as previously reported (17) with some modifications. Briefly, protein extracts were incubated with either a 1:200 dilution of rabbit anti-PI3-kinase p85 antibody (17) with some modifications. Briefly, protein extracts were incubated with a guinea pig anti-porcine insulin antibody (Zymed Laboratories Inc., San Francisco, CA) as primary antibody followed by a fluorescein isothiocyanate-conjugated rabbit anti-guinea pig IgG secondary antibody (Zymed Laboratories Inc.). After several washes with PBS, samples were incubated for 10 min at 37 °C with 2 μg/ml proteinase K (Boehringer Mannheim) in PBS, washed several times with water, and cover-slipped using the Prolong antifade kit (Molecular Probes, Inc., Eugene, OR).

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RESULTS

HGF Expression in Adenovirus-transduced Mouse Islets—Murine islet expression of HGF was analyzed using reverse transcription-PCR and Western blot in adenovirus-transduced mouse islets harvested 24 h after infection. As shown in Fig. 1, HGF was highly expressed in AdvHGF-transduced islets both in terms of mRNA (Fig. 1A) as well as protein (Fig. 1B) as compared with non-transduced or AdvLacZ-transduced islets. These results indicate that AdvHGF-transduced murine islets effectively produce HGF.

Insulin Secretion by Adenovirus-transduced Mouse Islets—To determine whether insulin secretion by mouse islets was adversely affected by adenovirus infection, glucose-stimulated insulin secretion was examined 24 h after adenoviral transduction. Insulin release was corrected for islet protein as described under “Materials and Methods” and in the legend to Fig. 2. The insulin secretary response from islets transduced with 250 m.o.i. of AdvLacZ or AdvHGF was similar to non-transduced islets (Fig. 2). This indicates that adenovirus-transduced islet function at 250 m.o.i. is not adversely affected. In contrast, glucose-stimulated insulin secretion was markedly blunted in islets transduced with 500 m.o.i. of either AdvLacZ or AdvHGF (Fig. 2). These studies suggest that 250 m.o.i. is an optimal and perhaps maximal m.o.i. for the studies described below.

Islet Transplantation of Adenovirus-transduced Mouse Islets into Diabetic SCID Mice—We next compared the performance of uninfected and AdvLacZ- and AdvHGF-transduced islets in vivo using a marginal mass islet renal transplant model in STZ-induced diabetic SCID mice. As is clear in Fig. 3A, 300 uninfected IE (125-μm diameter) were insufficient to maintain euglycemia during the 8-week period of this study. Similarly, 300 AdvLacZ-transduced IE were unable to sustain a blood glucose value under 300 mg/dl (Fig. 3A). In marked contrast,
however, 300 AdvHGF-transduced IE were able to immediately reduce blood glucose concentrations in the diabetic SCID renal transplant model (Fig. 3A). Furthermore, this dramatic decrease in the blood glucose was sustained for the complete 8 weeks of the study. These glucose concentrations were significantly lower \((p < 0.01)\) than in mice transplanted with 300 uninfected or AdvLacZ-transduced IE. In addition, random, non-fasting plasma insulin levels at day 54 after the transplant were significantly \((p < 0.025)\) increased in SCID mice transplanted with AdvHGF-transduced islets \((1.62 \pm 0.24 \text{ ng/ml, } n = 8)\) compared with mice transplanted with AdvLacZ-transduced islets \((0.90 \pm 0.11 \text{ ng/ml, } n = 10)\). After removal of the kidney containing the AdvHGF islet grafts on day 56, blood glucose levels immediately returned to pre-transplant diabetic levels, confirming that the transplant was responsible for reducing the blood glucose (Fig. 3A). Representative photomicrographs of kidney sections containing the grafts and stained for insulin are shown in Fig. 3B. Grafts were easily and abundantly visualized in each of the eight kidneys containing AdvHGF-transduced islets. This was in marked contrast to kidneys containing AdvLacZ-transduced islets in which the islet graft was either undetectable despite aggressive sectioning \((8 \text{ of } 10 \text{ mice})\) or minuscule \((2 \text{ of } 10 \text{ mice})\). These studies collectively indicate that AdvHGF-transduced islets, when transplanted under the kidney capsule into SCID diabetic mice, clearly improve blood glucose control and graft survival compared with mice transplanted with uninfected or AdvLacZ-transduced islets.

