*Original Article*

# Angiopoietin-1 Production in Islets Improves Islet Engraftment and Protects Islets From Cytokine-Induced Apoptosis

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**Successful islet transplantation depends on the infusion of sufficiently large quantities of islets, but only a small fraction of implanted islets become engrafted. The underlying mechanisms remain elusive. To probe the mechanism of islet revascularization, we determined the effect of angiopoietin-1 (Ang-1), a proangiogenic and antiapoptotic factor, on the survival, function, and revascularization of transplanted islets using a syngeneic model. Islets were transduced with adenoviruses expressing Ang-1 or control LacZ, followed by transplantation under the renal capsule. Diabetic mice receiving a marginal mass of 150 islets pretransduced with Ang-1 vector exhibited near normoglycemia posttransplantation. In contrast, diabetic mice receiving an equivalent islet mass pretransduced with control vector remained hyperglycemic. At 30 days posttransplantation, mice were killed and islet grafts retrieved for immunohistochemistry. Islet grafts with elevated Ang-1 production retained significantly increased microvascular density, improved glucose profiles, and increased glucose-stimulated insulin release. Cultured islets expressing Ang-1 displayed improved viability and enhanced glucose-stimulated insulin secretion in the presence of cytokines. In contrast, control islets exhibited increased apoptosis and diminished glucose-stimulated insulin release in response to cytokine treatment. These results indicate that Ang-1 confers a cytoprotective effect on islets, enhancing islet engraftment and preserving functional islet mass in transplants. *Diabetes* 56:2274–2283, 2007**

The Edmonton protocol depends on the infusion of sufficiently large quantities of islets (>10,000 IE/kg) to achieve sustained insulin independence, requiring multiple cadaver pancreata for one diabetic recipient (1). However, only a fraction of implanted islets (<30% islet mass) can survive and become engrafted (1–6). In addition to immune rejection, the rate and extent of islet revascularization has been viewed as a significant factor that may contribute to the loss of functional islet mass posttransplantation (7,8). Unlike whole-organ transplantation in which grafts are implanted as vascularized tissue and intragraft blood flow is readily resumed posttransplantation, islets are transplanted as single islets or islet clusters, which are considered avascular after collagenase digestion, a relatively harsh procedure that severs the arterial and venous connections of islets and presumably damages intraislet endothelium and extracellular matrix of islets. As a result, microvascular perfusion to newly implanted islets does not resume immediately after transplantation, and it can take up to 2–3 weeks to reestablish a functional microvasculature in islet grafts (8–13). Thus, newly implanted islets can be deprived of oxygen and nutrients, resulting in inflammation and apoptosis and contributing to the loss of functional islet mass posttransplantation.

To address this limitation, we used a gene transfer approach to deliver angiogenic genes such as vascular endothelial growth factor (VEGF) into islets (14,15). Elevated production of angiogenic factors locally in islet grafts is shown to promote graft angiogenesis and enhance islet revascularization, contributing to significantly improved glycemic control and better preservation of islet mass in diabetic recipient mice (15). Likewise, Lai et al. (16) transplanted islets expressing VEGF under control of the rat insulin promoter under the kidney capsule of diabetic mice, demonstrating that glucose-stimulated VEGF expression promoted islet revascularization along with increased blood flow to islet grafts. Sigrist et al. (17) used a different approach by which murine islets were embedded in VEGF-containing collagen and encapsulated before transplantation into the peritoneal cavity, showing that local VEGF release from encapsulated islet grafts significantly increased the viability of transplanted islets, resulting in sustained physiological glycemic control in diabetic mice. These studies validate the concept that islets preconditioned ex vivo with augmented angiogenic function are associated with improved revascularization.

Although considered avascular, freshly isolated islets contain residual endothelial cells. Three independent studies show that a fraction of these intraislet endothelial cells participate in revascularization and become integral parts of newly formed microvasculature within islet grafts (18–21). These results spurred us to examine the effect of Ang-1, a proangiogenic and antiapoptotic factor, on islet

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Ang-1, angiopoietin-1; IFN-γ, γ-interferon; IL, interleukin; MOI, multiplicity of infection; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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survival and revascularization. Expressed in various cell types including pancreatic islets, Ang-1 plays a significant role in angiogenesis and vasculogenesis by promoting the migration of pericytes for recruitment onto sprouting vessels for capillary formation and remodeling (22–25). In addition, Ang-1 binds to its endothelium-specific receptor tyrosine kinase Tie-2, which in turn reprograms endothelial gene expression to inhibit vascular leakage and protect endothelial cells from inflammation-induced damage (24–26). Genetic ablation of Ang-1 or its cognate receptor Tie-2 disrupts angiogenesis, resulting in defective vasculogenesis predominantly in heart, brain, and yolk sac (27–29). As a result, Ang-1 or Tie-2 gene knockout mice are embryonic lethal. Conversely, transgenic Ang-1 overexpression results in enhanced angiogenesis, as evidenced by increased size and branching of vessels that are resistant to inflammatory agent–induced damage in mice (30,31). In addition, there is evidence that Ang-1 stimulates endothelial cell proliferation (32). Based on the dual function of Ang-1 in endothelial survival and angiogenesis, we hypothesized that elevated Ang-1 production locally in islet grafts would enhance islet revascularization and protect islets from hypoxia-induced apoptosis, contributing to better preservation of islet mass and improved glycemic control in transplants. To address the effect of Ang-1 on islet revascularization in the absence of immune rejection, we tested this hypothesis in a syngeneic model.

## RESEARCH DESIGN AND METHODS

**Vectors.** The recombinant adenoviral vector Ad-EF1-Ang-1 expresses the human Ang-1 under the control of the elongation factor 1-α (EF1-α) promoter. To prepare adenoviruses, Ad-EF1-Ang-1 was propagated in HEK293 cells and purified by CsCl density centrifugation as previously described (15). The titer of Ad-EF1-Ang-1 was $1.1 \times 10^{11}$ plaque-forming units (pfu)/ml. The Ad-RSV-LacZ vector expressing β-galactosidase with a titer of $1.9 \times 10^{11}$ pfu/ml was used as the control.

**Islet isolation and transplantation.** Male inbred BALB/c mice (aged 8 weeks) (Charles River Laboratory, Wilmington, MA) were used as donors and recipients. Mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine, followed by pancreatic intraductal infusion of 3 ml cold Hank's buffer containing 1.75 mg/ml of collagenase-V (Sigma, St. Louis, MO). The pancreas was surgically procured and digested at 37°C for 18 min. Islets were purified on a discontinuous Ficoll gradient (Sigma) as previously described (15). Islets were resuspended in RPMI-1640 medium (GIBCO Laboratories, Grand Island, NY) and handpicked under an inverted microscope under sterile conditions.

For islet transduction by adenoviral vectors, aliquots of islets ($n$ = 150) were either mock treated with 100 μl PBS buffer or incubated with 100 μl Ang-1 or LacZ vector at a defined multiplicity of infection (MOI) (100 pfu/cell; one islet, ~1,000 cells) in 2 ml RPMI-1640 medium at 37°C for 16 h. After washing with Hank's balanced salt solution, transduced islets were used for transplantation. This vector dose has been shown to result in ~70% transduction in cultured islets without a detectable effect on islet structural and functional integrity (15,33).

