XIAP Overexpression in Human Islets Prevents Early Posttransplant Apoptosis and Reduces the Islet Mass Needed to Treat Diabetes


The Edmonton Protocol for treatment of type 1 diabetes requires islets from two or more donors to achieve euglycemia in a single recipient, primarily because soon after portal infusion, the majority of the transplanted cells undergo apoptosis due to hypoxia and hypoxia reperfusion injury. X-linked inhibitor of apoptosis protein (XIAP) is a potent endogenous inhibitor of apoptosis that is capable of blocking the activation of multiple downstream caspases, and XIAP overexpression has previously been shown to enhance engraftment of a murine β-cell line. In this study, human islets transduced with a XIAP-expressing recombinant adenovirus were resistant to apoptosis and functionally recovered following in vitro stresses of hypoxia and hypoxia with reoxygenation (models reperfusion injury). Furthermore Ad-XIAP transduction dramatically reduced the number of human islets required to reverse hyperglycemia in chemically diabetic immunodeficient mice. These results suggest that by transiently overexpressing XIAP in the immediate posttransplant period, human islets from a single donor might be used to effectively treat two diabetic recipients. Diabetes 54:2541–2548, 2005

The recent introduction of the Edmonton Protocol has demonstrated that islet transplantation is a viable route to achieve insulin independence in a population of patients with type 1 diabetes (1). Despite its promise, islet transplantation remains restricted to patients with severe hypoglycemia or glycemic lability and is currently unsuitable for the majority of patients with type 1 diabetes for several reasons. Most recipients require two or more islet transplant procedures (combined mass of >10,000 islet equivalents [IEQs]/kg body wt) in order to become insulin independent, which is a serious drawback given the prevalence of diabetes and the limited cadaveric organ donor pool (2,3). Also, the risks associated with islet transplantation appear to increase with the number of infusions and with the total packed cell volume of cumulative grafts (4).

Expansion of clinical islet transplantation has been limited by the large requirement for donor tissue. The fact that most patients must receive >10,000 IEQs/kg to become insulin independent suggests that a large portion of the infused islets fail to engraft sufficiently. In fact, in murine models of islet transplantation, it has been determined that even under ideal circumstances, >60% of syngeneic islet graft mass is lost due to apoptosis (5). In clinical islet transplantation, it has been estimated that more than two-thirds of the implanted islets never become functional (2).

This early profound loss in islet mass can be attributed to several factors. Within a healthy pancreas, islet function is maximized by the intimate proximity of the β-cells and circulating blood, and, as a result, β-cells require a microenvironment with highly oxygenated blood (pO2 of 40 mmHg) and abundant nutrients (6). The current method of human islet isolation and purification destroys the capillary network in islets, causing the rapid onset of hypoxia (7). Islet hypoxia immediately after transplantation into the portal circulation further extends the postisolation hypoxic period (pO2 of 5–10 mmHg or <1% O2), and the revascularization process leads to reperfusion injury and death in islets (6). Thus, the majority of the islet graft rapidly fails to engraft after injection and undergoes apoptosis, which begins within hours posttransplant and continues for up to 2 weeks (8,9). The immediate physiological burden faced by transplanted islets is also exacerbated by high levels of tissue factor expression in islets (10,11). The Uppsala Group has demonstrated that this causes an instant blood-mediated inflammatory response to transplanted islets, with platelet deposition and subsequent macrophage-mediated islet destruction (10,11).

Since the majority of the islet tissue is lost to apoptosis for the reasons listed above, intervention with ant apoptotic agents may substantially enhance preservation of...
islet mass following transplantation (5). Both extracellular and intracellular pathways of apoptosis have been implicated in β-cell death, and many studies have described inhibition of a variety of apoptosis-associated proteins, including cFLIP (prevents caspase-8 activation), A20 (inhibits NF-κB activation), Bcl-2, and Bcl-XL (mitochondria-associated antiapoptotic proteins) (12–18). In terms of preventing islet apoptosis and graft loss in the early posttransplant period, results thus far have been largely disappointing. Only A20 has been shown to moderately reduce the islet mass required for syngeneic islet transplantation (16,17). Despite extensive investigation of these apoptosis prevention strategies in rodent models of islet transplantation, none of these antiapoptotic gene transfer strategies have been reported to be successful in enhancing human islet engraftment.