To further define the function of the transplanted AdvHGF-transduced islets, glucose tolerance tests were performed on day 49. As shown in Fig. 4, basal fasting blood glucose levels were significantly lower \((p < 0.05)\) in SCID mice transplanted with 300 AdvHGF IE \((96 \pm 6 \text{ mg/dl})\) as compared with SCID mice transplanted with 300 uninfected \((161 \pm 20 \text{ mg/dl})\) or AdvLacZ-transduced IE \((160 \pm 18 \text{ mg/dl})\). Importantly, after intraperitoneal glucose injection, glucose tolerance was markedly and significantly \((p < 0.01)\) improved in SCID mice receiving AdvHGF islets as compared with uninfected or AdvLacZ islets. As compared with normal SCID mice, glucose tolerance in AdvHGF mice was slightly impaired, although these differences were only significant at the basal and 180-min time points \((p < 0.01 \text{ and } p < 0.05, \text{ respectively})\) (Fig. 4). These results collectively indicate that at 7 weeks after transplant of an equivalent mass of islets, adenovirally HGF-enhanced islets lead to superior fasting glucose control and to superior glucose tolerance as compared with control islets and that glucose tolerance approaches that observed in normal mice.

**Beta Cell Death and Insulin Content in Adenovirally Transduced Islet Grafts on Day 1 after Transplant**—The first few hours and days after islet transplantation are characterized by substantial islet cell dysfunction and death \((18, 19)\). In our transplant studies, improvement in blood glucose values was easily apparent on day 1 in animals receiving AdvHGF-transduced islets. This temporally rapid effect on graft survival

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**Fig. 2.** Glucose-stimulated insulin secretion in uninfected and AdvLacZ- and AdvHGF-transduced islets. Murine islets were isolated and exposed to 250 or 500 m.o.i. of AdvLacZ or AdvHGF for 60 min. Twenty-four hours after infection, groups of 10 islets were incubated with 5.5 or 22.2 mM glucose for 30 min, and insulin was measured by radioimmunoassay. Results are the means \(\pm \text{ S.E. of } 4\)–6 different experiments performed in triplicate. The data are presented as percentage above control, where insulin secretion by uninfected islets exposed to 5.5 mM glucose \((541 \pm 113 \text{ pg of insulin/\mu g of protein/30 min})\) is considered 100%. *, \(p < 0.05\) versus the corresponding value at 5.5 mM glucose.

**Fig. 3.** Immediate and sustained reversal of blood glucose in STZ-induced diabetic SCID mice after renal subcapsular transplantation of a marginal mass of AdvHGF-transduced IE. A, twenty-four hours after infection, 300 uninfected or AdvLacZ- or AdvHGF-transduced IE were transplanted under the kidney capsule of STZ-induced diabetic SCID mice. Non-fasting blood glucose was measured by glucometer. Results are the means \(\pm \text{ S.E. of } 4\) transplants with 300 uninfected IE, 10 transplants with 300 AdvLacZ IE, and 8 transplants with 300 AdvHGF IE. The post-transplant blood glucose in mice receiving AdvHGF-transduced islets was significantly lower \((p < 0.01)\) than the blood glucose values obtained after the transplant of either uninfected or AdvLacZ-infected islets, as determined using analysis of variance for repeated measures. Unilateral nephrectomy \((\text{UNX})\) was performed in SCID mice transplanted with 300 AdvHGF-transduced IE at day 56 post-transplant, and the blood glucose immediately returned to pre-transplant diabetic levels. B, representative photomicrographs (both at 100× magnification) of kidney sections containing the grafts \((\text{day } 56 \text{ after transplant})\) and stained for insulin as described under “Materials and Methods.” The transplant could be identified in only 2 of 10 AdvLacZ kidneys and was small and difficult to find. In contrast, grafts were large and easily identified in all eight AdvHGF kidneys.
suggested that in addition to the well characterized effects of HGF to increase beta cell proliferation (10, 20–22) and glucose-stimulated insulin secretion (11), HGF may also confer a previously unrecognized survival benefit on beta cells in the transplant setting. To address this question, we transplanted 300 AdvLacZ- or AdvHGF-transduced IE under the renal capsule of SCID diabetic mice and sacrificed the animals 24 h later. The number of dead beta cells in the grafts at 24 h was quantitated. As is obvious in Fig. 5, the number of condensed, pyknotic beta cell nuclei 24 h after transplant was significantly (p < 0.05) reduced in AdvHGF grafts compared with AdvLacZ grafts. Furthermore, the insulin content in the AdvHGF grafts was also 2.5-fold higher (p < 0.05) than in the AdvLacZ grafts (Fig. 5C). These results demonstrate that HGF-enhanced islets have a survival advantage as early as day 1 after transplantation. In addition, the increase in insulin content in AdvHGF-transduced islet-containing grafts might also reflect an increase in insulin expression induced by HGF in the beta cells of the graft, as previously observed in vivo in RIP-HGF mice (10, 11).