To determine the efficiency of transduction in islets, islets pretransduced with LacZ vector were harvested and dispersed to single-islet cells by incubating islets in trypsin-EDTA solution (Invitrogen, Grand Island, NY) for 10 min at 37°C, followed by staining with X-gal. The transduction efficiency, defined as the number of β-galactosidase–positive cells out of total islet cells, was determined.

For islet transplantation, inbred BALB/c mice were rendered diabetic by intraperitoneal injection of 180 mg/kg streptozocin as previously described (15). One week post–streptozocin treatment, diabetic mice (blood glucose levels ~400 mg/dl) were randomly assigned to three groups and were transplanted under the renal capsule with a marginal islet mass (150 islets) that was pretransduced with Ang-1 ($n$ = 21) or control ($n$ = 17) vector or PBS buffer ($n$ = 13) using the established procedure (15). After transplantation, blood was sampled weekly from tail vein for blood glucose measurement using a Glucometer Elite (Bayer, Mishawaka, IN). All procedures were approved by the institutional animal care and usage committee of the Children's Hospital of Pittsburgh.

**Glucose tolerance test and glucose-stimulated insulin release.** Mice were fasted for 6 h and injected intraperitoneally with 50% glucose solution (Abbott Laboratories, Chicago, IL) at 3 g/kg body wt. Blood glucose levels were measured before and at 30-min intervals after glucose infusion. To determine glucose-stimulated insulin release, aliquots (100 μl) of tail vein blood were sampled before and 5 min after glucose infusion for the determination of plasma insulin levels by ultrasensitive mouse insulin enzyme-linked immunosorbent assay (ALPCO, Windham, NH).

**Western blot analysis.** To verify Ang-1 production from Ad-EF1-Ang-1, aliquots ($n$ = 100) of islets were transduced with Ang-1 vector at MOI ranging from 0 to 200 pfu/cell. In addition, one aliquot of 100 islets was transduced with LacZ vector as the control. After 16 h incubation in 2 ml RPMI-1640 medium, conditioned media were collected and subjected to Centricon Plus-20 (Millipore, Bedford, MA). After centrifugation at 1,000$g$ for 30 min, aliquots of samples (30 μg) from each condition were resolved on 15% SDS-PAGE. Proteins were blotted onto nitrocellulose membrane, which was probed with goat anti-human Ang-1 antibody (1:100 dilution, sc-6320; Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with anti-goat IgG conjugated with horseradish peroxidase (1:10,000 dilution). Protein bands were visualized by chemiluminescent Western detection regents (Pierce, Rockford, IL). The intensity of protein bands was quantified by densitometry using the NIH Image software (National Institutes of Health, Bethesda, MD) as described (34).

To determine Tie-2 expression in islets, aliquots of 300 islets were homogenized in 100 μl ice-cold CER-I solution (Pierce) supplemented with 10 μl protease inhibitor cocktail (Pierce), followed by freezing in a dry ice bath and thawing in a 37°C water bath for three consecutive cycles. Total protein lysates (30 μg in aliquots) were subjected to immunoblot analysis using anti–Tie-2 antibody (1:200 dilution, SC-324; Santa Cruz Biotechnology) as described above.

**Immunofluorescent microscopy and morphometric analysis.** Diabetic recipient mice were killed under nonfasting conditions 30 days posttransplantation. Pancreatic islet graft–bearing kidneys were retrieved and fixed in 4% paraformaldehyde for 3 h at 4°C, followed by incubation in 30% sucrose overnight at 4°C. Samples were sent to the Image and Morphological Core at the Division of Immunogenetics, Children's Hospital of Pittsburgh. Islet graft–bearing kidneys were embedded in Shandon Cryomatrix (Thermo Electron, Waltham, MA) and snap frozen in liquid nitrogen–cooled isopentane (Fisher Scientific, Pittsburgh, PA). Cyrosections (10 μm) were cut and immunostained with rabbit anti-insulin (1:200, sc-9168; Santa Cruz Biotechnology) or rat anti-CD31 (1:10; BD Biosciences, Palo Alto, CA). After washing with PBS, sections were incubated with Cy3-conjugated goat anti-rabbit antibody or Cy5-conjugated goat anti-rat antibody (Jackson ImmunoResearch Lab, West Grove, PA).

Sections were viewed under a Nikon confocal microscope (Nikon D-ECLIPSE C1; Nikon, Tokyo, Japan). Images of islet grafts were captured and subjected to morphometric analysis using MetaMorph Image Analysis software (Molecular Devices, Downingtown, PA). Islet vascular density was determined by measuring the area of vessels within islet grafts that were positively immunostained by endothelium-specific anti-CD31 antibody. For each graft-bearing kidney, three nonconsecutive sections were viewed at ×400 magnification to determine the area occupied by CD31-positive endothelial cells within a given islet graft. The microvascular density of islet grafts was defined as the CD31-positive endothelial area divided by the insulin-positive area of islet grafts, as described (15).

**Apoptosis assay.** Aliquots of 100 freshly isolated islets from BALB/c mice were transduced with Ang-1 or control vector at an MOI of 100 pfu/cell in 2 ml RPMI-1640 medium at 37°C for 16 h. Islets were then seeded in collagen IV–coated 96-well plates (BD Biosciences, Bedford, MA) and incubated in 200 μl RPMI-1640 medium in the absence and presence of an inflammatory cytokine cocktail containing 5 ng/ml human interleukin (IL)-1β, 100 ng/ml γ-interferon (IFN-γ), and 10 ng/ml tumor necrosis factor (TNF)-α (Roche Diagnostics, Indianapolis, IN) for 48 h. Each condition was run in triplicate. Apoptosis in cultured islets was determined using the ApoPercentage Apoptosis Assay Kit (Biocolor, Newtownabbey, Ireland). This assay relies on the ApoPercentage dye that is selectively imported by apoptotic cells. Islets precultured under different conditions were incubated in the presence of ApoPercentage dye (5 μl in 200 μl RPMI-1640 medium) for 45 min at 37°C. Islets were washed three times with PBS to remove unincorporated dye. Apoptotic cells developed red color following intake of ApoPercentage dye and were detected under an inverted light microscope.

To quantify the extent of cellular apoptosis in cytokine-treated islets, ApoPercentage dye–stained islets were incubated in 100 μl ApoPercentage Dye Release Reagent with gentle shaking for 30 min at room temperature to allow the release of accumulated dye. The supernatants containing released dye in each sample were determined spectrometrically at 550 nm.

