Original Article

Caspase Inhibitor Therapy Enhances Marginal Mass Islet Graft Survival and Preserves Long-Term Function in Islet Transplantation

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Islet transplantation can provide insulin independence in patients with type 1 diabetes, but islets derived from two or more donors are often required. A significant fraction of the functional islet mass is lost to apoptosis in the immediate posttransplant period. The caspase inhibitor N-benzyloxycabonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-FMK) has been used therapeutically to prevent apoptosis in experimental animal models of ischemic injury, autoimmunity, and degenerative disease. In the current study, zVAD-FMK therapy was examined in a syngeneic islet transplant model to determine whether caspase inhibition could improve survival of transplanted islets. zVAD-FMK therapy significantly improved marginal islet mass function in renal subcapsular transplantation, where 90% of zVAD-FMK–treated mice became euglycemic with 250 islets, versus 27% of the control animals ($P < 0.001$). The benefit of zVAD-FMK therapy was further demonstrated after intraportal transplantation, where 75% of zVAD-FMK–treated animals established euglycemia with only 500 islets, and all of the controls remained severely diabetic ($P < 0.001$). zVAD-FMK pretreatment of isolated islets in the absence of systemic therapy resulted in no significant benefit compared with controls. Long-term follow-up of transplanted animals beyond 1 year posttransplant using glucose tolerance tests confirmed that a short course of zVAD-FMK therapy could prevent metabolic dysfunction of islet grafts over time. In addition, short-term zVAD-FMK treatment significantly reduced posttransplant apoptosis in islet grafts and resulted in preservation of graft insulin reserve over time. Our data suggest that caspase inhibitor therapy will reduce the islet mass required in clinical islet transplantation, perhaps to a level that would routinely allow for insulin independence after single-donor infusion. ***Diabetes*** **56:1289–1298, 2007**

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AUC, area under the curve; DAPI, 4′6-diamidino-2-phenylindole; IPGTT, intraperitoneal glucose tolerance test; STZ, streptozotocin; TUNEL, transferase-mediated dUTP nick-end labeling; zVAD-FMK, N-benzyloxycabonyl-Val-Ala-Asp-fluoromethyl ketone.

The introduction of the Edmonton Protocol in 2000 led to substantial improvements in 1-year insulin independence rates in clinical islet transplantation (1,2). However, recent long-term follow-up indicates marked reduction in graft function, with only 15% of islet recipients maintaining insulin independence at 5 years (3). Although single-donor islet transplant success has been reported in Minnesota in a limited number of patients, most centers still require at least two donors (4,5). The decline in insulin independence rates seen in clinical islet transplantation is currently not fully characterized, but it is likely to be complex. Detrimental factors include recurrent β-cell autoimmunity, subclinical allograft rejection, metabolic exhaustion, chronic islet toxicity of immunosuppressive drugs, and limitations from the intraportal site of islet delivery (2). Strategies designed to maximize survival and minimize immune reactivity of the initial islet mass are likely to have a major impact in enhancing long-term clinical outcomes.

A variety of approaches have been explored to prevent apoptotic destruction of islets in the experimental setting, and although promising data have been generated in vitro, demonstration of in vivo benefit to islet graft survival has been more elusive (rev. in 6). One molecule that has shown particular benefit is XIAP (X-linked inhibitor of apoptosis protein), a potent endogenous inhibitor of the downstream effector caspases 3, 7, and 9 that prevents apoptosis induced by both extrinsic and intrinsic signals (7). Its protective benefit has been proven in isolated human islets, where it minimized diabetogenic toxicity of immunosuppressive drugs in vitro and improved marginal mass islet graft function after transplantation into diabetic immunodeficient mice (8,9). The major drawback of this approach is that it requires gene therapy with adenoviral vectors, and safety concerns persist at the present time (8).

In the context of improving β-cell engraftment, effector caspase inhibition may be required only transiently in the first few days or weeks after transplantation. A series of small molecule peptidyl protease inhibitors have been used for >15 years as tools to investigate the activity of caspases. The most powerful among these is N-benzyloxycabonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-FMK), a pan-caspase inhibitor that is cell permeable and that irreversibly binds to the active site in caspases 1–10 and 12. Short-course zVAD-FMK therapy has been used in several animal models of disease to prevent apoptosis of the affected tissues, including autoimmune disease, aller-

gic inflammation, ischemia-reperfusion injury, and neurodegenerative disease (10–15). In the context of islet transplantation, the benefit of caspase inhibitors has only been investigated after in vitro pretreatment before transplantation. Montolio et al. (16) treated rodent islets in vitro with increasing zVAD-FMK concentrations (100–500 μmol/l) and found only marginal impact after transplantation of a syngeneic marginal mass. Pretreatment of human islets with the selective caspase-3 inhibitor zDEVD-FMK (benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone) for up to 2 days improved islet survival during culture and modestly improved islet survival posttransplant into streptozotocin (STZ)-induced diabetic nude mice (17). Based on the positive results obtained in other models of degenerative disease, the impact of therapeutic zVAD-FMK administration on islet survival in vivo after transplantation was investigated in the current study.

