Gene Transfer of Constitutively Active Akt Markedly Improves Human Islet Transplant Outcomes in Diabetic Severe Combined Immunodeficient Mice

Poornima Rao, Jennifer Roccisana, Karen K. Takane, Rita Bottino, Allan Zhao, Massimo Trucco, and Adolfo García-Ocaña

Akt is an important intracellular mediator of β-cell growth and survival in rodents. However, whether constitutive activation of Akt in human β-cells enhances the survival and function of transplanted islets is unknown. In the current study, we examined the efficacy of constitutive activation of Akt in improving human islet transplant outcomes using a marginal mass model in diabetic severe combined immunodeficient (SCID) mice. Human islets transduced with adenoviruses encoding constitutively active Akt1 (Adv-CA-Akt) displayed increased total and phosphorylated Akt and Akt kinase activity compared with control islets. Expression of CA-Akt in human islets induced a significant increase in β-cell replication and a significant decrease in β-cell death induced by serum and glucose deprivation or chronic hyperglycemia. Two control groups of islets (1,500 uninfected or adenovirus LacZ [Adv-LacZ]-transduced human islet equivalents [IEQs]) transplanted under the kidney capsule of streptozotocin-induced diabetic SCID mice were insufficient to correct hyperglycemia. Importantly and in marked contrast to these controls, 1,500 Adv-CA-Akt–transduced IEQs were capable of restoring euglycemia in diabetic SCID mice. Moreover, blood glucose normalization persisted for at least 6 months. Human plasma insulin at day 54 after transplant was 10-fold higher in Adv-CA-Akt islet recipients (2.4 ± 0.4 ng/ml) compared with those receiving Adv-LacZ islets (0.25 ± 0.08 ng/ml) (P < 0.05). In summary, expression of CA-Akt in human islets improves islet transplant outcomes in a subcapsular renal graft model in SCID mice. Akt is an attractive target for future strategies aimed at reducing the number of islets required for successful islet transplantation in humans. Diabetes 54:1664–1675, 2005

Recent studies have demonstrated that human islet allograft transplantation can be a successful therapeutic option in the treatment of patients with type 1 diabetes (1–3). However, despite this enormous advance, these studies have highlighted an obvious mismatch between the demand for and the availability of human islets for transplantation. This mismatch has prompted many laboratories to develop methods and strategies to increase β-cell proliferation, function, and survival in a transplant setting, so that smaller numbers of pancreatic islets could be used per recipient to achieve an optimal euglycemic state.

Akt (also named protein kinase B) has emerged as the focal point for many intracellular signal transduction pathways, regulating multiple cellular processes such as gene transcription, protein synthesis, cell survival, cell proliferation, and glucose metabolism (4–8). Activation of phosphatidylinositol 3′-kinase (PI3K) generates D3-phosphorylated phosphoinositides that bind Akt through a pleckstrin homology domain and induce the translocation of Akt to the plasma membrane. In the plasma membrane, Akt colocalizes with the constitutively active phosphoinositide-dependent kinase-1 and probably another unknown kinase that activate Akt by phosphorylation of residues Thr308 and Ser473 (in the Akt1 isoform), respectively. Constitutively active forms of Akt have been obtained by fusion of NH₂-terminal c-Src myristylation residues to Akt (9–12). The resultant myristylation targets Akt directly to the membrane where it is phosphorylated and constitutively activated inside the cell.

Glucose and growth factors such as glucagon-like peptide-1 (GLP-1), glucose-dependent insulinitropic polypeptide (GIP), IGF-I, or hepatocyte growth factor (HGF) have been shown to increase the phosphorylation/activation of Akt in insulinoma cells and mouse islets (11,13–18). Blockade of PI3K-Akt pathway in pancreatic β-cells has been shown to blunt proliferative and/or pro-survival effects induced by GLP-1, GIP, IGF-I, HGF, or glucose in these cells (11,13–18). On the other hand, transfection with a constitutively active form of Akt1 enhances the survival of rodent β-cells when they are glucose-deprived or treated with free fatty acids (12,18).
Transgenic expression of constitutively active Akt1 specifically in the mouse pancreatic β-cell has been reported from both Bernal-Mizrachi et al. (10) and Tuttle et al. (9). In both cases, the constitutively active kinase strikingly increased β-cell mass by 8- to 10-fold, resulting in hyperinsulinemia and decreased blood glucose levels. This increase in β-cell mass is at least in part mediated by an increase in β-cell size and probably increased isletogenesis. Bernal-Mizrachi et al. (10) also reported an increase in β-cell proliferation in adult mice that was not observed in Tuttle’s study (9). Importantly, in both studies, constitutively active Akt expression increases β-cell survival in vivo after treatment of transgenic mice with the diabeticogenic and cytotoxic agent streptozotocin (STZ). These studies strongly indicate that constitutively active Akt promotes hyperinsulinemia and β-cell hyperplasia and survival in vivo in rodents, and they strongly support the idea that activation of Akt could be useful as a therapeutic strategy for increasing islet mass and survival. However, no attempt has been made to explore whether activation of Akt in human β-cells enhances the survival and function of transplanted islets.

In the current study, we have analyzed the effect of constitutive activation of Akt in the proliferation and survival of human β-cells in vitro. More importantly, we have examined the efficacy of constitutive activation of Akt in improving human islet transplant outcomes in a marginal mass model of islet transplant in diabetic severe combined immunodeficient (SCID) mice. The results indicate that human islets with constitutive activation of Akt display increased proliferation and survival in vitro. Most importantly, they function more effectively than normal islets when transplanted into STZ-induced diabetic mice. Interestingly, this superior islet graft function and survival are maintained for a prolonged period of time. These results indicate that Akt activation in human islets may be a feasible therapeutic approach to enhance human islet transplant efficiency and duration while reducing the number of islets required for transplantation.