To determine the fraction of apoptotic cells in cytokine-treated islets,

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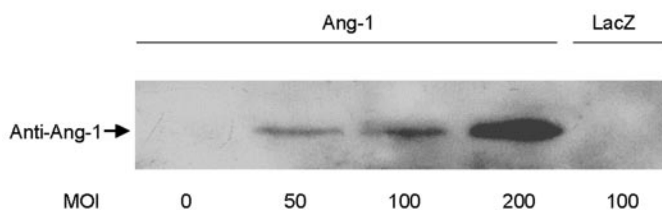


**FIG. 1. Characterization of Ang-1 vector. Freshly isolated islets ($n$ = 100) from BALB/c mice were transduced with Ang-1 vector at MOIs of 0, 50, 100, and 200 pfu/cell or 100 pfu/cell of LacZ vector. After 16 h of transduction, conditioned media were collected for measuring Ang-1 protein levels by immunoblot assay. The specific band corresponding to Ang-1 (molecular mass 57 kDa) is indicated by the arrow.**

aliquots of 100 BALB/c islets were either mock treated with PBS or transduced with Ang-1 or control vector at an MOI of 100 pfu/cell in 2 ml RPMI-1640 medium at 37°C for 16 h. Islets were gently washed three times in 1 min with $Ca^{2+}/Mg^{2+}$-free MEM (Mediatech, Herndon, VA), followed by incubation in trypsin-EDTA solution (Invitrogen, Carlsbad, CA) for 1 min in a 37°C $CO_2$ incubator. Islets were dispersed by gentle repetitive pepetting. Dispersed islet cells were incubated in six-well plates in the presence and absence of the inflammatory cytokine cocktail containing 5 ng/ml human IL-1β, 100 ng/ml IFN-γ, and 10 ng/ml TNF-α (Roche Diagnostics) for 48 h, followed by detection of apoptosis using the ApoPercentage Apoptosis Assay Kit (Biocolor). Apoptotic cells, stained red after intake of ApoPercentage dye, were counted in three to five nonoverlapping views for each condition in a light microscope at ×100 magnification.

To determine the expression levels of Bcl-2, a prosurvival factor, in cultured islets in the presence and absence of cytokines, aliquots of 500 islets were transduced with control or Ang-1 vector for 16 h, followed by a 48-h cytokine treatment as described above. Islets were homogenized in 100 μl ice-cold CER-I solution (Pierce) supplemented with protease inhibitor cocktail (Pierce). Aliquots of 30 μg protein were resolved on 4–20% SDS-PAGE and subjected to immunoblot analysis using anti–Bcl-2 antibody (1:100 dilution, sc-783; Santa Cruz Biotechnology).

**Immunofluorescent microscopy of intra-islet endothelium in cultured islets.** Aliquots of 30 freshly isolated islets were cultured in 2 ml RPMI-1640 medium supplemented with and without cytokine cocktail as described above. After 48 h of incubation, islets were centrifuged onto slides at 100 rpm for 6 min in the Shandon Cytospin-4 Zytozentrifuge (Thermo Electron) and subjected to immunohistochemistry using both rat anti-CD31 antibody (1:10; BD Biosciences) and rabbit anti-insulin antibody (1:200, sc-9168; Santa Cruz Biotechnology), followed by incubation with Rhodamine-conjugated goat anti-rat IgG (1:80; Jackson ImmunoResearch Lab) and Alexa-488–conjugated goat anti-rabbit IgG (Molecular Probe).

**Statistics.** Statistical analyses of data were performed by ANOVA post hoc tests using StatView software (Abacus Concepts, Berkeley, CA). Bonferroni pairwise comparisons were performed for statistical analysis of multiple groups. Data are expressed as means ± SEM. $P$ values <0.05 were statistically significant.

## RESULTS

**Characterization of Ang-1 vector.** To characterize the Ad-EF1-Ang-1 vector, aliquots of 100 freshly isolated murine islets were transduced with Ang-1 vector at different MOIs ranging from 0 to 200 pfu/cell or LacZ vector at 100 pfu/cell. After 16 h of incubation, conditioned media were subjected to immunoblot assay for the determination of Ang-1 protein levels. As shown in Fig. 1, transduction of islets with Ang-1 vector resulted in a dose-dependent increase in Ang-1 levels. In contrast, Ang-1 was undetectable in conditioned media of control vector–transduced islets. These results indicate that Ang-1 vector is capable of producing Ang-1 protein that is secreted into cultured media.

**Effect of Ang-1 production in islet grafts on glycemic control.** To determine the effect of Ang-1 production in islet grafts on glycemic control, inbred BALB/c mice with streptozocin-induced diabetes were stratified by blood glucose levels and randomly assigned to three groups to ensure a similar mean blood glucose level (429 ± 18 mg/dl) per group. Aliquots of 150 freshly isolated islets were transduced by Ang-1 ($n$ = 21) or control vector ($n$ = 17) at a fixed MOI (100 pfu/cell) for 16 h, followed by transplantation under the renal capsule in mice with streptozocin-induced diabetes. In addition, a group of diabetic mice ($n$ = 13) receiving mock-treated islets was used as control. As shown in Fig. 2*A*, diabetic mice transplanted with Ang-1 vector–transduced islets were restored to near normal (144 ± 13 mg/dl) 30 days after transplantation. In contrast, diabetic mice receiving the same number of islets that were transduced with control vector or mock treated remained hyperglycemic. In keeping with previous data (15,33), no significant differences in blood glucose profiles between control vector and mock treatment groups were detected.

To study the effect of Ang-1 production in islet grafts on glucose disposal rates, mice were challenged by intraperitoneal infusion of glucose (3 g/kg) at days 15 and 30 posttransplantation. As shown in Fig. 2*B*, diabetic recipient mice receiving Ang-1 vector–transduced islets exhibited significantly improved blood glucose profiles. After 30 min of glucose infusion, blood glucose levels in the Ang-1 group were elevated to 434 ± 27 mg/dl, followed by a decline to prechallenge levels within 2 h. In contrast, blood glucose levels in mock treatment and LacZ control groups were markedly raised to significantly higher levels (506 ± 20 and 552 ± 54 mg/dl) and remained hyperglycemic (328 ± 18 and 368 ± 59 mg/dl) after 2 h of glucose infusion. Similar results were produced on day 30 after islet transplantation.

To confirm islet graft function, nephrectomy was performed to remove graft-bearing kidneys in diabetic recipient mice 30 days posttransplantation. Nephrectomy resulted in the recurrence of severe hyperglycemia in the Ang-1 group. No significant differences in the degree of hyperglycemia were detected among Ang-1, LacZ, or mock treatment groups postnephrectomy (Fig. 2*A*). These results indicate that the improved glycemic control was attributable to islet graft function in the Ang-1 group.

**Effect of Ang-1 production in islet grafts on glucose-inducible insulin release.** To study the effect of Ang-1 production in islet grafts on islet function, plasma insulin levels were determined under basal and glucose-inducible conditions at 15 and 30 days posttransplantation. Diabetic recipient mice were fasted for 6 h, followed by intraperitoneal injection of 3 g/kg glucose. Before and 5 min after glucose infusion, aliquots (100 μl) of blood were collected from tail vein for the determination of plasma insulin levels. As shown in Fig. 2*C*, relatively higher plasma insulin levels were detected in diabetic mice receiving Ang-1 vector–transduced islets (0.86 ± 0.09 vs. 0.61 ± 0.04 ng/ml in the LacZ group and 0.65 ng/ml in the mock treatment group) under fasting conditions, but the difference did not reach a significant level. However, in response to glucose challenge, significantly higher plasma insulin levels were produced in diabetic mice that were transplanted with Ang-1 vector–transduced islets (1.89 ± 0.19 vs. 1.04 ± 0.09 ng/ml in the LacZ group and 1.26 ± 0.08 ng/ml in the mock treatment group, $P < 0.05$), which correlated with better glycemic control in the Ang-1 group.