Taken collectively, these results suggest that the best strategy to enhance islet survival in the harsh posttransplant environment would be to target the executioner caspases that function late in apoptosis, beyond the convergence point of both intracellular and extracellular signaling pathways. The inhibitor of apoptosis protein (IAP) family of apoptosis inhibitors includes a number of potent endogenous antiapoptotic genes, including X-linked IAP (XIAP), which binds to the active site of the main effector caspases 3, 7, and 9. Recent studies (19,20) examining the effect of XIAP overexpression in islet transplantation have been very promising. When XIAP was overexpressed in human islets, islet death and loss of function was prevented following treatment with the immunosuppressive drugs tacrolimus, sirolimus, and mycophenolic acid in vitro (20). The protective effects of XIAP were further magnified in the Tet-regulatable murine β-cell line, βTC-Tet, following Ad-XIAP transduction (19). XIAP overexpression markedly enhanced β-cell survival and functional recovery during periods of stress due to hypoxia, reoxygenation, and cytokine insult in vitro (19). These results were confirmed in vivo by the finding that the mean time to achieve normoglycemia in recipient mice was decreased nearly sevenfold in XIAP-overexpressing grafts (19). Based upon these positive results, the impact of adenoviral-mediated XIAP overexpression in human islets during periods of hypoxia, reperfusion, and during posttransplant engraftment was investigated in the present study.

**RESEARCH DESIGN AND METHODS**

**Human islet isolation.** Cadaveric donor pancreata were removed with prior informed written consent and stored in chilled University of Wisconsin solution or two-layer system before islet isolation. Islet isolation was performed as previously described for human islets (1,21,22). Immediately postisolation, islets were quantified with dithizone and assessed for viability formed as previously described for human islets (1,21,22). Immediately informed written consent and stored in chilled University of Wisconsin solution.

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**Animals.** Immuno deficient nonobese diabetic (NOD)-RAG−/− mice (NOD.129S7(476Rag1tm1Idd8)/J) were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions. Ethical approval was obtained from the animal welfare committee at the University of Alberta, and all mice were cared for according to the guidelines of the Canadian Council on Animal Care.

**Islet transplantation studies.** Diabetes was induced in 8- to 10-week-old male NOD-RAG−/− mice using a single intraperitoneal injection of streptozocin (200 mg/kg; Sigma). Animals were considered to be diabetic after two consecutive blood glucose measurements ≥250 mg/dl. Grafts containing a mass of 1,000 or 600 islets were transplanted under the left kidney capsule in confirmed diabetic mice. In all transplantation studies, the nonfasting blood glucose levels were measured every 1–3 days for 1 week to establish time to engraftment and on alternating days thereafter using a One-Touch Ultra Glucometer (Johnson & Johnson, New Brunswick, NJ). Islet grafts were considered to be functional when the nonfasting blood glucose returned to normoglycemic levels (<220 mg/dl). Euglycemic animals from
RESULTS

Expression of Ad-XIAP and Ad-LACZ in human islets.

