Adeno-Associated Virus-Mediated IL-10 Gene Therapy Inhibits Diabetes Recurrence in Syngeneic Islet Cell Transplantation of NOD Mice

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Islet transplantation represents a potential cure for type 1 diabetes, yet persistent autoimmune and allogeneic immunities currently limit its clinical efficacy. For alleviating the autoimmune destruction of transplanted islets, newly diagnosed NOD mice were provided a single intramuscular injection of recombinant adeno-associated viral vector encoding murine IL-10 (rAAV–IL-10) 4 weeks before renal capsule delivery of 650 syngeneic islets. A dose-dependent protection of islet grafts was observed. Sixty percent (3 of 5) of NOD mice that received a transduction of a high-dose (4 × 10^9 infectious units) rAAV–IL-10 remained normoglycemic for at least 117 days, whereas diabetes recurred within 17 days in mice that received a low-dose rAAV–IL-10 (4 × 10^8 infectious units; 5 of 5) as well as in all of the control mice (5 of 5 untreated and 4 of 4 rAAV-green fluorescent protein–transduced). Serum IL-10 levels positively correlated with prolonged graft survival and were negatively associated with the intensity of autoimmune immunity. The mechanism of rAAV–IL-10 protection involved a reduction of lymphocytic infiltration as well as induction of antioxidant enzymes manganese superoxide dismutase and heme oxygenase 1 in islet grafts. These studies support the utility of immunoregulatory cytokine gene therapy delivered by rAAV for preventing autoimmune disease recurrence in transplant-based therapies for type 1 diabetes. Diabetes 52:708–716, 2003

A lthough the cause of type 1 diabetes is complex and remains largely elusive to our appreciation (1), recent scientific endeavors have suggested that an immunological imbalance of CD4+ T helper (Th) cell responses, with tilting toward the cytotoxic Th1 type, results in elimination of insulin-secreting pancreatic β-cells (2). Hence, a fine tuning of the immune system toward Th2-like immunity may represent an attractive and reasonable therapeutic strategy for type 1 diabetes. On the basis of this notion of Th responses, a number of immunomodulatory approaches have been explored during the past decade, studies that display convincing potential for improving the curative outcome of islet transplantation as well as for prevention of type 1 diabetes (3). Among these immunomodulatory agents, the Th2-like cytokine IL-10 has been one of the most extensively investigated and promising candidates for effective immune diversion for diabetes treatment (4).

The anti-inflammatory properties of IL-10 include inhibition of proinflammatory cytokine (e.g., IL-2, IL-12, γ-interferon [IFN-γ]) production from macrophages and lymphocytes and promotion of the IgG1 antibody response (5). By a variety of methods, the production or action of IL-10 has been suggested to be deficient in both human patients and experimental animals of type 1 diabetes (6,7). For example, IL-10–deficient NOD mice demonstrate accelerated diabetes (8). Conversely, treatment of NOD mice with recombinant IL-10 prevents the development of diabetes (9). Hence, depending on the time and site of administration, IL-10 can exert distinct effects on diabetes, a phenomenon that has been traditionally dubbed “paradoxical” (10). Taken collectively, the systemic administration of recombinant IL-10 (subcutaneously, intramuscularly, orally), IL-10 expressed from recombinant adeno-associated virus (rAAV) (11), or plasmid (12) all seem to protect against insulitis and islet destruction. In contrast, the local pancreatic production of IL-10 can either precipitate diabetes, as observed in transgenic animals (13,14), or fail to prevent the recurrence of diabetes in mice that receive islet cell transplants (15,16).