**Effects of Ang-1 on islet revascularization.** To study the effects of Ang-1 on graft engraftment, mice were killed 30 days posttransplantation. Islet grafts were retrieved and subjected to immunohistochemistry using antibodies against insulin and endothelium-specific marker CD31. As

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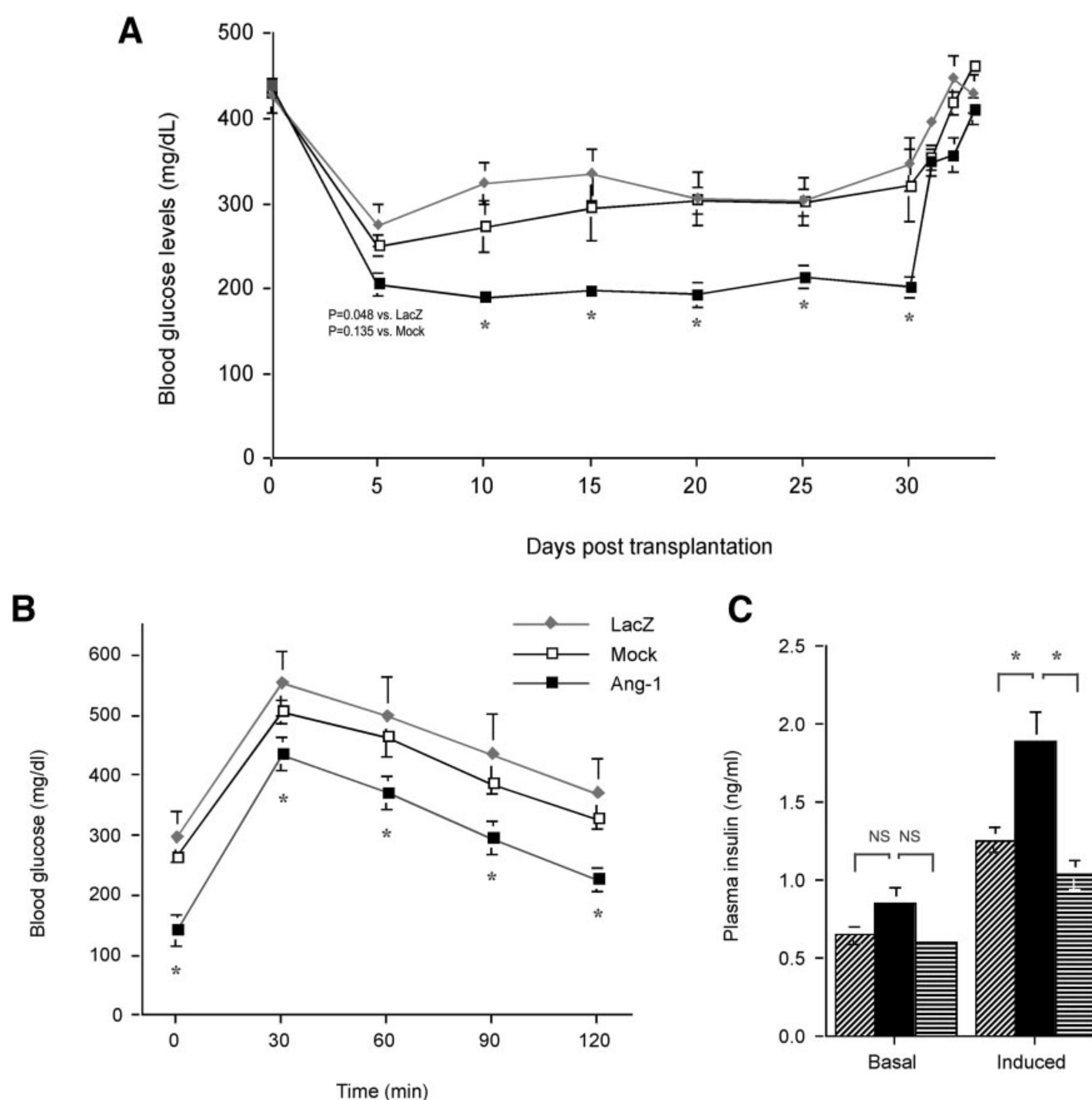


**FIG. 2. Effects of Ang-1 production in islet grafts on glycemic control. *A*: Blood glucose levels in the Ang-1 ($n$ = 21) (■), the LacZ control ($n$ = 17) (♦), and the mock treatment ($n$ = 13) (□) groups. Nephrectomy was performed on day 30, followed by blood glucose determination for 3 days. *B*: Glucose tolerance test. Diabetic recipient mice at day 15 posttransplantation were fasted for 6 h, followed by intraperitoneal injection of 3 g/kg glucose. Blood glucose levels were determined before and after glucose infusion at 30-min intervals. *C*: Glucose-stimulated insulin release in islet grafts. During glucose tolerance tests, aliquots (50 μl) of blood were collected from tail vein before and 5 min after glucose infusion. Plasma insulin levels were determined under basal and glucose-stimulated conditions. Similar results were reproduced at 30 days posttransplantation. *$P$ < 0.05 vs. mock treatment (▨) and LacZ (▤) controls. Black bar, Ang-1. NS, not significant.**

shown in Fig. 3, relatively higher numbers of intra-islet vessels were detected in engrafted islets in the Ang-1 versus the control group. This observation was confirmed by morphometric analysis of intragraft vessels. Islet grafts that were transduced with Ang-1 vector exhibited significantly higher vascular densities (approximately twofold) than those exhibited by control islet grafts in mock treatment and LacZ vector groups. In addition, we detected more islets engrafted under the kidney capsule in the Ang-1 group (Fig. 3). This difference in intra-islet vascular density between Ang-1 and control groups of diabetic recipient mice correlated with their differences in blood glucose profiles and glucose-stimulated insulin secretion (Fig. 2).

**Effect of Ang-1 on islet viability and function.** To characterize the underlying mechanism of Ang-1–mediated improvement in islet survival and engraftment, we studied the effect of Ang-1 on islet viability and function in response to adverse events such as cytokine-induced apoptosis. We hypothesized that elevated Ang-1 production in islets would protect islet cells from cytokine-induced apoptosis. To address this hypothesis, we transduced freshly isolated islets ($n$ = 100) with Ang-1 or control vector at 100 pfu/cell, followed by a 48-h incubation in the absence and presence of three inflammatory cytokines—IL-1β, IFN-γ, and TNF-α—at predefined concentrations (35). Treated islets were subjected to an apoptosis assay. Because of the loss of membrane integrity, apoptotic cells developed a purple red color after selective uptake of a synthetic dye that was included in culture media, as visualized under a light microscope. As shown in Fig. 4*A*–*E*, cytokine treatment resulted in marked accumulation of the dye in cultured islets, an effect of cytokine-induced apoptosis that was mitigated in response to elevated Ang-1 production in islet cells.