To study the effect of XIAP overexpression on β-cell survival, human islets were transduced with an adenoviral vector containing the Hu-XIAP cDNA (Ad-XIAP) or a control vector containing the βGAL cDNA (Ad-LACZ). In both cases, the transgene was driven by the chicken β-actin promoter, which gives stable high levels of gene expression (25). Immunoblot analysis of Ad-XIAP–transduced human islets revealed that following transduction, the islets expressed high levels of the recombinant human XIAP protein, which varied slightly among different preparations of islets (Fig. 1A). Since the Hu-XIAP gene was overexpressed in human islets, we were unble to quantify the number of Hu-XIAP–expressing islets by immunohistochemistry without generating a low background staining of endogenous XIAP protein. However, when a similarly grown and titered Ad-LACZ virus was used to transduce human islets in parallel at the same multiplicity of infection as Ad-XIAP, βGAL staining showed that >95% of the islets present in the sample were transduced, at least on the surface (Fig. 1C), while untransduced human islets exhibited no evidence of βGAL staining (Fig. 1B). To determine the penetrance of the adenoviral transduction in human islets, X-GAL–stained islets were embedded in agar and sectioned. As shown in Fig. 1E, most of the cell clusters were completely transduced by the Ad-LACZ vector. Immunohistochemical analysis for the ductal cell marker CK19 in X-GAL–stained islets illustrated that the larger cell clusters with no visible staining in the middle were in fact ductal in origin, suggesting that all of the smaller completely transduced clusters were islets (Fig. 1F). Immunohistochemical staining for insulin in these sections was not possible due to the destruction of insulin epitopes following the X-GAL staining procedure, but dithizone staining in Ad-LACZ– or Ad-XIAP–transduced islets consistently demonstrated >90% islet purity (data not shown). Ad-XIAP– or Ad-LACZ–transduced human islets showed no loss in their ability to secrete insulin in response to glucose stimulation.

Hypoxia and hypoxia reoxygenation–induced apoptosis is inhibited by XIAP overexpression. To examine the protective effects of XIAP during periods of hypoxia and following hypoxia reoxygenation injury, a well-characterized in vitro hypoxia reperfusion model system that has proven to induce apoptosis in β-cells was utilized.
Presence of adenovirus in supernatants of cultured human islets following transduction. In the clinical context, transduction of islets ex vivo is attractive since the majority of the adenoviral vector could be washed away, in theory, before portal vein infusion, reducing the risk of nontargeted gene transfer to the recipient. This hypothesis has not been verified, so culture supernatants from Ad-LACZ–transduced islets were collected and transferred to murine A9 fibroblast cells (which are very susceptible to adenovirus transduction) to determine whether infectious virions were present for up to 10 days posttransduction. Samples were collected every 2nd day after virus exposure, beginning on day 2 (at which point no washes had yet been performed after the addition of the vector on day 0). Following collection of the sample, the islets were washed twice with PBS and resuspended in fresh culture medium. Pretransduction culture supernatant was used as a negative control and had no infectious Ad-LACZ present, as indicated by the complete absence of βGAL staining in treated A9 cells. As expected, supernatants collected on day 2 had a high concentration of infectious Ad-LACZ, since none of the free virus had been washed away (Fig. 4). The amount of infectious Ad-LACZ in human islet culture supernatants decreased dramatically until no detectable βGAL staining was observed in A9 cells treated with day 10 supernatants (Fig. 4).
FIG. 3. XIAP overexpression protects transduced human islets from hypoxia and hypoxia reperfusion injury–induced loss of function, as demonstrated by glucose-stimulated insulin release assay. Hypoxia was achieved in untransduced and Ad-LACZ– and Ad-XIAP–transduced human islets by incubating flasks in 1% O₂, 5% CO₂ for 24 h. When human islet cultures maintained for 24 h in hypoxic conditions were transferred back to oxygenated conditions (mimics reperfusion, 20% O₂, 5% CO₂), glucose-stimulated insulin release assays were performed at time 0 (following hypoxia) and 24 h (following reperfusion). Human insulin levels were measured in triplicate for each experimental condition, and the glucose stimulation index was calculated by dividing the mean insulin secretion in high-glucose medium by the mean insulin secretion in low-glucose medium. The data are presented as means ± SE and are representative of three independent experiments assayed in triplicate.

DISCUSSION
The present study demonstrates that XIAP overexpression enhances human islet survival during the posttransplant engraftment period by preventing hypoxia and hypoxia reperfusion–induced apoptosis. Inhibition of effector caspases via XIAP overexpression significantly enhanced human islet survival posttransplant, allowing a 70% reduction in human islet mass needed to reverse diabetes—the most profound reduction in graft mass capable of maintaining euglycemia in mice to be reported to date (Fig. 5). Although it was not measured directly, the success of these XIAP-transduced marginal mass islet grafts supports the fact that apoptosis is a critical mediator of β-cell survival posttransplant. This is consistent with the data obtained using an in vitro system that mimics hypoxia and reperfusion injury. XIAP-transduced human islets were significantly less apoptotic and recovered functional response to glucose stimulation following these insults, suggesting that XIAP mediates islet survival in vivo by inhibiting apoptosis during hypoxia and reperfusion stress (Figs. 2 and 3). These data confirm that the protective effect of XIAP overexpression previously observed in murine β-cells is reproducible and relevant in human islets (19).