**RESEARCH DESIGN AND METHODS**

**Generation of recombinant adenoviruses.** Adenovirus containing the cDNA of a constitutively active form of Akt (Adv-Ca-Akt) or β-galactosidase were prepared according to methods we have previously described in detail (13), originally described by Becker et al. (19). The adenovirus LacZ (Adv-LacZ) was generously provided by Dr. Christopher Newgard (Duke University, Durham, NC). The Adv-Ca-Akt was a generous gift from Dr. Thomas Soderling (Vollum Institute, Portland, OR). The constitutively active form of Akt contains the NH2-terminal c-Src myristylation signal fused to Akt, which directs Akt to the plasma membrane leading to its constitutive activation, and a hemagglutinin tag for monitoring expression. Multiplicity of infection (MOI) was determined by optical density at 260 nm and by plaque assay. MOI calculations assume 1,000 cells per islet equivalent (IEQ). Human islets were kept in culture for 4–7 days before they were transduced with the adenoviruses as previously described in detail (13). Uninfected and adenovirus-transduced human islets were used 24 h after infection for the different experiments described below.

**Human islet isolation.** Human islets from pancreatic cadaver donors (aged 39 ± 7 years, n = 11) were provided by the University of Pittsburgh Human Islet Isolation Facility and were prepared according to Ricordi and Rastellini (20) as previously described in detail (21). Akt immunoblot analysis and kinase activity assays. Islet extracts were made in freshly prepared ice-cold lysis buffer (PBS with 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mmol/l sodium orthovanadate, 100 μg/ml phenylmethylsulfonyl fluoride, and 57 μg/ml aprotonin) incubated 30 min on ice and sonicated; the supernatant containing the cell lysate was separated; and protein concentrations were measured using the MicroBCA assay (Pierce, Rockford, IL). Twenty-five micrograms of protein from uninfected or adenovirus infected islets was 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 membrane (Millipore, Bedford, MA) using standard techniques. Blots were then probed with antibodies against Akt, phospho-Ser473-Akt, phospho-Thr308-Akt (Cell Signaling Technology, Beverly, MA), and actin (Stautechology, Santa Cruz, CA) following previously described methods (13,22). Chemiluminescence was detected using the ECL system (Amersham Pharmacia, Piscataway, NJ).

The Akt immunoprecipitation and kinase assay was carried out using a glycerogen synthase kinase (GSK)-3α/β phosphorylation kit (Cell Signaling Technology) following the manufacturer’s procedure and as previously described (13). Briefly, 200 μg of islet protein extracts obtained 24 h after adenovirus infection or uninfected islets were incubated with protein G/Akt monoclonal antibody complex for 2 h at 4°C. After several washes, immunoprecipitates were resuspended in kinase buffer containing 200 mmol/l ATP and 1 μg of GSK-3 fusion protein and incubated at 30°C for 30 min. The reaction was stopped with SDS gel loading buffer, and samples were analyzed by immunoblotting (as described above) with the phospho-GSK-3α/β (Ser 21/9) antibody.

**Glucose-stimulated insulin secretion in isolated human islets.** Insulin from uninfected and adenovirus-transduced islets was measured in triplicate for each glucose concentration tested, as previously described (12,22,23). Briefly, uninfected and adenovirus-transduced islets were preincubated in Krebs-Ringer bicarbonate buffer supplemented with 10 mmol/l HEPES, 1% BSA, and 5 mmol/l 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% BSA and 5 or 22 mmol/l glucose at 37°C in the 5% CO2 incubator. After 30 min of incubation, buffer was removed and frozen at −20°C until insulin measurement by radioimmunoassay (Linco Research, St. Louis, MO). Islets were then digested overnight in NaOH at 37°C, and protein was measured by the Bradford method after neutralization with HCl. Results are expressed as a percentage of insulin concentration obtained with uninfected islets incubated at 5 mmol/l glucose.

**Islet cell proliferation: [3H]thymidine and bromodeoxyuridine incorporation.** Human islet cell proliferation was assayed as reported previously (22). Briefly, uninfected or adenovirus-transduced islets were incubated in RPMI medium containing 5 mmol/l glucose and 1% penicillin and streptomycin for 24 h at 37°C. One microcurie of [3H]thymidine (Amersham Pharmacia) was then added to the medium for an additional 24 h. To quantitate [3H]thymidine incorporation, islets were washed with ice-cold PBS and precipitated in ice-cold 10% trichloroacetic acid twice for 10 min on ice. The resulting precipitant was resuspended in 0.1 N NaOH at 30°C for 30 min and neutralized with 0.1 N HCl. Aliquots were counted in a β-scintillation counter and assayed for protein using the Bradford assay.

For immunohistological confirmation of [3H]thymidine incorporation, human islets were labeled with bromodeoxyuridine (BrdU) using the same protocol as described above except that a 1,1000 dilution of BrdU (Amersham Pharmacia) (22) was used. The islets were processed and processed islet transplantation has been previously described (22). Briefly, serial 5-μm sections of the isolated islets were deparaffinized, rehydrated, microwaved in citrate buffer for 5 min, quenched with 3% H2O2 in methanol for 10 min at room temperature, rinsed, and blocked with 2% goat serum. Sections were then incubated with an anti-insulin antibody (Zymed, South San Francisco, CA) at 1:50 dilution and an anti-BrdU antibody (Amersham Pharmacia) at 1:5 dilution for 1 h at room temperature. Visualization of the staining was achieved using fluorescein isothiocyanate-conjugated anti-guinea pig IgG and tetramethylrhodamine isothiocyanate (TRITC)-conjugated rabbit anti-mouse IgG secondary antibodies (Sigma), respectively. Hoechst 33258 (Sigma) was used to detect all the nuclei in the sections.