To quantify the extent of apoptosis, we determined the

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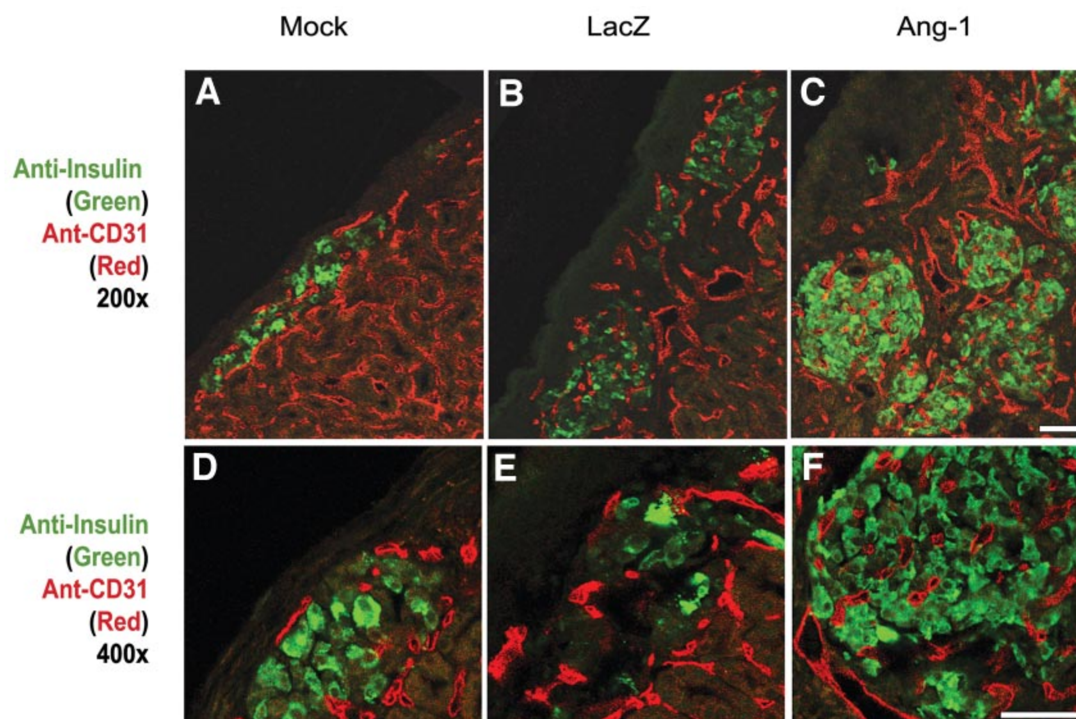


FIG. 3. Immunofluorescent microscopy of islet grafts. Islet grafts were retrieved from diabetic mice that were transplanted with mock-treated (*A* and *D*), control vector (*B* and *E*)–, and Ang-1 vector (*C* and *F*)–transduced islets. Frozen sections of islet grafts were immunostained with anti-insulin and anti-CD31. Bar, 50 μm. (Please see http://dx.doi.org/10.2337/db07-0371 for a high-quality digital representation of this figure.)

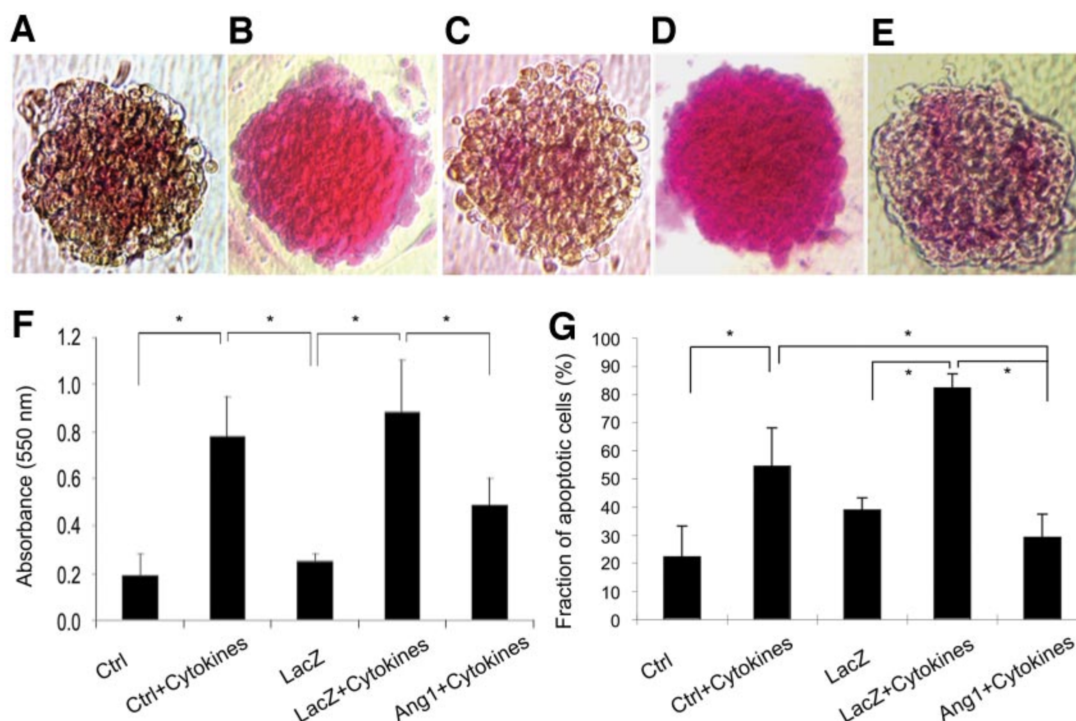


FIG. 4. Cytokine-induced apoptosis in cultured islets. Freshly isolated mouse islets ($n$ = 100) were transduced with 100 pfu/cell Ang-1 or LacZ vector for 16 h, followed by incubation in the absence and presence of cytokines for 48 h. Apoptotic cells (stained red) were detected by ApoPercentage dye. *A*: Control islets. *B*: Control islets treated with cytokines. *C*: LacZ vector–transduced islets without cytokine treatment. *D*: LacZ vector–treated islets in the presence of cytokines. *E*: Ang-1 vector–transduced islets with cytokine treatment. *F*: Quantitative analysis of apoptosis in islets. The extent of apoptosis was determined by incubating islets in ApoPercentage Dye Release Reagent, followed by determination of the supernatant containing preincorporated dye in apoptotic cells spectrometrically at 550 nm. *G*: Effect of Ang-1 on islet cell survival. Islets were either mock treated with PBS buffer or transduced with LacZ or Ang-1 vector, followed by dispersion into individual cells. After incubation in the presence or absence of cytokines for 48 h, the fraction of apoptotic cells out of total islet cells was determined using ApoPercentage Apoptosis assay. *$P < 0.05$. (Please see http://dx.doi.org/10.2337/db07-0371 for a high-quality digital representation of this figure.)