**Beta Cell Death Induced by STZ in RIP-HGF Transgenic Mice**—This novel protective effect of HGF on beta cells was confirmed independently using a different model system and a different inducer of cell death. We used our previously described RIP-HGF transgenic mouse model for this purpose (10). Fig. 6 shows the effect of intraperitoneal injection of 150 mg/kg STZ on beta cell death in normal and RIP-HGF islets. Pancreatic sections co-stained for insulin and TUNEL or propidium iodide displayed numerous TUNEL-positive or condensed pyknotic nuclei, respectively, in the islets of normal mouse pancreata compared with RIP-HGF transgenic pancreata (Fig. 6, A and C). Quantification of these TUNEL-positive beta cells or condensed pyknotic beta cell nuclei showed that the index of beta cell death was 3–5-fold higher (p < 0.01) in normal littermates as compared with RIP-HGF transgenic mice (Fig. 6, B and D). These observations independently confirm that HGF overexpression confers a survival advantage on beta cells and

**Fig. 4.** Glucose tolerance in normal and STZ-induced diabetic SCID mice transplanted with uninfected or AdvLacZ- or AdvHGF-transduced islets. At day 49 after the transplant, mice were fasted for 16 h and then injected intraperitoneally with glucose (2 g/kg of body wt). Blood glucose levels were measured from the snipped tail at the time points indicated in the figure with a portable glucometer. Results are the means ± S.E. Blood glucose values were significantly lower (p < 0.01) in both mice transplanted with AdvHGF-transduced islets and normal SCID mice than in mice transplanted with either uninfected or AdvLacZ-infected islets, as determined using analysis of variance for repeated measures. Blood glucose values were not significantly different in normal mice and mice transplanted with AdvHGF-transduced islets except at basal and 180 min (p < 0.01 and p < 0.05, respectively).

**Fig. 5.** Beta cell death and insulin content in AdvLacZ grafts and AdvHGF grafts obtained at day 1 after transplant. A, representative photomicrograph of renal subcapsular grafts obtained at day 1 after transplant with AdvLacZ- or AdvHGF-transduced islets. Sections were stained with propidium iodide (red) to detect condensed pyknotic nuclei and insulin (green) to identify beta cells. Notice that the graft with AdvHGF-transduced islets contained fewer condensed pyknotic nuclei (arrowheads) as compared with the grafts composed of AdvLacZ-transduced islets. B, the percentage of pyknotic condensed beta cell nuclei found in renal subcapsular grafts containing AdvLacZ- (n = 4) or AdvHGF-transduced islets (n = 4) obtained at day 1 after the transplant. At least 600 beta cells were counted per section. C, insulin content of AdvLacZ grafts and AdvHGF grafts harvested at day 1 after transplant. Insulin content was significantly higher in grafts containing AdvHGF-transduced islets than in grafts composed of AdvLacZ-transduced islets. This finding correlates with the decreased beta cell death observed in the grafts with AdvHGF-transduced islets. *, p < 0.05.
extend it to two types of cell death agonists, ischemia/transplantation and STZ.