**Quantitation of human β-cell death by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling and insulin staining.** Twenty-four hours after infection with the adenovirus, adenovirus-transduced and uninfected islets were incubated in RPMI medium without glucose and fetal bovine serum (FBS) for 24 h or in RPMI medium containing FBS and either 5 or 22 mmol/l glucose for 5 days (chronic hyperglycemia). At the end of the incubation period, islets were fixed in Bouin’s solution, embedded in paraffin, and sectioned (5 μm) with a microtome. β-Cell nuclei were identified enzymatically in situ labeling for DNA strand breaks using the terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) method using the In Situ Cell Death Detection kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s protocol. Subsequent to TUNEL, sections were
Akt and islet transplantation

Stained with the anti-insulin antibody as described above. Visualization was performed by using a TRITC-conjugated rabbit anti-guinea pig IgG secondary antibody. Hoechst 33258 was used to detect all of the nuclei in the sections. Islet transplantation. Human islets, uninfected or transduced with Adv-LacZ or Adv-CA-Akt as described above, were transplanted under the kidney capsule of STZ-induced diabetic SCID mice (BALB/cByJ) as previously described (13,23). Briefly, SCID mice were rendered diabetic by injecting 250 mg/kg body wt STZ i.p. Diabetes was confirmed by the presence of hyperglycemia (>300 mg/dl), polyuria, and weight loss. Random nonfasted blood glucose was measured from the snipped tail and analyzed for glucose levels using the portable glucometer. Twenty-four hours after transduction, islets were harvested, and protein was extracted. To detect Akt phosphorylation, antibodies against phosphorylated Ser473 and Thr308 were used. Actin was used as an internal control for loading.

RESULTS

Akt is overexpressed, phosphorylated, and activated in adenovirus-CA-Akt–transduced human islets. We first examined the ability of Adv-CA-Akt to transduce human islets and whether this transduction resulted in overexpression of phosphorylated Akt. As shown in Fig. 1A, 125–300 mg/dl), polyuria, and weight loss. Random nonfasted blood glucose was measured from the snipped tail by a Precision Q.I.D. portable glucometer (Medisense, Bedford, MA). After 3 consecutive days of hyperglycemia, SCID diabetic mice were transplanted with either 1,500 or 4,000 uninfected, 1,500 Adv-LacZ– or 1,500 Adv-CA-Akt–transduced IEQs (1 IEQ = 125-μm-diameter islet) beneath the kidney capsule, as previously described (13,23). Blood glucose levels were measured at days 1 and 7 and then weekly until the transplanted mice underwent unilateral nephrectomy. At day 54, retroorbital bleeds were performed to measure plasma human insulin levels with a human insulin-specific radioimmunoassay (Linco). All studies were performed with the approval of and in accordance with guidelines established by the University of Pittsburgh Institutional Animal Care and Use Committee.

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 injection of 2 g/kg body wt i.p. as previously reported (13,23). Blood samples were obtained from the snipped tail and analyzed for glucose levels using the portable glucometer.

Immunohistochemical staining for hemagglutinin, islet hormones, and cytokeratin-19. Kidneys containing islet grafts were fixed in Bouin’s solution, paraffin embedded, sectioned, and stained with the anti-insulin antibody (see above) and an anti-cytokeratin-19 (anti-Ck-19) antibody (Novocastra/Vector Labs, Burlington, Ontario, Canada) at 1:75 dilution and anti-somatostatin antibodies (Novacastra, U.K.) at 1:200 dilution. Visualization of the staining was achieved by using the avidin-biotin immunoperoxidase complex system (Biogenex). Sections were counterstained using hematoxylin.

To detect the presence of cells expressing CA-Akt in adenovirus-transduced islets and islet grafts, sections were stained with an anti-hemagglutinin tag monoclonal antibody (Cell Signaling Technology) at 1:500 dilution and anti-somatostatin antibodies (Novocastra, U.K.) at 1:200 dilution. Visualization of the staining was achieved by using the avidin-biotin immunoperoxidase complex system (Biogenex). Sections were counterstained using hematoxylin.

Statistical analysis. Results are expressed as means ± SE. Statistical differences were determined by a two-tailed unpaired Student’s t test. P < 0.05 was considered statistically significant.

Akt is overexpressed, phosphorylated, and activated in adenovirus-CA-Akt–transduced human islets. We first examined the ability of Adv-CA-Akt to transduce human islets and whether this transduction resulted in overexpression of phosphorylated Akt. As shown in Fig. 1A, 24 h after the transduction of human islets with 500 MOI of Adv-CA-Akt, ~40–50% of islet cells were trans-
duced with Adv-CA-Akt as determined by immunofluorescent staining with an antibody against the hemagglutinin epitope tag. Furthermore, the expression of Akt and its phosphorylation status was examined by Western blot analysis. As shown in Fig. 1B, exposure of human islets to increasing doses of Adv-CA-Akt (250–1,000 MOI) for 1 h at 37°C resulted in an increase in Akt expression at 24 h after transduction. Activation of Akt requires phosphorylation of residues Ser473 and Thr308 (5–8). Western blot analysis of extracts from human islets exposed to Adv-CA-Akt revealed an increase in the expression of Akt phosphorylated on Ser473 and Thr308 (Fig. 1B). This indicates that exposure of human islets to Adv-CA-Akt results in overexpression and increased phosphorylation of Akt.