Pancreatic transplantation represents an effective therapy, given successful immunosuppression, and when successful, a potential permanent cure for diabetes. However,
limited by the availability and quality of donor organs, many patients are deprived the opportunity of benefiting from this procedure. Islet transplantation offers an appealing alternative as it can be performed with high-quality islets selected and pooled from multiple donors. However, the success rate and long-term survival of islet grafts in human subjects has historically been very low (i.e., <8%) because of autoimmune/allogeneic immune rejection and cytotoxic effects of general immunosuppressants (17). T cells have been shown to be correlated with the recurrence of diabetes after islet transplantation (18,19). Studies involving the syngeneic transplant of islet cells placed under the renal capsule of diabetic NOD mice have provided a suitable model for evaluating the therapeutic value of candidate agents for preventing autoimmune rejection of grafts.

rAAV vectors have become increasingly recognized as a safe and efficient vehicle for gene therapy with multiple advantages over other therapeutic gene delivery systems, owing to their wide range of infection, low levels of inducing immune responsiveness, and potential for long-term gene expression (20). We and others have demonstrated the stable and persistent production of secreted proteins through rAAV-mediated gene delivery into skeletal muscle (21). In our previous study involving long-term production of secreted compounds and type 1 diabetes, systemic IL-10 production achieved by this means completely abrogated diabetes and substantially reduced pancreatic insulitis in NOD mice (11). We now report that immune modulation conferred by rAAV IL-10 gene therapy effectively and dramatically improved transplant survival and delayed recurrence of diabetes after syngeneic islet transplantation in NOD mice. Observed changes afforded by this therapy that may have contributed in part to this protection include a reduction in T-cells within graft inammation, as well as the induction of superoxide dismutase (SOD) and heme oxygenase 1 (HO-1) in islet β-cells.

RESEARCH DESIGN AND METHODS

Mice. NOD mice purchased from Taconic Farms (Germantown, NY) were housed in specific pathogen-free facilities at the University of Miami. All animal manipulations were approved by the Institution Animal Care and Use Committees at both the University of Florida and the University of Miami. Female NOD mice were obtained at 8–10 weeks of age and monitored for glycosuria until they became diabetic, as defined by two consecutive nonfasting blood glucose levels >250 mg/dl. These diabetic animals were kept on a subcutaneous insulin pellet for glucose homeostasis until islet transplantation. Donor islets utilized for transplantation studies were isolated from 4- to 6-week-old NOD male mice.

Plasmid construction and rAAV vector generation and administration. Murine IL-10 cDNA (a gift from Nora Suravnick, Scripps Institute, La Jolla, CA) was cloned into the pE2.2 plasmid, with expression driven by a fused cytomegalovirus-chicken β-actin promoter. rAAV serotype 2 production, titer determination, and infectivity were performed as previously described (11). Twenty-eight days before islet transplantation, diabetic female mice were untreated or received an injection into the caudal muscle of the pelvic limb of 4 × 10^6 infectious units (IU) of rAAV-green fluorescent protein (GFP), 4 × 10^9 IU rAAV-IL-10, or 4 × 10^9 IU rAAV-IL-10 per mouse, respectively.

Islet isolation. Islet cells were isolated by digestion of pancreatic tissues with collagenase and purified by using Ficoll density gradient centrifugation as previously described (22). Islet purity was assessed by dithizone staining, with islets counted and scored for size. An algorithm was used for the calculation of the 150-μm-diameter islet equivalent number.

Islet transplantation and blood glucose analysis. A total of 650 islet equivalents (per recipient) were transplanted under the left kidney capsule of diabetic NOD female mice. Tail-vein blood glucose was measured daily after transplantation using a strip glucometer (Elite; Bayer, Elkhart, IN). The day of diabetes recurrence was defined as the first of 2 consecutive days of nonfasting blood glucose >250 mg/dl. Graft survival was calculated as the number of days before diabetes recurrence.

