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Genetic Engineering of a Suboptimal Islet Graft with A20 Preserves β Cell Mass and Function[1]

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Transplantation of an excessive number of islets of Langerhans (two to four pancreata per recipient) into patients with type I diabetes is required to restore euglycemia. Hypoxia, nutrient deprivation, local inflammation, and the β cell inflammatory response (up-regulation of NF-κB-dependent genes such as *inos*) result in β cell destruction in the early post-transplantation period. Genetic engineering of islets with anti-inflammatory and antiapoptotic genes may prevent β cell loss and primary nonfunction. We have shown in vitro that A20 inhibits NF-κB activation in islets and protects from cytokine- and death receptor-mediated apoptosis. In vivo, protection of newly transplanted islets would reduce the number of islets required for successful transplantation. Transplantation of 500 B6/AF$_1$ mouse islets into syngeneic, diabetic recipients resulted in a cure rate of 100% within 5 days. Transplantation of 250 islets resulted in a cure rate of only 20%. Transplantation of 250 islets overexpressing A20 resulted in a cure rate of 75% with a mean time to cure of 5.2 days, comparable to that achieved with 500 islets. A20-expressing islets preserve functional β cell mass and are protected from cell death. These data demonstrate that A20 is an ideal cytoprotective gene therapy candidate for islet transplantation. ***The Journal of Immunology*****, 2003, 170: 6250–6256.**

β cell destruction in the early post-transplantation period is the leading cause of primary nonfunction of islet grafts. It is the loss of critical β cell mass during these first few days that determines the long term metabolic control of diabetic recipients. The etiology of early apoptotic and necrotic loss of islet grafts is multifactorial and includes damage incurred during islet isolation and culture procedures, local hypoxia, lack of nutrients, hyperglycemia, and the induction of nonspecific inflammatory reactions (1–4). Islet dysfunction and loss occur even in the most advantageous circumstances with excellent metabolic control (i.e., transplantation into nondiabetic recipients) (1, 5, 6). It is established that activation of inflammatory macrophages, local elaboration of cytokines (i.e., IL-1β, IFN-γ, and TNF-α), and generation of free radicals contribute to the induction of β cell apoptosis (7). Islets of Langerhans contribute to their own destruction by expressing proinflammatory, NF-κB-dependent genes such as *inos*, which leads to increased local production of the β cell toxin NO (7, 8).

Since the early work of Ballinger et al. (9–12) showing that islet transplantation could cure diabetes in rodents, islet transplantation for humans has been regarded as a potential cure for diabetes. The first series of successful islet transplantation in Edmonton, Canada, has established transplantation of islets of Langerhans as a viable therapeutic option for the cure of type I diabetes. This success comes at the cost of using a high number of islets derived from at least two pancreata and intense immunosuppression, precluding the wide-spread application of this approach, particularly for children (13). The deleterious effects of initial loss of β cells are manifest by the large number of islets (two or more pancreata per patient) required to achieve euglycemia in clinical transplantation, which stands in contrast to the observation that only 50% of the endogenous pancreas are required to maintain good metabolic control (13, 14).

Given the important role of β cell destruction in the initial loss of islet cell mass post-transplantation, genetically engineering islets to express cytoprotective genes may, through the creation of a death-defying islet, provide one therapeutic strategy to enable successful islet transplantation. Previous in vivo studies overexpressing the antiapoptotic proteins Bcl-2 and Bcl-x$_L$ in β cells have met with mixed success. Adenovirus-mediated expression of Bcl-2 in macaque islets decreased the islet cell mass required to achieve euglycemia in diabetic SCID mice (15). However, transgenic NOD mice expressing Bcl-2 in their β cells were not protected from inflammatory and streptozotocin-mediated destruction in vivo (16). In addition, transgenic expression of Bcl-x$_L$ in β cells of nonautoimmune mice impaired mitochondrial signaling for insulin secretion and induced diabetes (17). These data indicate that genetic engineering of islets with any given antiapoptotic protein may either not completely protect from β cell destruction or even interfere with the very function of β cells. An optimal gene therapy candidate should have antiapoptotic, anti-inflammatory properties and a wide, safe therapeutic window and should not interfere with the normal physiological functioning of the β cell.

The cytoprotective gene A20 could be an ideal gene therapy candidate. A20, a zinc finger protein, was originally identified as a TNF-inducible gene in HUVECs (18). A20 is expressed in a variety of cell types, including lymphocytes, smooth muscle cells, and hepatocytes, in response to a number of inflammatory stimuli

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(8, 19–24). The expression of A20 confers resistance to TNF-mediated apoptosis and necrosis and down-regulates the inflammatory response via inhibition of NF-κB at a level upstream of IκBα degradation (8, 19, 22, 23, 25–27). The potent antiapoptotic and anti-inflammatory function of A20 was dramatically confirmed in A20 $^{-}/^{-}$ knockout mice, which are born cachectic and die within 3 wk of birth as a result of unfettered liver inflammation (28).