Next, we examined whether phosphorylation of Akt in extracts from Adv-CA-Akt–transduced human islets resulted in increased Akt kinase activity compared with uninfected and Adv-LacZ–transduced human islets. As shown in a representative immunoblot in Fig. 1C, Akt activity is significantly four- to sevenfold increased in human islets exposed to 500 MOI of Adv-CA-Akt (7.6 ± 1.7-fold, n = 5, P < 0.05) compared with human islets exposed to the same MOI of Adv-LacZ (1.9 ± 0.9-fold, n = 5) or uninfected islets (1.0 ± 0.3-fold, n = 5). Taken together, these results demonstrate that adenovirus containing the cDNA of a constitutively active form of Akt is able to: 1) efficiently transduce human islet cells; and 2) increase Akt expression, phosphorylation, and activation in human islets.

Glucose-stimulated insulin secretion by adenovirus-transduced human islets. To determine an optimal dose of Adv-CA-Akt that induces overexpression and activation of Akt in human islets without adversely affecting insulin secretion, glucose-stimulated insulin secretion (GSIS) was examined 24 h after adenoviral transduction. The insulin secretory response from human islets transduced with 500 MOI of either Adv-LacZ or Adv-CA-Akt was similar to nontransduced islets, indicating that islet function is not adversely attenuated by this dose of adenovirus (Fig. 1D). In contrast, GSIS was negatively affected in islets transduced with 1,000 MOI of either adenovirus. Taken together, the results shown in Fig. 1 suggest that 500 MOI is an optimal and perhaps maximal MOI that can be used for subsequent experiments.

Adenovirus-CA-Akt increases proliferation in human islets in vitro. To determine whether constitutively active Akt could also induce an increase in the proliferation rates of human islet cells as has been reported to occur in rodent islets (10), we performed [3H]thymidine incorporation experiments in Adv-CA-Akt–transduced and control human islets. To eliminate variability in islet size, we selected islets of similar size and also expressed [3H]thymidine incorporation (counts per minute) per microgram of protein. As shown in Fig. 2A, human islets transduced with 500 MOI of Adv-CA-Akt displayed a twofold increase (P < 0.05) in [3H]thymidine incorporation compared with uninfected or Adv-LacZ–transduced human islets. These studies indicate that Adv-CA-Akt delivery to human islets increases islet cell proliferation. However, they do not provide any information on whether the proliferation is occurring in β-cells. To determine whether this is the case, sections from adenovirus-transduced and uninfected human islets were stained for insulin and BrdU. The percentage of insulin-positive cells, expressed as a function of total number of cells, in the different human islet prepara-
The number of BrdU-positive nuclei in sections from islets transduced with Adv-LacZ and Adv-CA-Akt at the indicated MOI. Islets were treated as described for the \[^{3}H\]thymidine experiments except BrdU was added instead of \[^{3}H\]thymidine. Arrowheads indicate BrdU-positive nuclei. Hoechst 33342 (blue) staining was performed to detect cell nuclei. The magnification used is x400 except for the lower right panel in which BrdU-positive \(\beta\)-cell nuclei are shown, which was taken at x1,000.

**C:** Quantitation of BrdU incorporation into uninfected human islets and Adv-LacZ- and Adv-CA-Akt–transduced human islets at the indicated MOI. The minimum number of nuclei counted per section was 1,000. \(*P < 0.05\) vs. uninfected and Adv-LacZ islets.

**D:** Quantitation of BrdU-positive \(\beta\)-cells as a function of the total number of human \(\beta\)-cells in sections of uninfected human islets or Adv-LacZ– and Adv-CA-Akt–transduced human islets. For these experiments, an MOI of 500 was used. Results are means \(\pm\) SE. \(*P < 0.05\) vs. uninfected and Adv-LacZ–transduced islets. Six different human islet preparations were examined.

Taken together, these results indicate that adenoviral delivery of constitutively active Akt stimulates human \(\beta\)-cell proliferation in vitro.

**Adenovirus-CA-Akt protects against \(\beta\)-cell death induced by serum and glucose deprivation or chronic high glucose levels in vitro.** Akt is a survival factor in rodent systems (9,10,12,18). To examine whether constitutive activation of Akt increases the survival of human islet \(\beta\)-cells in vitro, we exposed Adv-CA-Akt–transduced and control islets to two cell death inducers: combined glucose and serum deprivation and chronic high glucose levels (24–26).
First, the ability of constitutively active Akt to protect against glucose and serum deprivation-induced β-cell apoptosis was evaluated. As shown in the representative photomicrograph in Fig. 3A, exposure of uninfected or Adv-LacZ–transduced human islets to RPMI medium without glucose and serum for 24 h resulted in an increased number of nuclei displaying DNA fragmentation (TUNEL-positive nuclei) compared with islets grown in regular conditioned medium (Fig. 3A). Interestingly, and in striking contrast to uninfected or Adv-LacZ–transduced islets, Adv-CA-Akt–transduced human islets cultured for 24 h in the absence of glucose and serum show a striking reduction in the number of TUNEL-positive nuclei (Fig. 3A). Quantitation of the number of insulin- and TUNEL-positive nuclei in those sections indicate that survival in Adv-CA-Akt–transduced human islets from apoptosis induced by glucose and serum deprivation is increased by ~80% (Fig. 3B).