RESEARCH DESIGN AND METHODS

C57BL/6 and BALB/C mice were obtained from the University of Alberta Health Sciences Laboratory Animal Services and housed under conventional conditions. Ethical approval was obtained from the animal welfare committee at the University of Alberta, and all animals were cared for according to the guidelines of the Canadian Council on Animal Care.

Caspase inhibitor therapy. zVAD-FMK was obtained from Bachem (Torrance, CA). A stock preparation of 10 mg/ml was prepared in sterile DMSO to solubilize the compound. For in vitro and in vivo preparations, the stock solution was diluted into sterile saline to produce a water-soluble concentration of 1 mg/ml. All stocks were stored at −20°C until use. For control experiments, a solution containing DMSO in sterile saline at the same concentration as zVAD-FMK stocks was used.

Islet transplantation studies. Male BALB/C islets were isolated using established methods (18). To induce diabetes in syngeneic recipients, a single intraperitoneal injection of STZ (250 mg/kg; Sigma-Aldrich, Mississauga, ON, Canada) was administered to male BALB/C mice, and animals were considered to be diabetic after two consecutive blood glucose measurements ≥325 mg/dl using a One Touch Ultra Glucometer (Lifescan, Burnaby, BC, Canada). Before transplantation, islet preparations were split in half and incubated in zVAD-FMK–containing medium (100 μmol/l in Dulbecco's modified Eagle's medium; Invitrogen, Canada) or vehicle-containing medium for 2 h. After this incubation step, islets were washed, counted, and transplanted. Transplant recipients were given a single zVAD-FMK or vehicle injection (10 mg/kg body wt s.c.) on the day of transplant and for 5 days thereafter. Islets were transplanted under the left kidney capsule for renal subcapsular studies or into the portal circulation by direct trans-pancreatic puncture for intraportal studies. In some parts of this study, the graft-bearing kidney was harvested, and the islet graft was processed for immunohistochemical analysis or total insulin content. These animals were followed afterward to confirm that the islet graft had been functional, which was confirmed by the return to blood glucose levels >325 mg/dl on 2 consecutive days.

Glucose tolerance tests. Transplanted animals were fasted overnight and injected intraperitoneally with 50% dextrose in Ringer's solution at 2 g/kg body wt. The animals were monitored for blood glucose levels at 0, 15, 30, 60, 120, and 180 min postinjection. Area under the curve (AUC) calculations were completed using SigmaPlot 10 (SPSS, Chicago, IL).

Graft insulin content. Islet grafts were harvested from the kidney capsule. Aliquots of 500 isolated islets were also pelleted and analyzed after a 2-h incubation in zVAD-FMK–containing medium or vehicle medium. Samples were collected and stored at −80°C until bulk analysis could be performed as reported previously (19). Samples were neutralized using 0.25% BSA in PBS, pH 11, before analysis in triplicate using an insulin radioimmunoassay (Linco Research, St. Charles, MO).

Apoptosis assays. Apoptosis of islet cells within transplanted grafts was quantified using transferase-mediated dUTP nick-end labeling (TUNEL) staining (Dead-End apoptosis detection system; Promega, Madison, WI) as previously described (20). Nuclear counterstaining with 4′6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) was used to detect all cells present in the sample. Islet grafts were harvested, placed in formalin, processed, and embedded in paraffin. To quantify apoptosis in vivo, fields containing at least 500 cells were analyzed at 200× magnification. The number of TUNEL-positive cells (green or yellow) within the insulin-positive islet graft area of the section were counted and compared with the total number of DAPI-positive nuclei within that same field to determine percent apoptosis. Sections were prepared from three transplant recipients in each cohort, and at least four fields were analyzed in each section.

Toxicological analysis. Mice that were either untreated, STZ-administered, or STZ- and zVAD-FMK–treated (10 mg/kg body wt for up to 10 days) were subjected to cardiac exsanguination. Blood samples were allowed to clot for 1 h and then spun at 1,000 rpm for 10 min to separate serum. Serum samples were analyzed in triplicate for alanine aminotransferase, alkaline phosphatase, γ-glutamyltransferase, creatinine, blood urea nitrogen, and creatine phosphokinase by Veterinary Pathology Labs (Edmonton, AB, Canada).