Histopathology and immunohistochemistry. Graft-bearing kidney, liver, leg muscle, lung, ovary, pancreas, salivary gland, spleen, and lymph nodes were fixed in periodate-lysine-parafomaldehyde buffer, embedded in paraffin, and sectioned at 4 μm. All sections were stained with hematoxylin/eosin for histological assessment. Insulin (1:200; DAKO) staining was performed on graft-bearing kidney and pancreas. Graft-bearing kidneys were stained for macrophages and dendritic cells (CD11b, 1:200; Sorotec), T-cells (CD3, 1:500; Sorotec), B-cells (B220, 1:50; Pharmingen), manganese SOD (1:2,400; Stressgen), and HO-1 (SPA-895, 1:300; Stressgen). All slides were independently read and scored on a coded basis by two pathologists.

Cytokine measurement. Mouse serum was analyzed for the presence of IL-10 using the Lumene LabMAP system with kits (Biosource, LMC0101, LMB0001). The manufacturer's protocol was followed with the incorporation of a MultiScreen MABVN 1.2-μm 96-well filter plate and MultiScreen Vacuum Manifold (Millipore). Samples were read using the Lumune Max, with IL-10 concentrations interpolated using the Softmax program against the linear range on the standard curve (20–19,300 pg/ml).

RNA extraction and RT-PCR for rAAV-IL-10–specific transgene expression. Total RNA from muscles or cells was extracted with Trizol reagent and treated with RNase-free DNase (Life Technologies). RT and the first PCR were performed with primers P1 and P2 using AccessQuick RT-PCR system (Promega) for 35 cycles (Fig. 2B). The second PCR was performed with primers P3 and P4 using Platinum PCR Supermix (Life Technologies) for 35 cycles. Primer sequences were as follows: P1, 5′-AGTGCCTTCGAGCCCTGCCT-3′; P2, 5′-CTGGCTCCATGCCTTGTCC-3′; P3, 5′-GGCTCTGACTGACCGATT-3′; and P4, 5′-GGAGCTCTAGGAGCATTGG-3′.

Statistical analysis. Statistical analysis was performed with one-way ANOVA, and correlation analysis was performed with Pearson test. All data are presented as mean ± SE. Statistical significance is defined as P < 0.05.

RESULTS

Dose-dependent prevention of diabetic recurrence by rAAV-IL-10 administration. In all experimental groups, successful transplantation was achieved, as defined by normal blood glucose levels being observed within 3 days. Syngeneic islet grafts placed under the renal capsule of diabetic female NOD mice underwent a recurrent autoimmune attack capable of destroying the transplanted pancreatic β-cells. Hence, as expected, untreated mice lost islet grafts rapidly (Fig. 1) and returned to hyperglycemia within 17 days after transplantation (median 12 days, range 9–17 days). One control set of animals that involved skeletal muscle transduction of five mice with rAAV-GFP
at the dose of $4 \times 10^9$ IU was used to test the effects of viral introduction on transplant cell survival. As expected, these animals also showed rapid recurrence of diabetes at the same pace as untreated animals (range 7–12 days). For investigating the effects of systemic IL-10 on islet graft survival, a single intramuscular injection of rAAV–IL-10 was provided to animals at one of two doses, either $4 \times 10^9$ IU or $4 \times 10^8$ IU, 4 weeks before transplantation. This period of time allows for sufficient expression and secretion of transgene proteins such as α1-antitrypsin, IL-4, and IL-10 from skeletal muscle (11,21 and unpublished data). IL-10 provided a dose-dependent protection of islet grafts and prevention of diabetes recurrence (Fig. 1). Specifically, high-dose rAAV–IL-10 significantly prolonged islet transplant survival to a median of 117 days ($P < 0.01$). Two of five high-dose rAAV–IL-10 mice (40%) maintained normoglycemia for $>125$ days and were killed for the purpose of additional molecular and histological analysis. Nephrectomy of the graft-bearing kidney was performed in these two mice before they were killed, which reversed normoglycemia within 2 days. This confirmed that functional islet grafts were responsible for the sustained normal blood glucose levels. In contrast, the lower dose of rAAV–IL-10 offered no protection of transplanted islets (range 7–12 days) as compared with control treatments.