Next, we exposed adenovirus-transduced and uninfected human islets to increasing glucose concentrations (from 5 to 22 mmol/l) for 5 days. As shown in the representative photomicrograph of Fig. 3C, chronic high glucose levels increased the number of TUNEL-positive nuclei in uninfected or Adv-LacZ–transduced human islets compared with islets grown in regular conditioned medium containing 5 mmol/l glucose. However, islet sections from Adv-CA-Akt–transduced islets treated with high glucose for 5 days displayed reduced numbers of apoptotic nuclei compared with control islets (Fig. 3C). Quantitation of β-cell apoptotic rates in these sections revealed that transduction with Adv-CA-Akt increased survival of human β-cells treated with high glucose concentrations for 5 days by ~80% (Fig. 3D). In addition, transduction with Adv-CA-Akt did not significantly change baseline apoptotic levels at 5 mmol/l glucose (Fig. 3B and D). Taken together, these results indicate that constitutive activation of Akt confers a survival advantage to human islet β-cells exposed to two different cell death inducers in vitro: glucose and serum deprivation and chronic high glucose levels.

Adenovirus-CA-Akt markedly improves human islet transplant outcomes in diabetic SCID mice. The results presented above suggest that human islets transduced with Adv-CA-Akt might function more efficiently than normal islets in a transplant setting. To address this hypothesis, we compared the ability of a marginal mass, 1,500 IEQs of uninfected, Adv-LacZ– and Adv-CA-Akt–transduced human islets to normalize blood glucose in STZ-induced diabetic SCID mice, as previously performed using mouse islets (13,23). As shown in Fig. 4A, transplantaion of 1,500 uninfected or Adv-LacZ–transduced human IEQs under the kidney capsule of diabetic SCID mice did not induce euglycemia in these mice, indicating that this amount of islets was clearly insufficient to achieve sustained normal blood glucose levels. In marked contrast, when 1,500 Adv-CA-Akt IEQs were transplanted under the kidney capsule of diabetic SCID mice, they rapidly normalized blood glucose concentrations, and the mice remained euglycemic throughout the 56-day period of the study (Fig. 4A). The kidney bearing the graft was removed in three of six mice transplanted with Adv-CA-Akt–transduced islets at day 56 after transplantation. Blood glucose levels promptly returned to the original pretransplant values, indicating that the human islets engrafted under the kidney capsule were responsible for the euglycemia observed in these mice. Importantly, random, nonfasting total (human plus rodent) circulating insulin levels were significantly higher in mice transplanted with Adv-CA-Akt–transduced islets compared with mice transplanted with Adv-LacZ–transduced islets (2.90 ± 0.7 ng/ml [n = 6] vs. 0.80 ± 0.2 ng/ml [n = 5], P < 0.05). Furthermore, human insulin levels at day 54 after transplantation were 10-fold higher (P < 0.05) in SCID mice transplanted with Adv-CA-Akt–transduced islets (2.4 ± 0.4 ng/ml, n = 6) compared with mice transplanted with Adv-LacZ–transduced islets (0.25 ± 0.08 ng/ml, n = 5). In addition, blood glucose levels in 1,500-IEQ Adv-CA-Akt–transplanted mice were similar to the blood glucose levels reached with 4,000 uninfected IEQs, an amount that was sufficient to induce euglycemia in SCID diabetic mice.

To further define the function of the transplanted Adv-CA-Akt–transduced islets, we performed intraperitoneal glucose tolerance tests (IPGTTs) on day 49 in mice transplanted with adenovirus-transduced islets and in normal nondiabetic littermates. After an overnight fast, blood glucose levels were significantly (P < 0.05) lower in SCID mice transplanted with Adv-CA-Akt–transduced islets (81 ± 6 mg/dl, n = 6) as compared with SCID mice transplanted with Adv-LacZ islets (181 ± 27 mg/dl, n = 5) (Fig. 4B). Importantly, after glucose injection, glucose tolerance in mice transplanted with Adv-CA-Akt–transduced islets was similar to that in normal nondiabetic mice and was remarkably and significantly (P < 0.025) superior to that in the mice transplanted with Adv-LacZ–transduced islets (Fig. 4B). These results indicate that Adv-CA-Akt–transduced islets confer improved fasting and nonfasting blood glucose control and superior glucose tolerance when transplanted in diabetic SCID mice.

Immunohistochemical analysis of sections from kidneys containing the grafts obtained at 56 days after the transplant of 4,000 uninfected or 1,500 Adv-CA-Akt–transduced IEQs revealed the presence of insulin-positive cells and ductal structures staining for the ductal cell marker CK-19 (Fig. 4C–F). Furthermore, insulin-positive cells could be seen within the ductal structures, suggesting the presence of β-cell neogenesis from the ductal formations in both types of grafts (Fig. 4G–H).

To assess the long-term function of Adv-CA-Akt–transduced islet grafts implanted in diabetic SCID mice, we allowed one-half of the Adv-CA-Akt mice in Fig. 4 to retain their graft for 6 months. As shown in Fig. 5, normalization of blood glucose levels in diabetic mice transplanted with Adv-CA-Akt was sustained for the complete 6-month period of the study compared with the diabetic blood levels observed in mice transplanted with Adv-LacZ–transduced islets. Importantly, when uninephrectomy was performed in mice transplanted with Adv-CA-Akt–transduced islets at 6 months after the transplant, blood glucose levels increased to pretransplant levels, indicating that the graft was responsible for the normal blood glucose observed in these mice. These results indicate that the improvement in blood glucose induced by Adv-CA-Akt islet grafts in diabetic SCID mice is persistent, lasting at least one-fourth of the murine life span.