The in vivo benefit of XIAP overexpression compared with other antia apoptotic molecules is most likely related to its ability to potently block activation of late, effector apoptotic caspases, which prevents β-cell death in the multifactorial apoptotic environment present following portal vein infusion. This suggests that using XIAP in the clinical setting could immediately enhance the availability of islet transplantation by dramatically reducing the amount of islet tissue necessary to obtain insulin independence, effectively removing the need for multiple donor infusions. The recent announcement (32) of a successful case of living-donor islet transplantation in Japan has provided renewed hope for many patients with type 1 diabetes. Reversal of diabetes required transplantation of a significantly reduced number of Ad-XIAP–transduced human islets. Based upon the observation that XIAP-transduced human islets were protected from hypoxia and hypoxia reoxygenation death in vitro, we set out to evaluate the role of apoptosis in the acute posttransplant period in human islet transplantation. In our experience, a graft mass of 2,000 IEQs/mouse is necessary to reverse hyperglycemia in diabetic mice, which is in keeping with other reports (29–31). Marginal mass grafts containing 1,000 or 600 XIAP- or LACZ-transduced human islets were transplanted in streptozotocin-induced diabetic NOD-RAG−/− recipients (Fig. 5). Control grafts containing 1,000 Ad-LACZ–transduced human islets reversed hyperglycemia only 10% of the time (n = 10, Fig. 5A), while grafts containing only 600 islets never became functional (n = 7, Fig. 5A). In the presence of XIAP overexpression, islet grafts containing 600 islets were able to engraft rapidly and restore euglycemia in 89% of the diabetic mice (n = 9, Fig. 5A). The superior survival characteristics of the XIAP-transduced grafts were confirmed by measurement of serum human C-peptide levels in transplanted animals. Compared with serum from animals that had received 600 or even 1,000 Ad-LACZ–transduced human islets, the serum from animals receiving 600 Ad-XIAP–transduced islets showed significantly higher levels of human C-peptide, and these levels remained constant for several weeks posttransplant (Fig. 5B; animals in the control LACZ cohorts were hyperglycemic and had to be killed before the 21-day time point).

FIG. 4. Adenovirus levels in supernatants of cultured human islets decrease significantly over 10 days in culture. Culture supernatants from Ad-LACZ–transduced islets were harvested pretransduction, and at 2, 4, 6, 8, and 10 days posttransduction to measure the number of infectious particles present in supernatant samples, as determined by adding Ad-LACZ supernatant samples to murine A9 fibroblast cells and staining for β-GAL expression after 48 h. Samples collected before the addition of adenovirus had no detectable infectious virus (data not shown). Each sample was assayed in triplicate, and levels are expressed as means ± SE.

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diabetes. To bring living-donor islet transplantation to the mainstream and enhance recipient benefit for potential donor risk, success rates with marginal mass infusions must be improved, since only 50% of a donor's pancreas could be excised safely, and our data implies that potent apoptosis prevention with XIAP could have an immediate impact in these studies.