**Efficient and functional rAAV transduction of skeletal muscle.** For confirming the production of IL-10 and evaluating its concentration, serum samples collected at multiple time points until the day of diabetic recurrence or the animal was killed were measured by a microsphere-based assay. This assay provides a much more sensitive and accurate assessment of circulating cytokine levels than traditional enzyme-linked immunosorbent assay (11). Elevated IL-10 levels were identified in mice that received high-dose ($4 \times 10^9$ IU) rAAV–IL-10, whereas IL-10 was undetectable in mice that received saline, rAAV-GFP, or low doses ($4 \times 10^8$ IU) of rAAV–IL-10. This observation was confirmed by RT-PCR analysis of muscles of injection site, as vector-specific transgene expression was found only in muscles from animals that were treated with high-dose rAAV–IL-10 (Fig. 2C). Taken together with our previous observations, this suggests that a dose threshold of rAAV infection is required for sufficient production of recombinant therapeutic protein. Surprisingly, IL-10 levels showed a transient elevation, with the peak occurring 4 weeks after injection (i.e., the time of transplantation) (Fig. 2A), a finding inconsistent with the documented long-lasting transgene expression afforded by rAAV-mediated transfer in muscle (21). Furthermore, one animal that received high-dose rAAV–IL-10 did not have detectable IL-10 level and lost the islet transplant at 12 days (Figs. 1 and 2A).

The site of injection was examined to observe the local effects of transgene expression. The introduction of rAAV–

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**FIG. 2. Analyses of the efficacy and function of viral transduction in vivo.** A: Serum IL-10 from NOD mice treated with $4 \times 10^9$ IU rAAV–IL-10 ($n = 5$). The length of islet graft survival is shown for each animal. Untreated animals and animals treated with $4 \times 10^9$ IU rAAV–IL-10 or $4 \times 10^8$ IU rAAV-GFP had undetectable IL-10 levels in serum. B: Nested PCR strategy. Specifically, the first PCR was performed with primers P1 (downstream of the transcription start site) and P2 (within IL-10 coding region). The second PCR was performed with primers P3 (between P1 and the site of the splicing donor) and P4 (between ATG and P2). C: RT-PCR products using total RNA from the muscle injection site. Lane M, 100-bp marker; lanes 1 and 2, mice that received injection of $4 \times 10^9$ IU rAAV–IL-10 ($n = 2$); lanes 3 and 4, mice that received injection of $4 \times 10^9$ IU rAAV–IL-10 ($n = 2$); lane 5 and 6, mice that received injection of $4 \times 10^8$ IU rAAV-GFP ($n = 2$); lane 7, HEK 293 cells transduced with rAAV–IL-10; lane 8, untreated HEK 293 cells; lane 9, PCR of total RNA without reverse transcription; lane 10, RT-PCR without RNA template. The transgene specific product is 232 bp.
IL-10 into muscle induced a mild degree of perimyositis, an observation consistent with the known action of IL-10 on immunological recruitment and proliferation (23). This lymphocytic accumulation seemed to be transgene-specific and not vector-related, as rAAV-GFP transduction failed to induce abnormal muscle pathology and was similar to that of untreated controls (data not shown). Furthermore, myositis was dose-dependent as this was observed only in muscle that received high-dose but not low-dose rAAV–IL-10 (Fig. 3A–C). Additional phenotyping of infiltrating lymphocytes in inflamed muscles revealed a predominance of B-cells (Fig. 3D–F).