Next, we immunohistochemically analyzed sections from kidneys containing the grafts obtained at 6 months
after the transplant. Grafts were easily and abundantly visualized in the kidneys containing Adv-CA-Akt–transduced human islets incubated in RPMI medium devoid of glucose and serum and stained for insulin (red) and TUNEL (green). Twenty-four hours after transduction, islets were placed in RPMI containing 5 mmol/l glucose and 10% FBS or RPMI medium devoid of glucose and FBS for 24 h. Arrowheads indicate TUNEL-positive nuclei. As control, a section of Adv-LacZ–transduced human islets incubated in complete conditioned RPMI medium (5 mmol/l glucose and 10% FBS) is shown. B: Quantitation of TUNEL-positive β-cell (insulin-positive) nuclei in these sections of uninfected human islets and Adv-LacZ– or Adv-CA-Akt–transduced human islets incubated in conditioned medium or medium devoid of glucose and FBS (see above). C: Representative photomicrographs of sections of uninfected human islets and Adv-LacZ– and Adv-CA-Akt–transduced human islets incubated in RPMI medium containing 22 mmol/l glucose for 5 days and stained for insulin (red) and TUNEL (green). Arrowheads indicate TUNEL-positive nuclei. As control, a section of Adv-LacZ–transduced human islets incubated in complete conditioned RPMI medium (5 mmol/l glucose and 10% FBS) is shown. D: Quantitation of TUNEL-positive β-cell nuclei in these sections of uninfected human islets and Adv-LacZ– or Adv-CA-Akt–transduced human islets incubated in medium containing either 5 or 22 mmol/l glucose (see above). For these experiments, an MOI of 500 was used. Hoechst 33258 (blue) staining was performed to detect cell nuclei. Results are means ± SE. *P < 0.05 vs. uninfected and Adv-LacZ–transduced treated islets. Five different human islet preparations were examined.

FIG. 3. Quantitation of cell death in human islets after glucose and serum deprivation (A and B) and chronic exposure to 22 mmol/l glucose for 5 days (C and D). A: Representative photomicrographs of sections of uninfected human islets and Adv-LacZ– and Adv-CA-Akt–transduced human islets incubated in RPMI medium devoid of glucose and serum and stained for insulin (red) and TUNEL (green). Twenty-four hours after transduction, islets were placed in RPMI containing 5 mmol/l glucose and 10% FBS or RPMI medium devoid of glucose and FBS for 24 h. Arrowheads indicate TUNEL-positive nuclei. As control, a section of Adv-LacZ–transduced human islets incubated in complete conditioned RPMI medium (5 mmol/l glucose and 10% FBS) is shown. B: Quantitation of TUNEL-positive β-cell (insulin-positive) nuclei in these sections of uninfected human islets and Adv-LacZ– or Adv-CA-Akt–transduced human islets incubated in conditioned medium or medium devoid of glucose and FBS (see above). C: Representative photomicrographs of sections of uninfected human islets and Adv-LacZ– and Adv-CA-Akt–transduced human islets incubated in RPMI medium containing 22 mmol/l glucose for 5 days and stained for insulin (red) and TUNEL (green). Arrowheads indicate TUNEL-positive nuclei. As control, a section of Adv-LacZ–transduced human islets incubated in complete conditioned RPMI medium (5 mmol/l glucose and 10% FBS) is shown. D: Quantitation of TUNEL-positive β-cell nuclei in these sections of uninfected human islets and Adv-LacZ– or Adv-CA-Akt–transduced human islets incubated in medium containing either 5 or 22 mmol/l glucose (see above). For these experiments, an MOI of 500 was used. Hoechst 33258 (blue) staining was performed to detect cell nuclei. Results are means ± SE. *P < 0.05 vs. uninfected and Adv-LacZ–transduced treated islets. Five different human islet preparations were examined.
DISCUSSION

The recent demonstration that human pancreatic islet transplantation is both technically and immunologically feasible has revealed the need for large numbers of human pancreatic islets to treat an increasing number of patients with diabetes (1–3). This scarcity of human islets might potentially be reduced by increasing proliferation, differentiation, function, and survival of the donated human pancreatic islets. β-Cell–specific expression of constitutively active Akt1 in mice results in increased β-cell proliferation, differentiation, and survival (9,10). However, the effects of constitutive activation of Akt in human β-cell proliferation, survival, and transplant performance are entirely unknown. In the studies described herein, we document three novel observations: adenovirus-mediated expression of constitutively active Akt1 (Adv-CA-Akt) in human islets results 1) in increased human β-cell proliferation, 2) in enhanced survival in vitro, and 3) more importantly, in prolonged improvement in human islet transplant outcomes in diabetic SCID mice.

Activation of Akt is associated with increased proliferation in several cell types (8,27). However, whether activation of Akt is also associated with increased pancreatic β-cell replication remains controversial. Several pancreatic β-cell mitogens such as GLP-1, GIP, HGF, and glucose have been shown to stimulate Akt activity in these cells (15–18). In all of these cases, inhibition of the PI3K-Akt pathway reduced or blunted β-cell proliferation induced by these factors. In addition, studies performed by Dickson et al. (11) have demonstrated that adenoviral delivery of CA-Akt into INS-1 cells enhanced two- to threefold β-cell mitogenesis at 3 mmol/l glucose but did not significantly alter β-cell replication at 15 mmol/l glucose. Furthermore, Bernal-Mizrachi et al. (10) have recently reported that constitutive activation of Akt in the pancreatic β-cell of transgenic mice is associated with an increase in adult β-cell replication. On the other hand, Tuttle et al. (9) have observed that transgenic mice overexpressing constitutively active Akt in the pancreatic β-cell have β-cell proliferation rates unaffected. These, in some cases, conflicting studies have been performed in rodent pancreatic β-cells in vitro or in transgenic mice in vivo. However, the role of Akt activation on human β-cell proliferation has not been studied. To begin to evaluate this question, we have analyzed proliferation rates in control human islets and in islets transduced with Adv-CA-Akt by two different methods: [3H]thymidine and BrdU incorporation. In these proliferation studies performed at 5 mmol/l glucose, we...
observed that gene delivery of CA-Akt induced an increase in human islet cell proliferation, and, more importantly, constitutive activation of Akt resulted also in specifically increased /H9252/cell mitogenesis. These results indicate that activation of Akt could expand human pancreatic /H9252/-cells in culture, a result that has potential clinical value given the urgent need to increase the availability of human /H9252/-cells for transplantation.