By reducing the total number of apoptotic islets within a graft, one can speculate that XIAP may reduce immune stimulation caused by host antigen-presenting cells presenting graft antigens. This is an important distinction from previously reported genetic manipulations to human islets that have moderately enhanced marginal mass graft survival by targeting overexpression of β-cell growth factors like hepatocyte growth factor or erythropoietin or by overexpression of the insulin gene to enhance insulin

FIG. 5. Significantly fewer XIAP-transduced human islets are required to reverse hyperglycemia when transplanted into diabetic NOD-RAG−/− mice. A: Diabetes was induced in NOD-RAG−/− mice by streptozotocin injection. Two to three days later, diabetic animals (blood glucose >18 mmol/l) were transplanted with either marginal mass islet grafts containing 600 or 1,000 Ad-LACZ–transduced human islets or with 600 Ad-XIAP–transduced islets. A total of 600 XIAP-transduced human islets provided enough β-cell mass to restore euglycemia in 89% of the animals, while none of the animals receiving 600 and 10% of the animals receiving 1,000 LacZ control islets normalized (four independently islet donors were used in these experiments). B: Animals receiving 600 XIAP-transduced islets exhibited consistently high serum levels of human C-peptide compared with animals receiving βGAL control grafts with 600 or even 1,000 islets.
output per islet (29,33,34). These strategies function by enhancing the survival, growth, and/or function of the islets that manage to engraft but still exert little effect on apoptotic islets posttransplant. Thus, a large amount of donor antigen would theoretically still be presented to the recipient’s immune system, potentially limiting the possibility of immunosuppressive therapy reduction or tolerance induction. Minimizing islet apoptosis posttransplant should prolong graft longevity, resulting in a more quiescent immunological state, and thereby enhance long-term rates of insulin independence. If this could be achieved, islet transplantation would be potentially safer and therefore more available to a broader spectrum of patients with type 1 diabetes, including children.

The primary limitation in the clinical application of genetic modification to islets lies in the efficiency and reproducibility of gene transfer. Over the years, it has become apparent that adenoviral vectors represent the most reliable and efficient method to deliver genes to intact islets without the worrisome long-term effect of nontargeted genomic integration associated with lentiviral vectors. That being said, many groups have struggled to obtain transduction efficiencies >50%, which limits the opportunity to observe any protective effect, especially with a transgene such as XIAP, which functions intracellularly (29,34). One approach to improve transduction efficiency involves the use of more virus (i.e., increase the multiplicity of infection per cell), and while this does result in enhanced levels of transgene expression, the consequence is that the viral load itself becomes toxic to the islets (35). We hypothesized that transduction of islets very soon after isolation would enhance transduction efficiencies, since the microcapillary network in each islet would still be intact, allowing for improved adenoviral penetration. Though collaboration with the University of Alberta Clinical Islet Isolation Laboratory, we were able to obtain and transduce islets >1 h following the completion of the isolation procedure. This resulted in highly efficient gene transfer to the islets, with >95% of Ad-LACZ–stained islets exhibiting at least 75% X-GAL staining through the islet (Fig. 1E). This result is especially remarkable considering that only 10 virus particles/cell were used, while other groups have obtained at most 50% transduction efficiency in human islets using 500–1,000 virus particles/cell (29,34,35). Even with the 50- to 100-fold decrease in adenovirus used to transduce the islets in our experiments, infectious virus was still detectable up to 8 days posttransduction, after >20 wash steps, suggesting that adenovirally transduced islets must be cultured for at least a week before transplantation to ensure that no vector is inadvertently transferred to the recipient (Fig. 4). Islet mass generally decreases in the culture setting due to hypoxic and other stress; however, the use of an antiapoptotic agent like XIAP would tend to enhance islet survival during this period.

Our studies suggest that effector caspase inhibition may be only transiently required to improve islet engraftment in the first few days or weeks following transplantation. Since first-generation adenoviral vectors were used in this study, and it is known that expression levels from these vectors declines 60–90 days posttransduction, we believe that XIAP overexpression would naturally taper off post-transplant, removing the need for the design of complex vectors with drug-regulatable promoters (36). Our studies also suggest that XIAP gene transfer and overexpression could be circumvented altogether, should pharmaceutical agents targeted at preventing caspase activation become available, allowing the positive effects observed following XIAP overexpression to be reproduced using a transient drug therapy. Taken together, these data confirm that XIAP overexpression in human islets dramatically enhances engraftment and in so doing reduces the islet mass necessary to achieve euglycemia, suggesting that clinical application of this protocol could immediately and greatly enhance the availability and long-term outcome of islet transplantation for type 1 diabetes.

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