**Relationship of islet transplant protection, IL-10 concentration, and anti-AAV antibodies.** Evaluation of serum IL-10 levels at different time points revealed a significantly positive correlation between the length of islet graft survival and IL-10 levels at the time of transplantation ($r = 0.861, P < 0.001$; Fig. 4A). Systemic IL-10 produced at the proper time window was an important factor affecting how long islet grafts survived. Although several mice that received high-dose rAAV–IL-10 had undetectable IL-10 75 days after transplantation, the protection of islet grafts extended beyond 117 days. In contrast, all mice that had negligible IL-10 upon transplantation rejected islet grafts within 17 days. To uncover the reason for transient IL-10 production in NOD mice, we performed analysis that assessed the antibody responses to AAV. A trend toward negative association was observed between serum IL-10 levels and anti-AAV antibody titers at the time of transplantation (Fig. 4B). However, the correlation was not significant ($P = 0.1506$) because of the limited number of animals with detectable IL-10. Consistent with this view, the mouse that received high-dose rAAV–IL-10 yet had undetectable IL-10 in serum displayed the highest anti-AAV antibody response as well as an extremely high level of autoantibodies to insulin (1,000 ng/ml). In addition, three of five mice that were treated with high-dose rAAV–IL-10 developed anti–IL-10 autoantibodies (data not shown). These results suggested that the intensity of autoimmune response in NOD mice might be an important factor influencing the efficiency of therapeutic gene expression and the consequent efficacy of gene therapy.

**Reduction of islet insulitis by IL-10.** Histological analysis of islet grafts indicated that systemic IL-10 production significantly reduced the cumulative inflammation scores of islet grafts ($P < 0.005$), in comparison to rAAV-GFP and untreated controls (Fig. 5). IL-10 (low or high dose) did not prevent the occurrence of insulitis, as appreciable cellular infiltration was observed in all of the islet grafts, even in those animals with normal blood glucose levels. However, the invasive insulitis was far less in the islet grafts from mice that received rAAV–IL-10 injection. Even at 125 days after transplantation, two animals that received high-dose IL-10 treatment maintained a large mass of intact islet graft.

![FIG. 3. Histology of injected muscles. Representative longitudinal cross-sections of muscle (hematoxylin/eosin stained) from rAAV-GFP–treated mice with normal histology (A), 4 × 10⁸ IU rAAV–IL-10–treated mice with normal histology (B), or 4 × 10⁹ IU rAAV–IL-10–treated mice with moderate myositis and perimyositis (C). Muscle sections from 4 × 10⁹ IU rAAV–IL-10–treated mice were stained for T-cells with anti-CD3 antibody (D), B-cells with anti-B220 antibody (E), and macrophages and dendritic cells with anti-CD11b antibody (F). Magnification ×200.](image-url)
with minor peri-insulitis (Fig. 5C). Furthermore, although not subject to the same quantitative evaluation, insulin content seemed to be retained and at high levels in islet grafts from IL-10–treated mice (data not shown). Additional phenotyping of infiltrating lymphocytes identified T-cells, B-cells, macrophages, and dendritic cells that were normally found in pancreatic inflammation of NOD mice during diabetes progression (Fig. 6). However, high-dose rAAV–IL-10 seemed to reduce T-cell accumulation. Additional histological examination of multiple organs revealed no abnormal morphology or inflammation in liver, lung, heart, kidney, pancreas, ovary, salivary gland, spleen, and lymph nodes. Specifically, there was no alteration in the degree of sialitis in salivary gland and cellular configuration of spleen and lymph nodes, indicating the absence of effects on general immunity of systemic IL-10 produced from rAAV-transduced muscle at the dose used.

Induction of SOD and HO-1 in islet grafts by IL-10. Immunohistochemical staining for SOD and HO-1 showed negligible to minor levels of expression in islet grafts of untreated or rAAV-GFP–treated NOD mice. In contrast, in rAAV–IL-10–infected animals, SOD and HO-1 displayed a marked increase in islet transplants (Fig. 7). In the two mice that received high-dose rAAV–IL-10 and were normoglycemic for >125 days, SOD and HO-1 expression in islet grafts colocalized with insulin staining. Surrounding exocrine tissue, fibrotic tissue, and infiltrating monocytes did not seem to produce any visible amount of SOD and HO-1. As control grafts were examined at the time of diabetes recurrence and grafts from high-dose rAAV–IL-10–treated mice were evaluated in mice that maintained normoglycemia, it is possible that SOD and/or HO-1 in the control grafts was depleted by insulitis and graft injury. To clarify this possibility, we subsequently evaluated the levels