We have demonstrated that A20 is a natural part of the physiological stress response of islets (8). In islets A20 exhibits a dual anti-inflammatory and antiapoptotic function. A20 protects islets from cytokine-mediated apoptosis and blocks activation of the transcription factor NF-κB (8). We reasoned that if A20 could protect in vivo, it would decrease the loss of islet cell mass and function in the early post-transplantation period, reducing the number of islets required to achieve euglycemia. To address this question, a suboptimal syngeneic islet graft was genetically modified to express A20 and transplanted into diabetic B6/AF$_1$ mice. Overexpression of A20 in these suboptimal islet grafts protected them from cell death and preserved the functional β cell mass, resulting in a cure rate comparable to that achieved with double that number of islets.

Materials and Methods

Mice

B6/AF$_1$ male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Procedures were performed according to the recommendations of the institutional animal care and use committee.

Ex vivo gene transfer into mice islets

The recombinant adenovirus vector (rAd.)[3] expressing human A20 (rAd.A20) and the control β-galactosidase (rAd.β-gal) were gifts from Dr. V. Dixit (Genentech, South San Francisco, CA) and Dr. R. Gerard (University of Texas South Western, TX) (27, 29). Mouse islets were isolated with Liberase (Roche, Indianapolis, IN) as previously described (8). Following isolation, islets were counted, washed in RPMI 1640, and plated into 24 wells at a density of 1000 islets/ml in glucose-, serum-, and antibiotic-free RPMI 1640. Mouse islets were then incubated for 1 h with the appropriate rAd. at a multiplicity of infection (MOI) of 5:1 at 37°C in 5% CO_2. One hour of incubation with rAd. and the absence of serum were required for optimal infection. However, the glucose concentration (0–20 mM) did not affect the efficacy of gene transfer. The islets were washed in RPMI 1640, loaded into sterilized polyethylene tubing (internal diameter, 0.58 mm; BD Biosciences, Sparks, MD), and kept on ice until transplantation.

Islet transplantation

To induce diabetes, B6/AF$_1$ mice were injected with 160 mg/dl streptozotocin (Sigma-Aldrich, St. Louis, MO) in 10 mM citrate buffer, pH 4.2, and blood glucose levels were determined using a Glucometer Dex (Bayer Corp., Elkhart, IN). Mice with a blood glucose value ≥350 mg/dl and a body weight of 20–25 g were selected as transplant recipients. Mice were anesthetized with 2.5% avertine (200 μl/100 g; Aldrich, Milwaukee WI), and the kidney was accessed by a left flank incision and brought into the wound by gentle blunt dissection. A small nick was made in the kidney capsule with the bevel of a 20-gauge needle over the inferior renal pole. The sterile polyethylene tubing containing the islets was then connected to a 0.5-ml Hamilton syringe (Fisher Scientific, Pittsburgh, PA) and advanced under the capsule through the nick toward the superior pole of the kidney where the islets were deposited. The wound was closed in two layers with absorbable sutures. Blood glucose levels were analyzed on postoperative days (POD) 2, 5, 7, 10, 15, and 20. Nephrectomy was performed on POD 5 and 20.

Immunohistochemical analysis of islet grafts

The position of the graft area on the kidney was marked with India ink (Eberhard Faber, Lewisburg, TN), and the kidney was fixed in 60% methanol/10% acetic acid with 30% chloroform for 24 h. The tissue was processed and embedded in paraffin using standard protocols. The entire graft area was serially cut, and contiguous regions (spanning ~75 μM) were examined for the expression of insulin (1/10 dilution; DAKO, Carpinteria, CA), active caspase 3 (1/50 dilution; Cell Signaling, Beverly, MA), and general morphology by H&E. A20 expression was revealed by the polyclonal rabbit antiserum (A20-NT) used at a dilution of 1/800, this Ab recognizes an N-terminus peptide sequence of human A20 (IRERTPED IFKPTN) (8). The expression of the A20 protein in rAd.A20-infected islets was confirmed by Western blotting analysis using standard techniques. Immunohistochemical labeling was detected by biotinylated secondary Ab (Vector Laboratories, Burlingame, CA) enhanced with ABComplex/HRP (DAKO) and was revealed by diaminobenzidene (Vector Laboratories). To determine the ratio of insulin- and active caspase 3-positive cells, two representative areas (A and B) from each graft were chosen, and contiguous sections (within 75 μM) were analyzed. Each area was divided into three regions, and the total numbers of insulin- and active caspase 3-positive cells were counted (×600 magnification).

Statistics

All statistical analysis was performed using Student's *t* test on Instat (version 2.01) software (GraphPad, San Diego, CA).