Statistics. SigmaPlot 10 and SigmaStat 3.5 (SPSS, Chicago, IL) were used for all statistical analysis in this study, and results are expressed as the means ± SE. Student's t tests for paired and unpaired data were used to compare results in each experimental condition, and ANOVA was used to analyze multiple groups. Euglycemic survival curves were analyzed with the log rank test.

RESULTS

zVAD-FMK therapy promotes marginal islet mass function at the renal subcaspular site. To investigate the impact of caspase inhibitor administration on islet survival posttransplant, we designed a protocol that was based on a successful dosing strategy used to reduce kidney damage in a model of lupus nephritis (12). Because the majority of islet death occurs in the first few days posttransplant, we designed our study such that zVAD-FMK would only be administered until day 5 posttransplant, after which point the rate of apoptosis is so minimal that zVAD-FMK therapy would not be justified (21). Our study also implemented a pretransplant incubation period of 2 h in 100 mmol/l zVAD-FMK, which would reduce isolation-induced apoptosis and improve islet survival in the first few hours posttransplant by "loading" the islets with caspase inhibitor (16,17). For all transplant studies, zVAD-FMK was injected into recipients at least 2 h before transplantation, so that the caspase inhibitor would be circulating during the implantation and able to prevent apoptosis immediately. Initially, nonmarginal mass islet grafts containing 500 islets transplanted under the kidney capsule (renal subcapsular) were performed. As shown in Fig. 1*A*, zVAD-FMK–treated animals rapidly achieved euglycemia posttransplant. When a marginal mass of 250 islets was transplanted under the kidney capsule, the benefit of zVAD-FMK therapy was clearly demonstrated by the significant decrease in blood glucose levels compared with vehicle controls ($P = 0.002$ by ANOVA), which was not observed when zVAD-FMK was only used in vitro pretransplant (Fig. 1*B*). Recovery nephrectomies performed in randomly selected zVAD-FMK–treated or control animals (500 renal subcapsular) at either 1 week or 6 months posttransplant demonstrated that the islet graft was maintaining euglycemia (Fig. 1*C*). zVAD-FMK therapy dramatically improved islet graft function posttransplant because return to euglycemia was achieved in 100% of zVAD-FMK–treated animals receiving 500 islets ($n = 18$), compared with 83% of control animals ($P = 0.023$ by long rank, $n = 18$) (Fig. 1*D*). After transplantation of a marginal mass of 250 islets, 90% zVAD-FMK–treated animals returned to euglycemia ($n = 10$), whereas only 27% of control animals became euglycemic ($P = 0.008$ by log rank, $n = 11$) (Fig. 1*D*). zVAD-FMK pretreatment of islets without therapeutic injections did not result in a significant benefit compared with controls ($n = 6$) (Fig. 1*D*).

A brief period of zVAD-FMK therapy posttransplant promotes long-term renal subcapsular islet graft function. To explain the chronic decline in β-cell reserve after islet transplantation, a constant functional demand on a marginal islet mass likely leads to metabolic dysfunc-