FIG. 5. The effect of rAAV–IL-10 gene delivery on islet grafts under renal capsules. A–C: Representative hematoxylin/eosin–stained kidney sections with islet transplants from rAAV-GFP–treated mice (A; 11 days after transplantation, time of hyperglycemia recurrence), low-dose rAAV–IL-10–treated mice (B; 7 days after transplantation, time of hyperglycemia recurrence), and high-dose rAAV–IL-10–treated mice (C; 125 days after transplantation, normoglycemic). Magnification ×200. D: Insulitis scores of islet transplants from various groups. Data are means ± SE of three to five animals of each group. *P < 0.005 versus the rAAV-GFP–treated group.

FIG. 6. Phenotypes of lymphocytic infiltration around islet grafts. Representative kidney sections from low-dose rAAV–IL-10–treated mice (A–C) and high-dose rAAV–IL-10–treated mice (D–F) stained for T-cells with anti-CD3 antibody (A and D), B-cells with anti-B220 antibody (B and E), and macrophages and dendritic cells with anti-CD11b antibody (C and F).
of SOD in healthy syngeneic isografts at 140 days after transplantation in streptozotocin-induced diabetic C57BL/6 mice that were not exposed to rAAV–IL-10. No staining for SOD was observed in these grafts, whereas significant insulin staining was present (data not shown). Therefore, it seems that IL-10 overexpression was necessary for SOD induction.

**DISCUSSION**

These studies demonstrate that systemic IL-10 production, conferred by a single administration of rAAV–IL-10 before islet transplantation, provided a degree of immunomodulation capable of inhibiting the autoimmune rejection of islet grafts. Although previous studies have identified agents that demonstrate some degree of therapeutic efficacy in this model, few have been reported with this combination of the duration in prolonging islet graft survival, safety, and convenience of treatment. Indeed, this novel gene therapy–based approach eliminated the need for repeated administration as well as the documented side effects related to immunosuppressive agents and adjuvants that have shown a modest degree of success using this transplantation model.

In terms of the mechanisms underlying this protection, these results suggest that IL-10 might protect islet transplants from autoimmune destruction via two pathways. First, we observed a reduction of lymphocytic infiltration, predominantly that of T-cells, in transplanted islet grafts. In addition, we saw induction of antioxidant and cytoprotective enzymes SOD and HO-1 in islet cells. Questions surrounding the mechanism(s) underlying this protection and the need to perform additional studies with increased numbers of animals both will be the subject of future investigations.

Although subject to controversy and perhaps too much dogma, CD4+ T-cells can be divided into two types, commonly referred to as Th1 and Th2 cells. Th1 cells produce cytokines such as IL-2 and IFN-γ, which in broad terms promote the “cellular” immune response. Prototypic cytokines of Th2 cells include IL-4 and IL-10, factors that promote the “humoral” immune response. Th1 and Th2 cells are also known to cross-regulate by releasing IFN-γ and IL-10 (24). Th1 cells play a central role in both allograft rejection and autoimmune destruction by promoting cytotoxic T-cells and releasing cell-destructive substances, such as reactive oxygen species, that induce apoptosis or necrosis (19,25). Therapies aimed at an immune shift from Th1 to Th2 have proved effective in the facilitation of organ transplants. Although allogeneic immune responses are present in most human subjects who receive pancreatic islet transplants, because of the difficulty of finding donors with perfectly matching major histocompatibility complex haplotypes, these patients can also show recurrence of autoimmune diabetes even in the case of identical twin donors (26). Like human subjects, autoimmune inflammation also results in the rejection of syngeneic islet grafts in NOD mice. This animal model shares striking resemblance to human diabetes in regard to pathophysiology, disease development, and autoimmune rejection of islet transplants (1). Some drugs that have been shown to suppress allograft rejection have not demonstrated efficacy for improving syngeneic islet grafts in NOD mice (27).
In addition, many treatments are still administered with immunological adjuvants of low clinical applicability (e.g., Complete Freund's Adjuvant, Incomplete Freund's Adjuvant, Bacille Calmette-Guerin) (28,29), are subject to side effects of general immunosuppressants (30), or require continuous treatment (31,32).