Results

Overexpression of A20 in islets is not toxic

We first evaluated whether forced expression of A20 would be toxic to islets and interfere with their metabolic function. An optimal number of freshly isolated islets ($n = 500$) were infected ex vivo with the rAd.A20 at an MOI of 5:1 and transplanted into diabetic B6/AF$_1$ mice (blood glucose, >350 mg/dl). The MOI of 5:1 was chosen based upon our in vitro data demonstrating that this MOI of rAd.A20 was sufficient to inhibit NF-κB activation and protect from apoptosis (8). Noninfected (NI) islets or islets infected with rAd.β-gal were used as controls. Blood glucose levels were monitored daily after transplantation. Mice transplanted with 500 rAd.A20-infected islets were euglycemic by POD 5. The mean blood glucose (±SEM) fell significantly from 442 ± 40 to 124 ± 12 mg/dl ($n = 5$; $p < 0.01$). These results were not significantly different ($p > 0.1$) from those obtained in mice receiving NI islets or islets infected with rAd.β-gal. The mean blood glucose of these mice fell from 500 ± 5 and 349 ± 13 mg/dl to 135 ± 16 and 108 ± 8 mg/dl, respectively ($n = 5$ and 3) by POD 5 (Fig. 1*A*). Mice nephrectomized on POD 5 became hyperglycemic (blood glucose, >350 mg/dl; data not shown) within 24 h, demonstrating that euglycemia was dependent upon the engrafted islets. The expression of A20 in islet grafts was confirmed on POD 5 by immunohistochemistry (refer to Fig. 3*C*) and Western blot analysis (Fig. 1*B*). These data demonstrate that A20-expressing islets restore euglycemia as efficiently as control islets; hence, A20 is not toxic to islets and does not interfere with their normal metabolic function at this MOI.

Establishing a suboptimal islet transplant mass

We aimed to determine the in vivo cytoprotective effect of A20 in islets, by addressing whether A20 would enhance the survival and function of a suboptimal islet mass transplanted into syngeneic diabetic mice (30, 31). We defined the suboptimal islet mass as the number of transplanted islets that would have a <25% success rate in restoring euglycemia within 2–5 days. Transplantation of 500 islets resulted in a cure rate of 100%, with a mean time to cure of 5 days (Figs. 1*A* and 2). Transplantation of 250 islets resulted in a cure rate of 20%. The mean blood glucose on POD5 (430 ± 60; $n = 10$) was not significantly changed ($p = 0.188$) from pretransplant levels (Fig. 2). To confirm that the transplantation of 250 islets constituted a suboptimal islet mass, diabetic mice were transplanted with 150 islets, which resulted in no cure (e.g., blood glucose >300 mg/dl; $n = 4$; Fig. 2). These experiments establish that the transplantation of 250 islets constitutes a suboptimal, marginal

[3] Abbreviations used in this paper: rAd., recombinant adenovirus vector; β-gal, β-galactosidase; MOI, multiplicity of infection; NI, noninfected; POD, postoperative day.

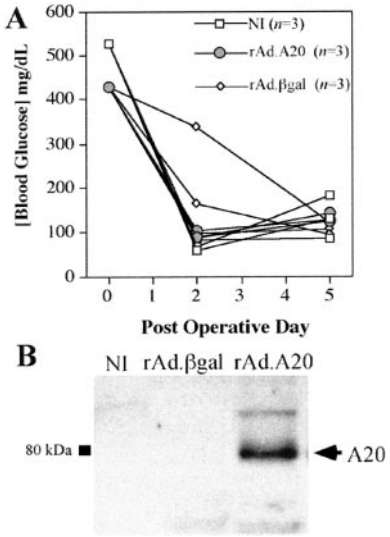

FIGURE 1. *A*, Adenovirus-mediated expression of A20 in islets is not toxic and does not perturb the metabolic function of β cells. Streptozotocin-induced diabetic (blood glucose, >300 mg/dl) $B6AF_1$ mice were transplanted with 500 NI islets or islets infected with rAd.A20 or rAd.β-gal at an MOI of 5:1. Euglycemia (blood glucose, <200 mg/dl) was achieved in all three groups by POD 5. The blood glucose values from three mice per group are shown. *B*, Expression of A20 in islets was confirmed by Western blot analysis showing high expression of 80-kDa A20 protein.