Cell Viability in INS-1 Cells Treated with STZ and HGF in Vitro—The cellular basis of the HGF protective effect against beta cell death is difficult to study in vivo. We therefore sought to develop an in vitro model of HGF-induced beta cell cytoprotection in which to study the cellular mechanisms underlying this phenomenon. We thus examined the ability of HGF to confer protection of INS-1 cells in vitro against STZ-induced cell death. Cell viability was assessed in INS-1 cells treated with 25 ng/ml HGF and 0.5 or 1 mM STZ in complete medium for 24 h (Fig. 7, white bars). As shown in the figures, STZ at 0.5 and 1 mM dramatically reduced cell viability in INS-1 cells as assessed by both MTT assay (Fig. 7A) and cell number (Fig. 7B). However, HGF treatment significantly increased the number of viable INS-1 cells 24 h after the addition of 0.5 or 1 mM STZ (Fig. 7, A and B). These results document the cytoprotective effect of HGF in a third independent system and indicate that INS-1 cells may serve as a useful model system for studying cellular mechanisms underlying the anti-cell death effects of HGF in beta cells.

PI3-kinase Activity Is Induced by HGF in INS-1 Cells, and Its Blockade Leads to Decreased Cell Viability—HGF is known to increase PI3-kinase activity in human islets and other cell types in vitro (23–25). In addition, the PI3-kinase/Akt pathway is involved in the survival effects of HGF in several non-beta cell systems (26–29). To investigate whether the PI3-kinase/Akt pathway is involved in the protective effect of HGF in the STZ-induced cell death in INS-1 cells, we examined PI3-kinase activity in cell extracts from INS-1 cells treated with HGF. As shown in Fig. 8A, after stimulation of INS-1 cells with 25 ng/ml HGF for 10 min, a significant increase in PI3-kinase activity was observed. Furthermore, mouse islets incubated for 10 min with 25 ng/ml HGF also exhibited an increase in PI3-kinase activity (Fig. 8A). Moreover, transgenic HGF-overexpressing islets from the RIP-HGF mouse also showed increased PI3-kinase activity (Fig. 8A). PI3-kinase stimulation results in phosphorylation and activation of Akt. Akt activity was therefore examined in INS-1 cells incubated with 25 ng/ml HGF for 30 min. As expected, HGF rapidly increased protein kinase B activity (Fig. 8B). These results confirm that PI3-kinase/Akt signaling pathway is activated by HGF in pancreatic beta cells.

Second, we demonstrate a novel and striking additional benefit of HGF on the beta cell; in addition to its ability to stimulate proliferation and induce key glucose-sensing and insulin secretory proteins in beta cells, it also has a rapid,

FIG. 6. Overexpression of HGF in RIP-HGF transgenic mice increases survival of pancreatic beta cells after treatment with streptozotocin (STZ) in vivo. Adult mice (3–6 months old) were injected intraperitoneally with STZ (150 mg/kg of body weight), and 12 h later pancreata were removed, fixed, embedded, and stained as described under “Materials and Methods.” Representative photomicrographs of pancreatic sections from RIP-HGF transgenic mice (TG) and normal littermates (NL) stained for TUNEL (purple nuclei) and insulin (brown) (A) or propidium iodide (PI, red) and insulin (green) (C) are shown. There are fewer TUNEL or pyknotic condensed beta cell nuclei in the islets from RIP-HGF transgenic mice than in the islets from normal littermates. Quantitation of STZ-induced beta cell death in vivo in normal (n = 4) and RIP-HGF transgenic (n = 4) mice by TUNEL and insulin (B) or propidium iodide and insulin staining (D) reveals that the index of beta cell death was significantly (p < 0.01) reduced in RIP-HGF transgenic mice as compared with normal littermates. At least 1000 beta cell nuclei were counted per pancreatic section.