IL-10 is known as a major Th2-driving and Th1-suppressing cytokine. IL-10 inhibits IL-1, tumor necrosis factor-α, and IL-12 production by antigen-presenting cells and suppresses IL-2 secretion from Th1 cells. It also plays a key role in differentiation and function of T-regulatory cells, which may figure prominently in control of immune response and tolerance (5). Decreased IL-10 and IL-4 production has been associated with a higher risk of type 1 diabetes in human subjects, including identical twins (6). In NOD mice, the disappearance of IL-10 from pancreas precedes diabetes onset (33). Endogenous IL-10 inhibited spontaneous diabetes development in BDC2.5/NOD transgenic mice, whereas anti–IL-10 receptor treatment triggers disease onset (34). Elevated IL-10 levels have been correlated with islet graft survival in autoimmune diabetes models (18,19). On the basis of these results, IL-10 seems to represent an attractive candidate for immunotherapy to prevent autoimmune diabetes development and recurrence.

Numerous studies involving IL-10 therapy have shown a distinct pattern of site-specific impact on immune response. Systemic delivery of IL-10 thus far has consistently conferred protection to pancreatic islets as well as islet transplants (9,12,18), whereas local introduction of IL-10 into islets has seemed to be detrimental rather than helpful (13–16). However, given the relatively short half-life of this cytokine, the extension of recombinant IL-10 treatment to clinical application is limited because of the need for repeated administration. Although plasmid-mediated delivery of IL-10 has been reported to be effective, IL-10 production afforded by this approach usually lasted for only a few weeks (12). Taking advantage of the unique property of rAAV vectors in efficient muscle transduction and stable protein expression, our study demonstrated that systemic production of IL-10 afforded by a single rAAV-mediated gene delivery could reduce autoimmune inflammation, increase antioxidant gene expression in islet grafts, and inhibit recurrence of diabetes in NOD mice that received syngeneic islets.

rAAV-mediated gene transfer to skeletal muscle has been reported to afford long-term expression of secreted proteins lasting >1 year (21). It is interesting that such sustained expression was not observed in our study. rAAV–IL-10 provided a dose-dependent production of IL-10 in circulation. Even with the higher dose, appreciable amounts of IL-10 could be found for >4 months after transduction in only two of five mice. This phenomenon may be attributed to several factors. The finding of IL-10 mRNA expression of rAAV in transduced muscle by RT-PCR does not completely eliminate the possibility that lymphocyte infiltration at the site of injection destroyed the rAAV-infected muscle fibers. Exogenous production of high levels of IL-10 may upregulate the expression of IL-10 receptor in various tissues, resulting in accelerated use and breakdown of the cytokine. Another potentially important factor is the autoimmune genetic background of NOD mice. Strain-related differences have been documented in plasmid-liposome-mediated gene delivery systems (35), in antibody response to DNA and peptide immunization (36).