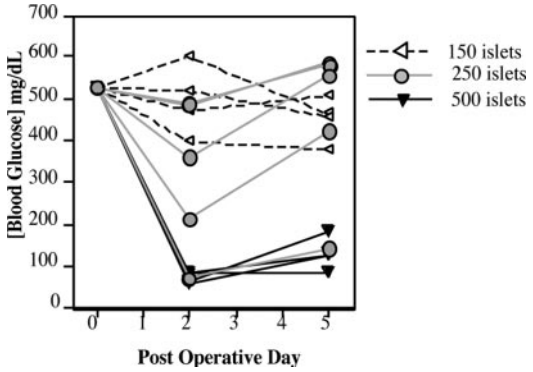

FIGURE 2. Transplantation of 250 islets is a suboptimal islet mass that is unable to restore euglycemia. Diabetic mice were transplanted with 500 (n = 5), 250 (n = 10), or 150 (n = 4) freshly isolated islets, and blood glucose was monitored daily. All mice transplanted with 500 islets were euglycemic by POD 5 (blood glucose, <200 mg/dl). Twenty percent of mice transplanted with 250 islets were euglycemic by POD 5, but none of the mice transplanted with 150 islets had achieved euglycemia. The blood glucose levels from four or five mice per group are shown.

islet mass, which is unable to restore metabolic control in diabetic mice.

A20 preserves islet cell mass and function in the early post-transplantation period

To test whether A20 could protect islets from destruction in this early post-transplantation period, 250 rAd.A20-infected islets were transplanted into diabetic mice, and blood glucose levels were monitored for a 20-day period. Mice transplanted with 250 NI islets or islets infected with rAd.β-gal were used as controls. The cure rate for mice transplanted with 250 NI or rAd.β-gal-infected islets was approximately 20%, with 2 of 10 and 4 of 17 cures in each group, respectively (Fig. 3*A*). Blood glucose levels in these groups remained significantly high (≥300 mg/dl; $p < 0.001$) for the entire observation period, except on POD2 when the mean blood glucose of animals receiving NI islets made a transient downward trend, perhaps as a consequence of insulin release from dying β cells (Fig. 3*B*). In marked contrast the cure rate for the A20 group was 75% (n = 25), with a mean time to cure of 5.2 ± 1.1 days (Fig. 3*A*). The mean blood glucose levels for the cured mice receiving 250 rAd.A20-infected islets was not significantly different from that observed in mice receiving 500 NI islets on both POD 5 and POD 20 (POD 5: 196 ± 25 mg/dl (n = 19) vs 151 ± 32 (n = 5), p = 0.23; POD 20: 149 ± 8 vs 152 ± 11 mg/dl, p = 0.84; Fig. 3*B*). Mice that were not euthanized at 20 days for pathology studies were followed up and remained euglycemic (>40 days post-transplant). We confirmed that euglycemia was entirely dependent upon the islet graft. Mice nephrectomized on POD 20 (n = 4) became diabetic within 24 h (blood glucose, ≥350 mg/dl; data not shown). Moreover, diabetic mice not receiving an islet graft remained hyperglycemic (blood glucose, ≥350 mg/dl; n = 10; data not shown) for >30 days, demonstrating that euglycemia did not simply relate to the regeneration/recovery of endogenous islets.

The expression of A20 in islet grafts was confirmed on POD 5 and POD 20 by immunohistochemistry (Fig. 3*C*). Strong A20 labeling was detected in the cytoplasm of 20–30% of cells in the insulin-positive region of the graft (Fig. 3*C*). We believe this percentage is an underestimate of gene transduction, since islets transduced with rAd.β-gal at comparable MOI showed 70–80% of cells expressing the transgene. This discrepancy is probably related to the sensitivity of the A20 Ab. A20-transduced islets exhibited normal morphology and stained strongly for insulin, with little evidence of infiltrating mononuclear cells, demonstrating the absence of an inflammatory reaction against the rAd.-infected cells at these time points (Fig. 3*C*).

A20 preserves β cell morphology, maintains insulin expression, and protects from apoptosis

We next conducted a systematic immunohistochemical analysis of the islet grafts on POD 5, the day by which most control grafts had failed and most A20 expressing grafts had cured. For these studies, rAd.A20- and rAd.β-gal-infected islet grafts from (n = 3/group) were analyzed at two transverse areas (A and B) across three regions (Fig. 4*M*) for graft morphology, insulin labeling, and apoptosis.

Morphologically, failed rAd.β-gal grafts exhibited an absence of islet structures, with extensive regions of hyalinization (Fig. 4*A*) and disrupted and often small, but still recognizable, islet structures (Fig. 4, *C* and *E*). The morphology of these grafts is consistent with the results of previous studies demonstrating widespread apoptotic and necrotic destruction of islet grafts in the early post-transplant period (1, 6). In contrast, cured rAd.A20 grafts showed highly preserved, well-rounded islet structures (Fig. 4, *G*, *I*, and *K*). Insulin staining in rAd.β-gal islet grafts reflected the damage indicated by H&E staining and was either absent or showed weakly stained cells within fused islet structures (Fig. 4, *B*, *D*, and *F*). The presence of fused islets has been recognized as a pathological feature of islet graft damage in the early post-transplantation period (1, 6). The morphology of the failed A20-expressing grafts (~25%) was similar to that of failed rAd.β-gal islet grafts, i.e., decreased insulin reactivity with poor morphology (data not shown). In contrast, cured rAd.A20 islet grafts showed intense insulin labeling defining preserved single islet structures (Fig. 4, *H*, *J*, and *L*). Quantitative analysis of insulin-positive cells in the three regions per area (regions 1, 2, and 3) of all grafts confirmed the