DISCUSSION

The studies of Shapiro and co-workers (1, 2) demonstrate the efficacy of human islet transplantation for Type 1 diabetes. They have also highlighted an acute shortage of human beta cells available for transplantation into the millions of Type 1 diabetics who could benefit from such therapy. We and others previously demonstrated that HGF induces islet proliferation in vitro and also in vivo in the RIP-HGF transgenic mouse (10, 20–22). We have also demonstrated that islets of RIP-HGF mice are larger and more plentiful than normal, 2) display increased expression of three proteins critical to beta cell differentiation and function (insulin, glucokinase, and the Glut-2 transporter), 3) sense glucose and secrete insulin more robustly than control islets, 4) therefore imbue on their recipients enhanced glucose tolerance in vivo as assessed using an intraperitoneal glucose tolerance test, and 5) function in a fashion superior to normal islets when transplanted into diabetic mice (10, 11). In short, HGF, both in vitro and in a transgenic setting, simultaneously enhances both the proliferation and function of beta cells and does so in a quantitatively very significant fashion. These observations suggested to us that ex vivo delivery of HGF gene might have therapeutic efficacy in enhancing islet transplant outcomes.

In the studies described herein, we demonstrate two broad types of novel observations. First, we demonstrate that adenoviral gene delivery of HGF to completely normal murine islets before transplantation markedly improves graft performance in the diabetic recipient. If applicable to human islet transplantation, this approach could significantly reduce the number of human islets required to correct diabetes in patients with Type 1 diabetes. We believe this is the first successful example of beta cell gene therapy with a growth factor leading to normalization of blood glucose in the setting of islet transplantation. Second, we demonstrate a novel and striking additional benefit of HGF on the beta cell; in addition to its ability to stimulate proliferation and induce key glucose-sensing and insulin secretory proteins in beta cells, it also has a rapid,
potent and beneficial effect to enhance beta cell survival in the immediate post-transplant period.

In these experiments, we employed adenovirus to deliver HGF to islets. We selected adenovirus because it is easy to prepare and is highly efficient (12). In rat and human islets, the infection rate has been reported to vary between 20 and 50% depending on the m.o.i. and the detection method used (13, 14). In the present studies, the infection rate in mouse islets (29%) as assessed by standard X-gal-staining methods in islets infected with AdvLacZ, was in the range of those previously reported (13, 14). It is important to note that HGF is a secreted paracrine factor and likely influences a greater number of islet cells than the 30% that have been targeted by the adenovirus. It likely exerts its effects in islet cells that have HGF receptors such as the beta cell (21).

As shown in Fig. 1, Western blot analysis of HGF expression in AdvHGF-transduced islets reveals the presence of two forms of HGF, the single chain precursor and heterodimeric mature HGF. HGF is produced as an inactive precursor that awaits activation by extracellular proteases. Several proteases have been reported to date to activate HGF in vitro, including blood coagulation factor XIIa, urokinase, tissue-type plasminogen activator, and a serum-derived serine protease named HGF activator (31–34). In the rat endocrine pancreas, tissue-type plasminogen activator has been detected preferentially in somatostatin cells (35). Furthermore, it has been shown that HGF activation is up-regulated specifically in injured tissues (36). Taken together, these studies suggest that in a transplant setting in which the islets are implanted into a hostile environment (hypoxia, and nutrient...
deprivation), inactive HGF might likely be activated by pro-
teases inside or surrounding the graft.

We employed SCID mice as recipients, for we anticipated using this model for human islet xenografts in the future. An additional benefit of SCID mice is that they should not display immune intolerance to adenovirus, potentially allowing for pro-
longed expression of HGF in the graft. Although we have not tested this hypothesis, others have demonstrated the presence of adenovirus in islet grafts up to 20 weeks after islet trans-
plant in syngeneic immunocompetent mice (37). Thus, in our studies the model system was designed as a “proof of principle” model to ask the question, Can HGF, delivered to normal islets by gene transfer before transplantation, enhance the engraf-
ment and performance of normal islets in diabetic recipients? We also employed a model of marginal beta cell transplant mass so that improvements over a grossly inadequate base line could be observed. The results of these studies are very clear; that is, adenoviral delivery of HGF to otherwise normal CD-1 murine islets dramatically improves islet performance in a transplant setting, resulting in better diabetes control in their diabetic SCID mouse hosts.