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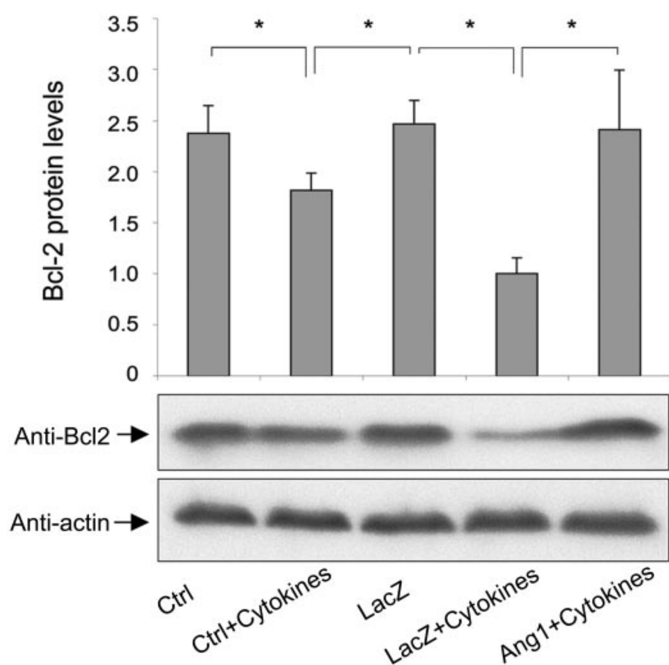


FIG. 5. Bcl-2 expression in cultured islets. Aliquots of islets ($n$ = 500) were mock treated (control [Ctrl]) or transduced with 100 pfu/cell of LacZ or Ang-1 vector for 16 h, followed by a 48-h incubation in culture media without and with cytokines. Bcl-2 protein expression levels in treated islets were determined by semiquantitative immunoblot assay using actin as a control. *$P$ < 0.05.

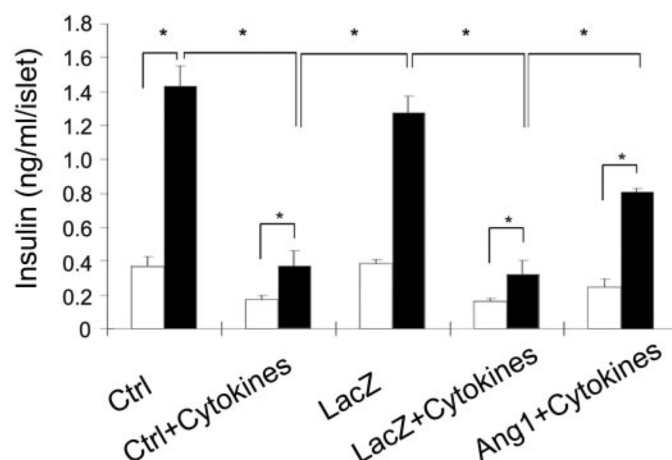


FIG. 6. Effect of Ang-1 on islet function. Aliquots of 100 islets isolated from BALB/c mice were either mock treated (control [Ctrl]) or transduced with Ang-1 or control vector at an MOI of 100 pfu/cell in 2 ml RPMI-1640 medium at 37°C for 16 h. Islets were then seeded in collagen IV–coated 96-well plates and incubated in 200 μl RPMI-1640 medium in the absence and presence of cytokines for 48 h. Each condition was run in triplicate. Glucose-stimulated insulin secretion was studied by shifting glucose concentration of culture media from 5 (basal [□]) to 20 mmol/l (induced [■]). After 15 min, aliquots (50 μl) of media were sampled for the determination of insulin concentrations. *$P$ < 0.01.

amount of dye accumulated in islet cells. As shown in Fig. 4*F*, islets pretreated with cytokines were associated with significantly increased apoptosis, as a threefold higher amount of dye was detected in cytokine-treated islets in comparison with that found in control islets. However, elevated Ang-1 production resulted in a significant reduction in dye accumulation in islet cells, although Ang-1 did not reduce dye accumulation in islet cells to basal levels. These results suggest that Ang-1 confers a cytoprotective effect on islet viability in response to cytokine treatment.

To determine the beneficial effect of Ang-1 on islet cells in response to cytokine treatment, islets were transduced with Ang-1 or control vector, followed by gentle dispersion into single cells. After incubation in the presence or absence of proinflammatory cytokines for 48 h, dispersed islet cells were subjected to the apoptosis dye–staining assay, as described above. In this assay, apoptotic cells were stained red and the fraction of apoptotic cells out of total dispersed islet cells determined. As shown in Fig. 4*G*, cytokine treatment caused a significant increase in cellular apoptosis, as ~50–70% of cells were stained red in the mock treatment and LacZ groups after cytokine treatment. However, elevated Ang-1 production in islets diminished the apoptotic effect of cytokines on dispersed islet cells to basal levels. Islets are known to contain ~70–80% of β-cells. These results indicate that Ang-1 also exerts an antiapoptotic effect on β-cells in the presence of cytokine treatment.

To corroborate these findings, we determined the expression levels of Bcl-2, a gene associated with cell survival (36). As shown in Fig. 5, Bcl-2 protein levels were significantly reduced, which correlated with the induction of cellular apoptosis in cytokine-treated islets (Fig. 4). However, elevated Ang-1 expression in islets significantly ameliorated the deleterious effect of cytokines on Bcl-2 expression, as relatively higher Bcl-2 protein levels were detected in cytokine-treated islets pretransduced with Ang-1 vector (Fig. 5).

To study the beneficial effect of Ang-1 on islet function, we determined glucose-stimulated insulin release in cytokine-treated islets in the presence and absence of Ang-1 production in islet cells. As shown in Fig. 6, cytokine-treated islets, as opposed to control islets, were associated with significantly reduced insulin secretion in response to glucose challenge. These results were consistent with previous data in the literature showing that proinflammatory cytokines are toxic to islet function (35,37,38). However, this impaired insulin secretion was significantly improved in cytokine-treated islets in the presence of Ang-1 production, although the amplitude of glucose-stimulated insulin release was not reversed to normal. These data indicate that Ang-1 helps preserve islet function in the presence of cytokines.

**Effect of Ang-1 on intra-islet endothelium.** To underpin the beneficial effect of Ang-1 on islet survival and islet engraftment, we investigated the impact of Ang-1 on intra-islet endothelium. Islets were transduced by Ang-1 or control vector at a fixed MOI of 100 pfu/cell, followed by incubation in culture media in the presence and absence of proinflammatory cytokines for 48 h. Islets were subjected to immunohistochemistry using anti-CD31 and anti-insulin antibodies, respectively. As shown in Fig. 7, cytokine treatment resulted in significant damage to intra-islet endothelium, as the microvascular network within cytokine-treated islets was severely truncated when compared with that in mock-treated islets. In contrast, the intra-islet microvasculature was better preserved in islets with elevated Ang-1 production.

To support the above observation, we determined the protein levels of Tie-2, an endothelium-specific marker in islets, in the presence and absence of cytokines. As shown in Fig. 8, cytokine treatment markedly decreased Tie-2 protein levels, correlating with the diminution of the intra-islet vascular network in cytokine-treated islets (Fig.