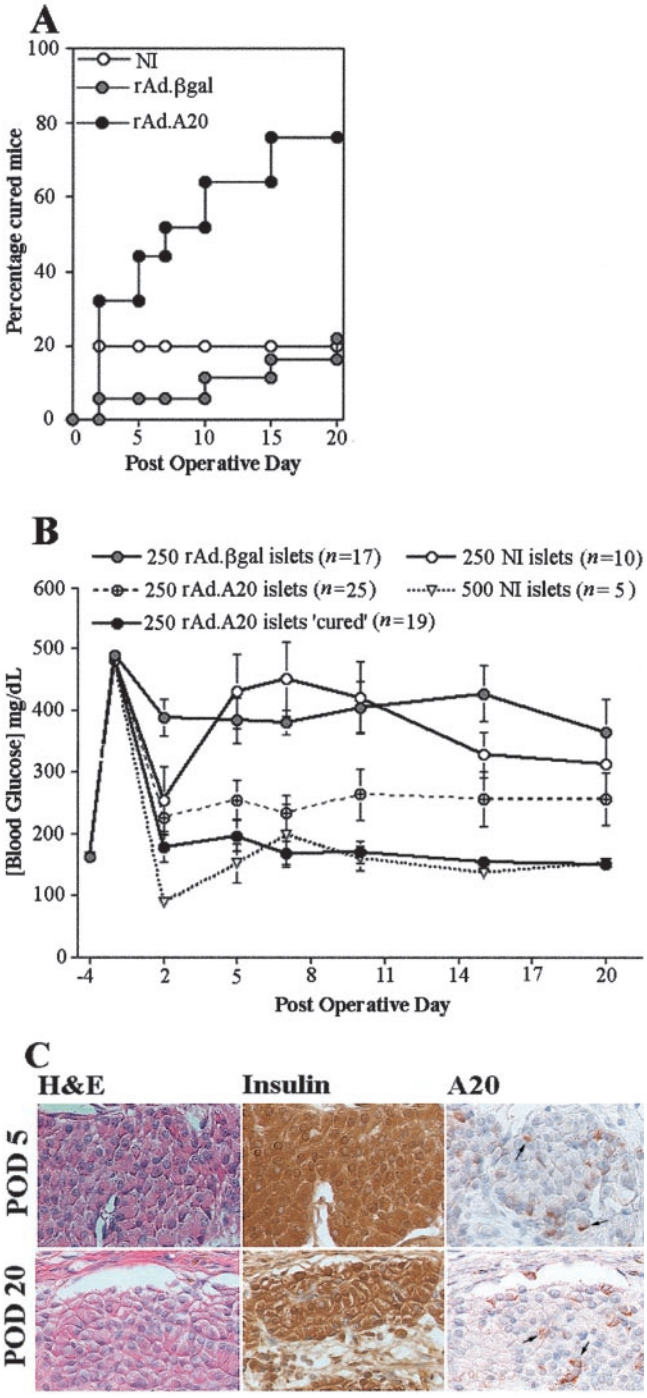

FIGURE 3. Overexpression of A20 in islets preserves islet cell mass and function. *A*, Diabetic $B6AF_1$ mice were transplanted with 250 NI islets ($n = 10$) or islets infected with rAd.A20 ($n = 25$) or rAd.β-gal ($n = 17$) at a MOI of 5:1. Blood glucose was monitored on days 2, 5, 7, 10, 15, and 20 following transplantation. The data represent the cumulative percentage of cured mice (defined as blood glucose levels <200 mg/dl on two consecutive readings). An ~20% cure rate was achieved in mice transplanted with NI or rAd.β-gal infected islets. In contrast, the cure rate reached 75% in mice transplanted with rAd.A20 infected islets. *B*, Mean blood glucose values of mice transplanted with NI, rAd.β-gal-infected, and rAd.A20-infected islets. Blood glucose values of cured mice receiving 250 rAd.A20 infected islets (19 of 25 mice) were similar to those of mice transplanted with 500 NI islets and were <200 mg/dl. *C*, Expression of A20 in islets on POD 5 and 20. Islet grafts infected with rAd.A20 exhibited normal morphology, architecture, and dense insulin labeling, as demonstrated by H&E and insulin staining. Distinct cytoplasmic expression of A20 (arrows) was demonstrated up to 20 days post-transplantation. Magnification, ×400.

marked loss of insulin-positive cells within the engrafted rAd.β-gal islets compared with rAd. A20 islet grafts (Fig. 4*M*). A20-expressing grafts exhibited a significant ($p < 0.0005$) ~4-fold increase in the mean number of insulin-positive cells across the three regions (mean ± SEM number of insulin-positive cells across the three regions, 262.44 ± 19.3 for rAd.A20-infected islets vs 58.88 ± 10.6 for rAd.β-gal-infected islets). Large areas containing erythrocytes, erythrocyte lakes, with a rim of endocrine tissue distributed around the lakes were seen in all grafts studied and did not correlate with graft function (Fig. 4, *E* and *F*). These areas may correlate with graft remodeling and vascularization and have been previously described (6). The normal morphology of the engrafted A20-expressing islets is indicative of the potent cytoprotective effect of A20.