Therapeutic interventions aimed to inhibit /H9252/-cell death will help to increase cell viability and function during and after islet isolation and islet transplantation. It has been extensively reported that Akt activation plays a pivotal role in cell survival in many cell systems (5,6,8). Among these studies, it has been shown that blockade of the P3K/Akt pathway in rodent /H9252/-cells blunts the pro-survival effects induced by glucose, HGF, GIP, GLP-1, or IGF-I in these cells (12,13,16–18). In addition, transfection of MIN-6 cells with a constitutively active form of Akt en-

FIG. 4. A: Blood glucose levels in STZ-induced diabetic SCID mice after renal subcapsular transplant of uninfected, Adv-LacZ- and Adv-CA-Akt–transduced human islets. Twenty-four hours after transduction, 4,000 and 1,500 uninfected or 1,500 Adv-LacZ– or 1,500 Adv-CA-Akt–transduced human IEQs were transplanted under the kidney capsule of STZ-induced diabetic SCID mice. Results are means ± SE of six mice transplanted with six different human islet preparations per condition. In the group of mice transplanted with 1,500 Adv-LacZ IEQs (n = 5) one hyperglycemic transplanted mouse died at 28 days after the transplant. The posttransplant blood glucose levels in mice transplanted with 1,500 Adv-CA-Akt–transduced IEQs or 4,000 uninfected IEQs were significantly lower (P < 0.05) than the blood glucose values obtained after the transplant of either 1,500 uninfected or 1,500 Adv-LacZ–transduced human IEQs. At day 56, unilateral nephrectomy (UNX) was performed in three mice transplanted with Adv-CA-Akt–transduced islets, and the blood glucose returned immediately to pretransplant diabetic levels. Blood glucose was measured in the other three mice transplanted with Adv-CA-Akt–transduced islets for up to 6 months (see Fig. 5). B: IPGTT in normal non-diabetic SCID mice and in STZ-induced diabetic mice transplanted with 1,500 Adv-LacZ– or 1,500 Adv-CA-Akt–transduced islets. At day 49 after the transplant, mice were fasted for 16 h and then injected with 2 g/kg body wt glucose. Blood glucose was measured from the snipped tail at the points indicated with a portable glucometer. Results are means ± SE. Blood glucose values were significantly lower (P < 0.05) in mice transplanted with Adv-CA-Akt–transduced islets compared with mice transplanted with Adv-LacZ–transduced islets. In fact, blood glucose values in mice transplanted with Adv-CA-Akt–transduced islets were similar to those of normal, non-diabetic SCID mice. C–H: Immunohistochemical detection of insulin- and CK-19–positive cells in kidney sections containing islet grafts from mice transplanted with 4,000 uninfected (C, E, and G) or 1,500 Adv-CA-Akt–transduced (D, F, and H) IEQs. Notice the presence of insulin-positive cells within ductal structures in the grafts (arrows in G and H).
hances the survival effect of glucose in these cells (18). Furthermore, expression of constitutively active Akt in the β-cell of transgenic mice confers striking resistance to cell death induced by the diabetogenic and cytotoxic agent STZ (9,10). Collectively, these rodent studies suggest that Akt activation could be an important pro-survival event for improving human β-cell resistance to cell death. To assess this hypothesis, we studied the effect of constitutive activation of Akt on human β-cell survival in vitro by using two different cell death inducers implicated in the massive β-cell loss that occurs immediately after islet transplantation (28) and in β-cell failure in type 2 diabetes (29): glucose and serum deprivation and chronic hyperglycemia. We found that gene transfer of CA-Akt protects human β-cells against cell death induced by both types of insults. Glucose and serum deprivation increased apoptotic rates more than eightfold in human β-cells in uninfected or Adv-LacZ–transduced islets. In marked contrast, Adv-AC-Akt–transduced human islets displayed markedly reduced number of apoptotic β-cells, similar to the apoptotic rates found in islets incubated in normal conditioned medium. In addition, incubation of control (uninfected or Adv-LacZ–transduced) human islets in 22 mmol/l glucose for 5 days resulted in a four- to sixfold increase in β-cell apoptosis. Again, CA-Akt transfer in human islets resulted in a striking decrease in the number of apoptotic β-cells. Contreras et al. (30) have recently demonstrated that activation of Akt is essential for the cytoprotective effect induced by the cholesterol-lowering drug simvastatin on isolated human islets in vitro. Furthermore, recent studies by Aikin et al. (31) have shown that inhibition of PI3K/Akt pathway enhances basal and cytokine-mediated cell death in human islets. These studies, together with the present observations, suggest that activation of Akt in human islets could be an important therapeutic strategy for enhancing human β-cell survival under normal and stress-induced conditions.