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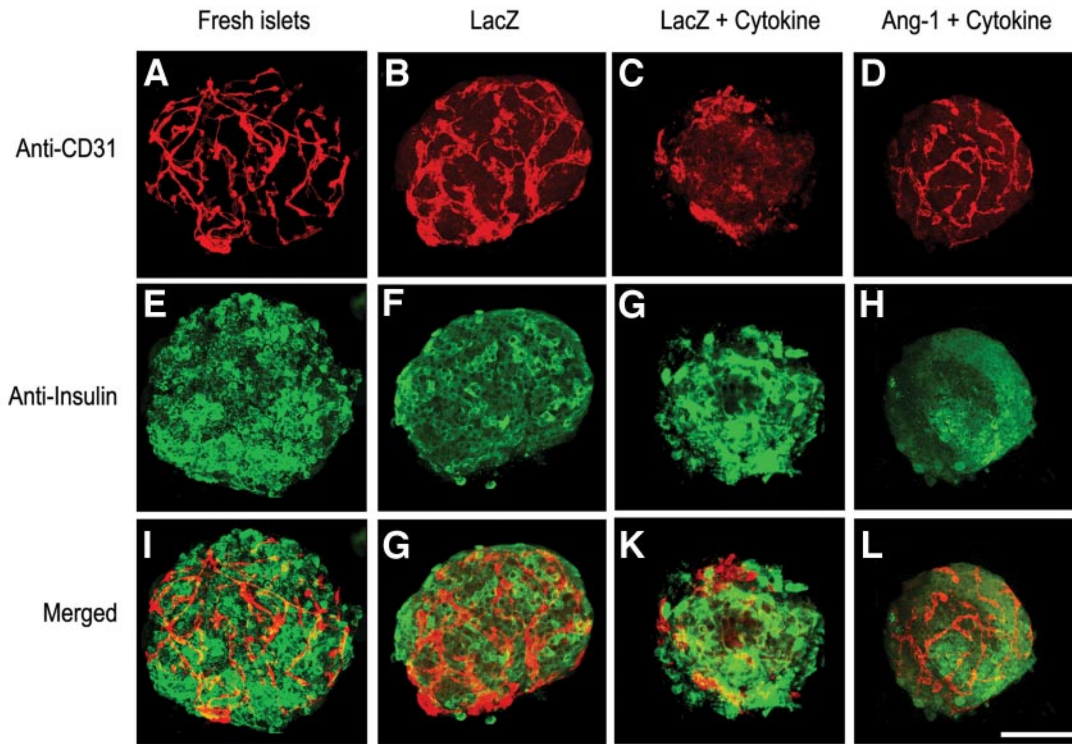


FIG. 7. Immunofluorescent microscopy of cultured islets. Freshly isolated BALB/c islets ($n = 30$) were transduced with 100 pfu/cell of LacZ or Ang-1 vector in culture medium in the absence and presence of cytokines. Forty-eight hours later, islets were subjected to immunocytochemistry using anti-CD31 (*A–D*) and anti-insulin (*E–H*) antibodies. *A*, *E*, and *I*: Freshly isolated islets without preculturing. *I–L*: Merged images. Bar, 50 μm. (Please see http://dx.doi.org/10.2337/db07-0371 for a high-quality digital representation of this figure.)

7). In contrast, Tie-2 expression was preserved at near-normal levels in the presence of elevated Ang-1 production in cytokine-treated islets (Fig. 8). Together, these results suggest that Ang-1 protects intra-islet endothelium from cytokine-induced damage.

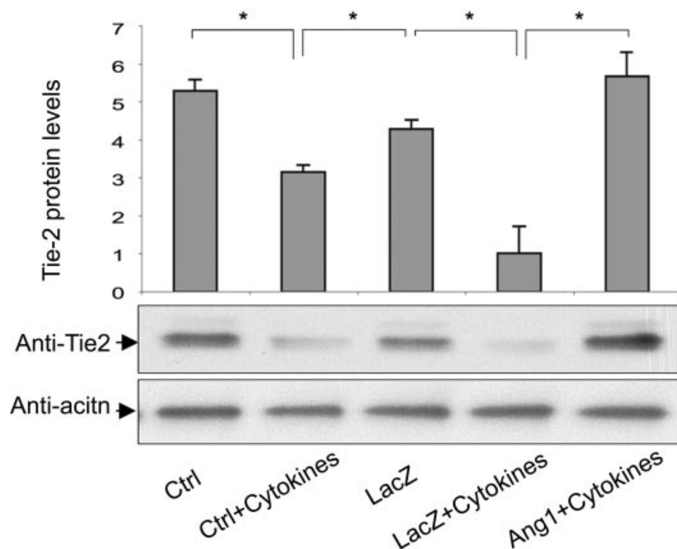


FIG. 8. Tie-2 expression in islets. Aliquots of 300 islets were either mock treated (control [Ctrl]) or transduced with Ang-1 or control vector at an MOI of 100 pfu/cell in 2 ml RPMI-1640 medium at 37°C for 16 h, followed by incubation in the absence and presence of cytokines for 48 h. Islets were homogenized in 100 μl of ice-cold CER-I solution containing 10 μl protease inhibitor cocktail, followed by freezing in a dry ice bath and thawing in a 37°C water bath for three consecutive cycles. Aliquots of 30-μg protein lysates were resolved on 4–20% gradient SDS-PAGE, which were subjected to immunoblot analysis using anti-Tie2 antibody. Data were from three independent experiments. *$P < 0.01$.

## DISCUSSION

In this study, we examined the effect of Ang-1, a proangiogenic and antiapoptotic factor, on islet survival, function, and revascularization posttransplantation in diabetic mice. To determine the net effect of Ang-1 on islet mass in the absence of variable immune rejections, we used a syngeneic islet transplantation model. We show that diabetic mice receiving a marginal islet mass that was pretransduced by Ang-1 vector exhibited significantly improved glycemic control and glucose-stimulated insulin secretion compared with diabetic mice receiving control vector–transduced islets. Furthermore, islet grafts with elevated Ang-1 production displayed a relatively higher content of intragraft capillaries than that displayed by control islet grafts. To account for the beneficial effect of Ang-1 on marginal islet transplantation, we investigated the effect of Ang-1 on the survival and function of cultured islets in the absence and presence of cytokines. We show that cultured islets were associated with markedly increased apoptosis in the presence of cytokines, correlating with severely impaired glucose-stimulated insulin secretion in cytokine-treated islets. This deleterious effect of cytokines on islet viability and function was significantly mitigated, as evidenced by the reversal to near-normal levels of islet viability and function in cytokine-treated islets with elevated Ang-1 production. These results indicate that Ang-1 confers a significant beneficial effect on islet survival and function, contributing to improved glycemic control and better preservation of islet mass in diabetic mice.

Although a number of proangiogenic factors including

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VEGF have been implicated in islet engraftment, a complete picture of islet revascularization remains obscure. A prevailing notion is that islet revascularization depends on angiogenesis emanating from recipient endothelium in local tissue surrounding newly transplanted islets. However, recent elucidation of donor-derived endothelial cells in nascent capillaries of islet grafts changed this view. In addition to recipient endothelium, residual intra-islet endothelial cells are shown to participate in islet revascularization (18–21). One potential mechanism is that these residual intra-islet endothelial cells initiate angiogenesis upon activation by angiogenic signals, contributing to the formation of intragraft capillaries. Unfortunately, a vast majority of islet endothelial cells are either destroyed by collagenase digestion during islet isolation or damaged by ischemia-induced apoptosis after islet transplantation (18,19). Furthermore, prolonged culturing of islets also results in the loss of intra-islet endothelial cells (20). Indeed, Linn et al. (18) showed that only a fraction of endothelial cells building the new microvasculature of engrafted islets are derived from the donor. It is anticipated that factors capable of protecting residual intra-islet endothelial cells from hypoxia-induced damage would enhance islet revascularization and preserve islet mass in transplants.