To determine whether the increased β cell mass in the A20-expressing grafts related to protection from apoptosis, we analyzed the rate of apoptosis in the A20- and β-gal-expressing grafts on POD 5. Apoptosis was evaluated by morphology (presence of apoptotic bodies) and by immunohistochemistry through labeling for active caspase 3 expression. Caspase 3 is a downstream integrator of multiple apoptotic pathways and is involved in the final effector phase of apoptotic cell disintegration, making it a robust marker for apoptosis (32).

Our data demonstrate that apoptosis, as indicated by active caspase 3 expression, is present in all grafts analyzed, but with a marked increase in β-gal vs A20 islet grafts. (Fig. 4*N*). The mean percentage of caspase-positive to insulin-positive cells counted in areas A and B in the A20-expressing grafts was 8.4 ± 2.8% (mean ± SEM)/graft. This percentage was increased by ~6-fold in β-gal islet grafts, where it reached 51.8 ± 44.5%/graft. The mean reflects the sum of the numbers from all three grafts analyzed per group.

Discussion

Our goal in this study was to determine whether genetic engineering of islets with A20 will protect them from destruction in the early post-transplant period. We reason that the requirement for large numbers of islets in human transplants using the Edmonton protocol (two to four pancreata per recipient) reflects the high percentage of islet loss in the early engraftment period. Studies using animal models suggest that up to 60% of the transplanted islet mass may be destroyed in this period (6). Islet apoptosis and necrosis induced by hypoxia, lack of nutrients, and local inflammatory reactions in vivo may be critical to the loss of islet graft function and mass in the early post-transplant period. If one could prevent both the apoptotic and necrotic losses of islets, it would be possible to transplant fewer islets and still achieve a successful outcome. This would have the dual benefit of reducing the need for large numbers of islets per recipient and alleviate the chronic shortage of islets available for human transplantation.

A potential gene therapy candidate for islet transplantation should be cytoprotective, have a wide, safe therapeutic window, and not interfere with the normal physiological functioning of the β cell. We had previously shown that A20 is inducible in islets and has dual antiapoptotic and anti-inflammatory functions (8). However, we had not tested whether A20 overexpression would interfere with islet function in vivo. The first part of this study was devoted to this quest. Our data demonstrated that an optimal mass of A20-expressing islets (500 islets/mouse) restores euglycemia as efficiently as unmodified islets. These data demonstrate that rAd.-mediated expression of A20 in islets does not interfere with the physiological function of the β cell, i.e., insulin secretion. This is the first demonstration that A20 completely fulfills the requirements for a gene therapy candidate in islet transplantation.