A key question that emerges from these initial in vitro studies is whether constitutive activation of Akt can improve human islet engraftment and performance when transplanted in diabetic mice. The results obtained in the studies presented herein are very clear: gene transfer of constitutively active Akt to a marginal mass of human islets dramatically improved human islet graft function and survival in a transplant setting in diabetic SCID mice. Importantly, this euglycemic effect in the mice transplanted with CA-Akt–transduced islets was long lasting. Contreras et al. (30) have recently reported that treatment of human islets with simvastatin improved islet transplant outcomes in 58% of the transplanted diabetic SCID mice. Furthermore, this improvement in the islet transplant outcomes was completely abolished when islets were pretreated with the PI3K/Akt pathway inhibitor, LY294002. Taken together, these studies suggest that activation of Akt in human islets could decrease the number of islets needed for successful transplantation.

In the present studies, we have used an adenovirus system to deliver a constitutively active form of Akt1 into human islets. The rate of infection achieved with this system is similar to that achieved in other studies (30–70%) in which rodent or human islets have been transduced with adenoviruses (13,32,33). Because Akt is an intracellular protein, one can argue that with the use of the adenoviral system only 30–70% in which rodent or human islets have been transduced with adenoviruses one can argue that with the use of the adenoviral system only 30–70% of the cells will acquire the potential positive benefits that constitutively active Akt may promote in human islet cells. Regarding the importance of these findings for human islet transplantation, three points are relevant. First, the present studies serve as a “proof of principle” studies designed to determine whether activation of Akt in human islets is beneficial for these islets. The answer to this question is surely affirmative. Second, unlike rodent islets, human islets seem to display a more peripheral distribution of β-cells (Figs. 1–3) (22), which could result in increased efficiency of adenoviral transduction in β-cells. Third, despite transducing only a portion of the cells in the islet, human islets infected with Adv-CA-Akt display increased proliferation, enhanced survival, and improved human islet transplant outcomes, as shown in Figs. 2–5. This is very significant, because one can readily envision future vector approaches that will result in enhanced efficiency of transduction, improved β-cell specificity, an adjustable level of expression, and a regulatable temporal expression pattern.
In the current studies, we detect the presence of CA-Akt in graft cells at 6 months after the transplant. Because overexpression of CA-Akt increases human islet cell proliferation and survival in vitro, one might worry that CA-Akt gene delivery into human islets might cause abnormal proliferation or tumorigenesis of graft cells. Although, we are not aware of any report indicating that continuous Akt activation is implicated in generation of human insulinomas, amplification and overexpression of Akt2 in a subset of human pancreatic ductal adenocarcinomas has been reported (34). Several points are relevant here. First, as mentioned above, the current study was intended as a “proof of principle” to analyze whether Akt activation in human islets is beneficial for improving islet transplant outcomes. Second, diabetic SCID mice transplanted with Adv-CA-Akt–transduced human islets do not apparently display hypoglycemia, a crude measure of islets tumors. Third, although we observed the presence of a large number of ductular structures in the CA-Akt islet graft at 6 months after the transplant, we did not find any invasion of the graft into the kidney. In fact, similar observations have been reported by others in transplants performed with human islets not treated with CA-Akt (35).

In summary, our studies demonstrate that adenoviral delivery of CA-Akt to human islets ex vivo improves islet transplant survival and performance in STZ-induced diabetic SCID mice. These studies demonstrating that CA-Akt expression can increase human β-cell proliferation and survival reveal two critical components required for expanding human β-cells in vitro and in vivo. These experiments also suggest that CA-Akt could play an important role in preventing early failure and promoting expansion of the islet graft. These beneficial effects could be mediated by constitutively active Akt or by other factors secreted by Adv-CA-Akt–transduced cells that could also affect nontransduced cells in a paracrine way. Controlled activation of Akt may be of therapeutic use for expanding and protecting isolated human islets, improving islet transplant outcomes, and reducing the number of islets required for successful islet transplantation.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (DK-067351 and DK-068836), the Juvenile Diabetes Foundation International, and the American Diabetes Association (Junior Faculty grant).

We are grateful to Dr. Andrew F. Stewart and Dr. Rupangi C. Vasavada for the frequent and thoughtful discussions of the ideas in this article. We thank Vasumathi Reddy and Darinka Sipula for superb technical assistance.

FIG. 6. Immunohistochemical detection of hemagglutinin and different islet and ductal cell markers in kidney sections containing Adv-CA-Akt grafts. Kidney sections from mice transplanted with 1,500 Adv-LacZ or 1,500 Adv-CA-Akt human IEQs obtained 6 months after the transplant were stained for insulin as described in RESEARCH DESIGN AND METHODS. The transplant could not be identified in any of the Adv-LacZ kidneys despite sectioning throughout the entire organ. In contrast, grafts were large and easily identified in all Adv-CA-Akt kidneys. Representative photomicrographs of sections from Adv-CA-Akt kidneys are shown displaying positive staining for insulin (A, E, and F), CK-19 (B), glucagon (C), and somatostatin (D). Notice the presence of insulin-positive cells inside of ductal structures in the grafts (arrows in E and F), suggesting β-cell neogenesis. G and H: Kidney sections from mice transplanted with 1,500 Adv-CA-Akt human IEQs obtained 6 months after the transplant were stained for hemagglutinin (red), insulin (green), and Hoechst 33258 (blue) as described in RESEARCH DESIGN AND METHODS. Arrows indicate hemagglutinin-positive cells. I and J: No hemagglutinin staining was observed in graft sections obtained at 56 days after the transplant of 4,000 uninfected IEQs.
REFERENCES