These observations raise several new questions. One key question was, Why is the beneficial effect of HGF gene delivery so apparent at the very earliest time point examined, day 1 post-transplant? It has been shown recently that the early failure of islet cell grafts is due to rapid cell death of the large majority of transplanted beta cells in the immediate post-
transplant period (18, 19). Thus, the very rapid normalization of blood glucose observed in Fig. 3A suggests that HGF may have an additional beneficial effect on beta cells to enhance beta cell survival in the immediate post-transplant period. As described above, this proves to be correct. Although HGF has been demonstrated in other tissues to have pro-survival and anti-ap-
optotic effects (26–29), this has not been demonstrated in beta cells previously. In addition to this pro-survival effect, it is also possible that the enhanced glucose-stimulated insulin secre-
tion we have previously described in RIP-HGF islets also con-
tributes to this early enhancement of graft function. Future studies are required to define the relative contribution of the several beneficial effects of HGF on the beta cell (increased proliferation versus improved function versus enhanced sur-
vival) to the improved islet graft performance induced by AdvHGF.

A second key question is, What is the cellular mechanism responsible for the protective effect of HGF on beta cells? HGF has been shown to induce anti-apoptotic actions via the PI3-
kinase/Akt signaling pathway in several different cell types subject to a variety of cell death agonists (26–29). In addition, overexpression of active Akt1 in the pancreatic beta cell in transgenic mice confers complete resistance to experimental diabetes induced by STZ (38–39). In our studies, we found that HGF increases PI3-kinase and protein kinase B/Akt activities in INS-1 cells in vitro. Importantly, the increased survival effect induced by HGF in INS-1 cells treated with STZ was completely blunted when these cells were treated with wort-
mannin, indicating that activation of PI3-kinase and protein kinase B/Akt seems to be essential for HGF to induce its pro-
teptive effect on beta cells. These results demonstrate for the first time that HGF through PI3-kinase/Akt activation in-
creases pancreatic beta cell survival. Additional studies exploring the potential targets in the cell death pathways activated/ inactivated by HGF in pancreatic beta cells through the PI3-
kinase/Akt signaling pathway are in progress.

A third key question is, Would this approach, which is effective in a highly artificial immunodeficient renal graft mouse model, have similar efficacy in a more authentic setting that includes an intact immune system and the vicissitudes of immuno-suppression and autoimmunity and alloimmune consider-
ations that would apply to human islet transplant and portal delivery of islets as is also employed in humans? Preliminary studies indicate that AdvHGF is similarly effective in a rat allograft setting as well.2

A fourth key question is, What applicability does this have to human islets and human islets with diabetes? Hayek and co-work-
ers (20, 21, 40) show that HGF is indeed a potent stimulator of proliferation in human beta cells. This together with the RIP-
HGF mouse data (10, 11) and the data described herein suggest that such a beneficial effect may be observed in human islets as well. In part, the reason we employed the SCID mouse model is so that we could adapt these experiments to human islets. On balance, there is no obvious reason why this approach could not be used with human islets or, indeed, for other beta cells or beta cell surrogates derived from stem cell approaches to engineering islets (30, 41, 42). If strictly translatable to the Edmonton protocol setting, these approaches would reduce by at least 50% the number of human pancreata required to transplant a single patient with diabetes. Indeed, because this is the early stage of AdvHGF therapy, one can envision approaches that might fur-
ther enhance the outcomes such that one donor pancreas could eventually serve multiple diabetic recipients.

In summary, our studies demonstrate that adenoviral deliv-
ery of HGF to murine islets ex vivo improves islet transplant survival and performance in STZ-induced diabetic mice. The in vivox beta cell survival effect induced by HGF appears to play an important role in preventing early failure of the islet graft. In vitro, the PI3-kinase/Akt pathway seems to be important for HGF-mediated survival effect in pancreatic beta cells. Gene delivery of HGF to human islets may improve islet transplant outcomes and reduce the numbers of islets required for suc-
cessful islet transplant in humans.

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Adenovirus-mediated Hepatocyte Growth Factor Expression in Mouse Islets Improves Pancreatic Islet Transplant Performance and Reduces Beta Cell Death
Adolfo García-Ocaña, Karen K. Takane, Vasumathi T. Reddy, Juan-Carlos Lopez-Talavera, Rupangi C. Vasavada and Andrew F. Stewart

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