In support of this hypothesis, we show that Ang-1 prevented cytokine-elicited damage to intra-islet endothelium in cultured islets. These results are consistent with previous data that Ang-1 possesses a potent vascular protective mechanism via selective binding to its endothelium-specific receptor Tie-2, which in turn promotes endothelial cell survival and prevents vascular inflammation and apoptosis (24–26). Consistent with this notion, we show that the levels of endothelium marker Tie-2 protein were maintained at near-normal levels in cytokine-treated islets in the presence of Ang-1 production. Likewise, the expression levels of Bcl-2, a prosurvival factor, in islets were decreased in response to cytokine treatment and were restored to normal in the presence of Ang-1. Interestingly, previous data indicate that Ang-1 does not affect Bcl-2 gene expression in cultured endothelial cells (39–41). This observation together with our data suggests that Ang-1 exerts a beneficial effect on the survival of β-cells, which accounts for >70% of cells in islets. In accordance with this interpretation, we show that Ang-1 protected dispersed islet cells from cytokine-induced apoptosis. This effect correlated with the cytoprotective effect of Ang-1 on the preservation of glucose-stimulated insulin release in cytokine-treated islets.

Indeed, Ang-1 has been shown to promote the survival of nonendothelial cell types such as cardiac and skeletal myocytes via the integrin family of cell adhesion receptors (42). Likewise, Ang-1 suppresses cellular apoptosis of mouse cortical neurons (43). It has been shown that Ang-1 binds directly to integrin subunits $\alpha5\beta1$ via the receptor-binding fibrinogen-like domain of Ang-1 (25,44,45). These results are reminiscent of our observation that Ang-1 protected islets from inflammatory cytokine-induced toxicity, which supports the notion that Ang-1 exerts a beneficial effect on nonendothelial cell types via a Tie-2–independent mechanism (25,42,46). Interestingly, integrin receptors are expressed in islets and play important roles in islet architecture, development, and function (47–50). Further studies are needed to determine whether integrin receptors mediate the antiapoptotic effect of Ang-1 on islet cells.

Recently, Källskog et al. (51) showed that transplanted islets under the kidney capsule are associated with increased lymphangiogenesis. Using Evans blue as a tracer, they visualized the presence of lymphatic capillaries in the graft stroma but not within the endocrine cells of engrafted islets. Like angiogenesis, lymphangiogenesis depends on coordinated actions of angiogenic signals that include VEGF-C and its cognate receptor VEGFR-3, which is specifically expressed in lymphatic endothelium (52). Lymphangiogenesis is normally coupled with angiogenesis and plays important roles in wound healing (52). Using an ear skin model of mice, Cho et al. (53) show that Ang-1 accelerates wound healing via enhanced angiogenesis and lymphangiogenesis. These results underscore the importance of Ang-1 in lymphangiogenesis. It would be of interest to determine the potential effect of Ang-1 on islet graft lymphangiogenesis and to address the physiological significance of lymphangiogenesis in terms of islet graft revascularization, survival, and function.

Nygvist et al. (20) showed that prolonged culturing of islets for 4 days is associated with a significant reduction in islet microvascular density. This finding seemed at variance with our studies, showing that 48 h culturing did not result in significant loss of intra-islet endothelial cells. This discrepancy may derive from different methods and culturing conditions. Nyqvist et al. used Tie-2 green fluorescent protein transgenic islets to visualize intra-islet endothelium in cultured islets, whereas we mounted islets via cytospinning on slides, followed by anti-CD31 immunostaining. Our method provided a gross measurement of intra-islet vessels projected on a single surface, which is different from the cross-sectional visualization of islet microvasculature in the cultured islets in Nyqvist's studies. It is plausible that our method did not allow the detection of marginal losses of intra-islet endothelial cells, as we limited our culturing condition to 48 h. However, we detected significant damage to intra-islet endothelium when islets were exposed to cytokines, an adverse effect that was reversed in response to Ang-1 production in cultured islets.

We acknowledge the limitation in estimating islet mass on the basis of glucose-stimulated insulin release in islet grafts. Although it has been suggested that glucose-stimulated insulin secretion best predicts functional islet mass in transplants (5), this approach is limited, as persistent hyperglycemia can potentially exhaust β-cells for insulin release in islet grafts, leading to underestimation of islet mass. Caution needs to be exercised when glucose-stimulated insulin release is used for inferring functional islet mass in a diabetic recipient because of the potential exhaustive effect of persistent hyperglycemia on β-cells. Indeed, we show that diabetic recipient mice in the control group, as opposed to the Ang-1 group, maintained hyperglycemia along with impaired glucose tolerance posttransplantation during the course of the study.

A second limitation is the lack of determination of intragraft blood flow. Although significantly higher levels of intra-islet vascular density were detected in islet grafts with elevated Ang-1 production and correlated with improved glycemic control in the Ang-1 group, whether Ang-1–mediated induction of islet revascularization is associated with increased blood flow remains to be determined.

Furthermore, our studies were limited by the lack of islet-specific Ang-1 transgenic mice. As a complementary approach, it would be of significance to transplant Ang-1–

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overexpressing islets isolated from Ang-1 transgenic mice to diabetic mice. Studies are needed to address whether transgenic overexpression of Ang-1 in islets is associated with enhanced islet revascularization.

Zhang et al. (54) showed that adenovirus-mediated transduction of islets induces the expression of multiple chemokines and chemokine receptors including monocyte chemoattractant protein-1 and CC chemokine receptor 2. This effect can cause local inflammation and trigger host-innate immune response in islet grafts. It would be of interest to address whether Ang-1 can suppress chemokine expression in cultured islets and mitigate local inflammation in islet grafts in diabetic recipients.

In conclusion, delayed and inadequate islet revascularization has been considered a contributing factor for the loss of islet mass posttransplantation. It has been estimated that <30% of transplanted islets can gain stable engraftment despite the infusion of >10,000 IE/recipient (3,55). This deficiency in islet engraftment appears to ensue in syngeneic and immune-deficient hosts, which is indicative of non–immune-mediated insults to transplanted islets, such as prolonged hypoxia (7,8,12,56) or instant blood-mediated inflammatory reaction (57–59). Furthermore, these adverse effects are compounded by immunosuppressants, which are shown to compromise the viability, function, and revascularization of newly transplanted islets (60–63). In this context, our present study is of significance, as we elucidate that Ang-1 promoted the survival of islets and residual intra-islet endothelium, contributing to improved glycemic control and better preservation of islet mass in diabetic recipient mice. This effect is likely due to a combined action of antiapoptotic and proangiogenic activities of Ang-1. This cytoprotective effect of Ang-1 may be explored as a novel mechanism for protecting newly implanted islets from ischemia reperfusion injury and preserving functional islet mass in transplants.


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