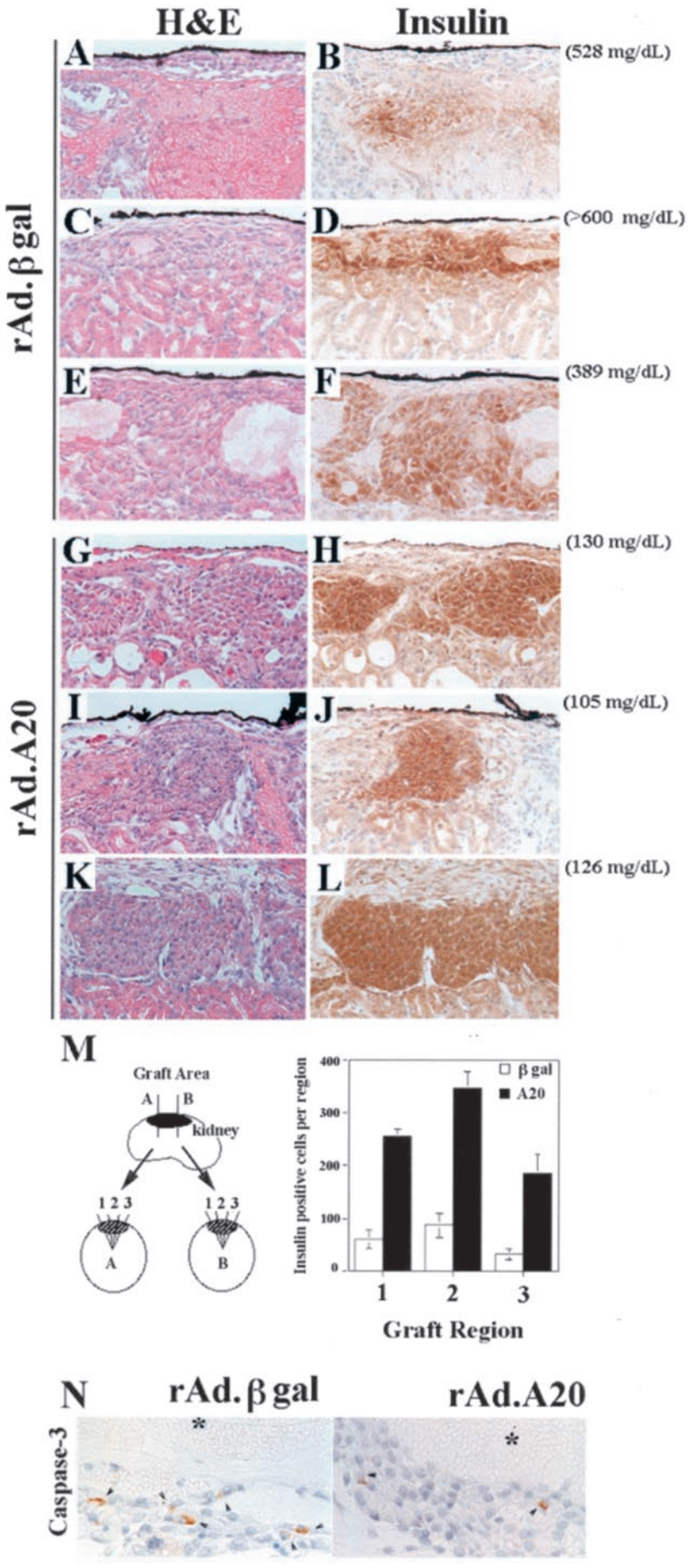

FIGURE 4. Overexpression of A20 preserves islet morphology, maintains insulin secretion, and protects cells from apoptosis. Diabetic $B6AF_1$ mice were transplanted with 250 NI islets or islets infected with rAd.A20 or rAd.β-gal at a MOI of 5:1 ($n = 3$/group). Islet grafts were explanted on POD 5, and graft morphology and insulin expression were analyzed by immunohistochemistry. Blood glucose levels at the time of harvest are indicated in brackets. Islet morphology was disrupted in all three rAd.β-gal infected grafts (*A*, *C*, and *E*) with evidence of erythrocyte lakes (*E*), whereas well-preserved and rounded islets were noted in the rAd.A20-infected islets (*G*, *I*, and *K*), as evidenced by H&E staining (magnification, $\times$400). Insulin labeling was absent to weak, delineating thin, disrupted, and fused islets in the rAd.β-gal-infected grafts (*B*, *D*, and *F*). In the rAd.A20-infected grafts, insulin staining was dense, delineating individual, large, well-rounded islets (*H*, *J*, and *L*). To quantify the number of insulin-positive cells, grafts were analyzed in two representative areas (A and B). Subsequently, the insulin-positive cells were counted across the three regions (magnification, $\times$600) within areas A and B. The data (*M*) are the mean number of insulin-positive cells per area for the three mice studied per group. A significantly higher number ($p < 0.0005$) of insulin-positive cells per region was detected in rAd.A20-infected islets compared with rAd.β-gal-infected islets (*M*). Caspase 3-positive staining (*N*) in rAd.β-gal- and rAd.A20-infected islets on POD 5 is shown. Arrows indicate caspase 3-positive cells. The asterisk indicates an erythrocyte lake. Data shown are representative of three grafts per group.

To determine whether A20 would protect islets in vivo, we studied a transplant model using a suboptimal islet mass (30, 31). We determined the number of islets that would result in a <25% cure rate when transplanted into diabetic recipients. In our hands, transplantation of 250 islets was insufficient to consistently restore euglycemia (20% cure rate), whereas double that number resulted in 100% success. These results are remarkably similar to clinical islet transplantation data. Successful islet transplantation in diabetic patients requires islets from at least two pancreata (the equivalent of ~400–500 islets in mice), whereas islets from a single pancreas (the equivalent of ~200–250 islets in mice) are usually unable to restore euglycemia (8). These data highlight the substantial apoptotic and necrotic loss of β cell mass in the early post-transplant period (1, 6, 30). Genetic engineering of islets with A20 dramatically improved the fate of the newly transplanted islets. This sole modification reversed the 80% fail rate observed in the control groups to a 75% success rate. The rapid normalization of blood glucose levels in mice receiving 250 A20-expressing islets was comparable to that seen in animals receiving twice that number of islets. The excellent metabolic function of the A20-expressing islets was reflected by their morphology. A20-expressing islets exhibited normal architecture, strong labeling for insulin, and the absence of features associated with islet dysfunction (e.g., necrotic cores, islet fusion, and poor insulin labeling). These data demonstrate that A20 was sufficient to protect the islet grafts from destruction in this early post-transplantation period.