In this study, a negative association was observed between IL-10 levels and anti-AAV antibody titers. One mouse that failed to demonstrate detectable IL-10 after high-dose rAAV–IL-10 treatment had extremely high amounts of anti-AAV antibodies as well as autoantibodies to insulin, an indication of intense autoimmunity in this particular animal. Three of five mice that received high-dose rAAV–IL-10 were found to develop autoantibodies to IL-10. Despite the shorter systemic IL-10 production than expected, three of five mice that received a transduction of rAAV–IL-10 stayed normoglycemic for >117 days and grafted islets maintained normal morphology with a mild degree of nondestructive lymphoid accumulation. These results suggest that the time window required for IL-10 induction of immune tolerance allowed transient IL-10 production during this period to provide persistent protection to islet transplants.

Histological analysis revealed that systemic IL-10 significantly reduced insulitis in syngeneic islet grafts and inhibited diabetes recurrence. One common concern of systemic IL-10 treatment has been suppression of the general immune system apart from local inflammation. Our study showed that rAAV-conferred IL-10 expression did not alter the levels of antibody to insulin. Additional histological examination found no change in the degree of sialitis in salivary gland and morphology of spleen and lymph nodes. Nevertheless, IL-10 expression in muscle did lead to local immune infiltration, a finding consistent with the well-known property involving IL-10 recruitment of lymphocytes (23). This problem may be solved by using a viral homologue of IL-10 that is known to possess the anti-inflammatory properties of cellular IL-10 but is devoid of immunostimulatory actions (37).

We observed a significant induction of SOD and HO-1 expression in islet grafts in high-dose rAAV–IL-10–treated mice. It seems that IL-10 overexpression is necessary for induction of SOD, as healthy syngeneic grafts in C57BL/6 mice that were not exposed to IL-10 treatment had no visible amount of SOD staining. The cell types that express SOD and HO-1 in islet transplants seemed to be β-cells as their immunostaining coincided with that of insulin. This suggests but does not formally conclude that SOD and HO-1 protect β-cells from oxidant radicals released from infiltrating mononuclear cells. Indeed, future studies by our laboratories will continue to investigate the importance of these molecules to β-cell preservation.

SOD and HO-1 are important enzymes in the host defense against oxidative stress and possess potent anti-inflammatory and cytoprotective properties. Induction of these molecules has proved effective in improving transplant survival of many organs, including heart, kidney, liver, neurons, and islets. One recent study demonstrated that HO-1 mediated the anti-inflammatory effect of IL-10 in protection against lipopolysaccharide (LPS)-induced septic shock in mice (38). CO, a product of the HO-1–catalyzed enzymatic reaction, is thought to be the major mediator of these actions and can in return increase LPS-induced expression of IL-10 in macrophages (39). It has also recently been shown that HO-1 and CO protected
pancreatic β-cells from apoptosis and improved islet function/survival after transplantation (22,40). A direct relationship between IL-10 and SOD has not been reported, but a number of studies have implicated the involvement of SOD in IL-10–mediated protection. IL-10 was shown to inhibit the generation of hydroxyl radicals in response to LPS (41) and protect endothelial function after an acute inflammatory stimulus by limiting local increases in superoxide (42). Our results suggest for the first time that SOD and HO-1 might be involved in IL-10–mediated protection of islet transplants. Although we do not have direct evidence for their protective roles, this question can be addressed in the future via a number of experimental approaches, including those involving localized rAAV-mediated expression of SOD and HO-1 in islet transplantation models as well as experiments involving transplantation of HO-1 knockout islets. SOD-deficient mice die early as a result of the vital role of SOD in development (43), and HO-1 knockout mice on NOD background have yet to be developed. Hence, our findings open a new avenue for many upcoming investigations and point to the potential therapeutic use of SOD and HO-1 in improving islet transplants for curing type 1 diabetes.

In summary, these studies support the utility of cytokine immunotherapy, specifically that involving IL-10 afforded by rAAV-mediated gene transfer, for prevention of recurrent autoimmunity in islet transplantation for type 1 diabetes. Given the anti-inflammatory and immunoregulatory properties of IL-10, this novel gene therapy might be applicable not only to diabetes but also to other diseases associated with dysregulated immune responses.

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REFERENCES