Multiple processes are involved in the induction of islet destruction during the early post-transplant period (1–7). We have examined the protective effects of A20 against some of these offenders in vitro. We have shown that A20 protects islets from apoptosis induced by cytokines, ligation of the Fas death receptor, or direct overexpression of Fas-associated death domain (8). We have also confirmed this antiapoptotic effect in endothelial cells. Overexpression of A20 in endothelial cells protects from both TNF- and Fas-mediated apoptosis by inhibiting the activation of caspases 8, 3, 6, and 9 (33). Cytokines can also induce β cell apoptosis via generation of NO (7, 34). NO promotes cytochrome *c* release from the mitochondrion, activating procaspase 9 and the apoptotic program (35). In islets, NO generation is controlled by de novo gene transcription of the inducible NO synthase by the transcription factor NF-κB (36). We have previously shown that A20 inhibits cytokine-mediated activation of the transcription factor NF-κB and suppresses the induction of inducible NO synthase and NO generation (8).

We have not as yet evaluated in vitro whether A20 will protect islets from apoptosis induced by hyperglycemia, hypoxia, nutrient deprivation, β cell toxins (streptozotocin and alloxan), or necrotic cell death. Work addressing the cytoprotective function of A20 against these noxious stimuli is ongoing. Interestingly, it has been reported that hyperglycemia can induce islet apoptosis in vitro via activation of the Fas death receptor; to the extent that this might occur in vivo, we would expect A20 to prove protective for the reasons discussed above (37). Moreover, supportive evidence for a protective effect of A20 against necrotic forms of cell death has been demonstrated in other studies (25). While we have not directly evaluated the protective effect of A20 against these other destructive pathways, our in vivo data strongly support a broad cytoprotective function of A20 in islets. We base this on the preservation of normal islet morphology and function as well as the lower apoptotic rate observed in the A20-expressing islet grafts.

Aside from directly inducing β cell destruction, the metabolic milieu of the newly transplanted islets has a profound effect on their function and the long term recovery of stable metabolic control (2, 30, 38). The reduced numbers of intact islets with decreased intensity of insulin labeling in the β-gal-expressing islet grafts underscores this metabolic derangement. These parameters were all manifestly improved in A20-expressing islet grafts demonstrating that expression of A20 sustains normal metabolic islet function in the face of a deleterious hyperglycemic and proinflammatory environment.

Our combined data from this present and previous work (8) demonstrate that A20 has a potent cytoprotective effect when overexpressed in primary islets. However, analysis of A20 expression in rAd.A20-transduced islets demonstrated that the percentage of transduced cells ranged from only 20–30% of the islet cells. This may relate to the sensitivity of our detection system (e.g., our Ab only recognizes cells expressing very high levels of A20, thus underestimating the percentage of transduced cells), since islets transduced with rAd.β-gal at comparable MOI showed 70–80% of cells expressing the transgene.

In conclusion, our data demonstrate that a single gene therapy-based approach, using A20 overcomes the initial barriers preventing successful islet transplantation. Expression of A20 in islets reduced by half the number of islets required for achieving euglycemia, rendering a suboptimal islet mass (20% cure rate) sufficient (75% cure rate). We propose that the protective effect of A20 can be related to inhibition of islet dysfunction and destruction at multiple levels. This includes protection from cytokine-mediated damage, inhibition of death receptor-dependent activation of caspase cascades, and blockade of NF-κB activation.

We propose that genetic engineering of islets with A20 will relieve the severe shortage of islet for clinical transplantation and enhance their function in the early post-transplantation period. To this end, transient expression of A20 is sufficient and preferable, given the concerns and risks for increased carcinogenesis associated with the expression of antiapoptotic proteins (39). The use of recombinant adenoviruses fulfills this criterion by achieving high, transient expression of the transgene (40). We recognize that issues related to toxicity and development of an immune response against currently used adenoviruses still hampers their widespread use in gene-based therapy (41–43). However, we anticipate that use of rAd.A20 at low MOI and ex vivo, as suggested in this study, is safe with the knowledge that adenoviruses were routinely used as vaccines in >5 million individuals (44). Additionally, whether the expression of A20 will modulate the antiadenoviral response is not known; it may positively impact upon transgene expression in vivo. Experiments are planned to address this question. In any case, the generation of novel safer and tissue-specific viral vectors for gene transfer and the development of nonviral means of protein delivery to cells will facilitate clinical translation of A20-based therapies (45–48).

Whether A20 will also protect islets from T cell-mediated immune destruction (allograft rejection and recurrence of autoimmunity) will probably require longer expression of the transgene. We are developing a recombinant adeno-associated virus expressing A20 that should allow integration of A20 in the genome; hence, long term expression.

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