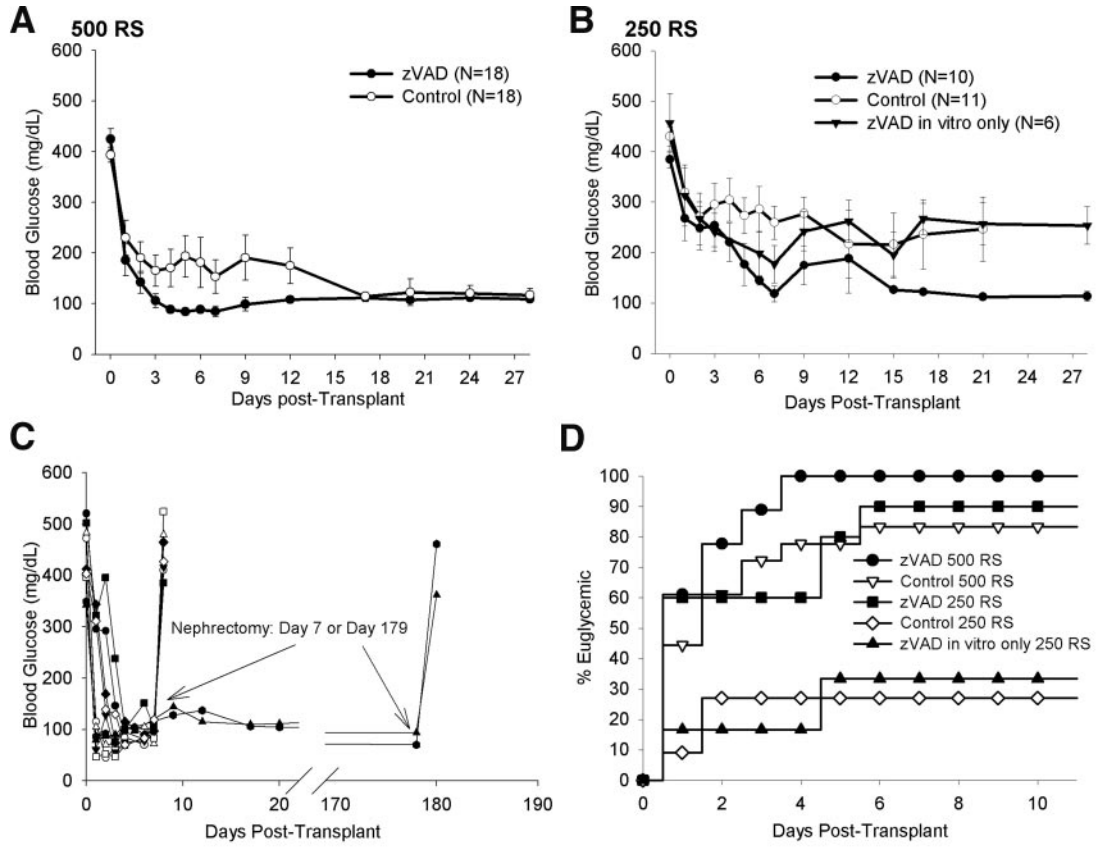

FIG. 1. zVAD-FMK therapy promotes survival of renal subcapsular marginal mass islet grafts. *A*: Diabetes was induced in BALB/C mice by STZ injection. Syngeneic nonmarginal mass islet grafts containing 500 islets that had been precultured for 2 h in zVAD-FMK–containing medium (100 μmol/l) or control medium were transplanted under the kidney capsule (renal subcapsular) in diabetic recipients. Recipients received subcutaneous injections of zVAD-FMK (10 mg/kg) from days 0 to 5 posttransplant. *B*: Marginal mass islet grafts containing 250 islets were transplanted under the kidney capsule in syngeneic diabetic BALB/C recipients using the same zVAD-FMK in vitro culture and in vivo therapeutic regimen outlined in *A*. zVAD-FMK–treated animals maintained significantly lower blood glucose values compared with vehicle controls ($P = 0.002$). zVAD-FMK treatment in vitro without in vivo injections did not significantly improve blood glucose levels compared with controls. *C*: Recovery nephrectomies performed in randomly selected zVAD-FMK–treated or control animals at either day 7 or 179 posttransplant resulted in 100% return to hyperglycemia. Closed symbols, 500 renal subcapsular islet grafts combined with ZVAD treatment ($n = 6$); open symbols, 500 control renal subcapsular islet grafts ($n = 4$). *D*: Return to euglycemia posttransplant was achieved in 100% of zVAD-FMK–treated animals receiving 500 islets ($n = 18$), compared with 83% of control animals receiving 500 islets ($n = 18$, $P = 0.023$). After transplantation of a marginal mass of 250 islets, 90% zVAD-FMK–treated animals returned to euglycemia ($n = 10$), whereas only 33% of animals receiving zVAD-FMK treatment in vitro only ($n = 6$) and 27% of control animals became euglycemic ($n = 11$, $P = 0.008$).

tion and eventually exertion-induced β-cell death (22). To investigate this theory, euglycemic animals that had received 500 or 250 islets under the kidney capsule and zVAD-FMK or vehicle therapy were followed long term (>1 year) using intraperitoneal glucose tolerance tests (IPGTTs). Results shown in Fig. 2*A* demonstrate that there was very little difference in IPGTT profiles at 14 days, 28 days, and 1 year posttransplant in zVAD-FMK–treated animals receiving a nonmarginal islet mass of 500. In contrast, control animals exhibited an increase in peak blood glucose values during IPGTT at 1 year posttransplant, even though these animals remained euglycemic (Fig. 2*B*). When the AUC was calculated, zVAD-FMK–treated animals had a significantly lower AUC compared with control animals at 1 year posttransplant, despite only receiving zVAD-FMK for the first 5 days posttransplant ($P < 0.01$) (Fig. 2*C*). No significant difference in fasting body weight between zVAD-FMK–treated and control animals was observed at any time point over the 1 year follow-up period (data not shown). When euglycemic animals that had received a marginal islet mass of 250 were followed in the long term, the result was similar (Fig. 3). Because only 27% of control animals achieved euglycemia with 250 islets, there were not enough animals in this cohort to be followed in the long term. As shown in Fig. 3*B*, there was no significant difference in AUC for either zVAD-FMK–treated or control euglycemic animals at 14 days posttransplant, which is consistent with the results obtained using 500 islets. However, there was a significant increase in AUC for zVAD-FMK–treated animals at 28 days posttransplant, which returned to 14-day levels by 1 year posttransplant ($P < 0.01$) (Fig. 3*B*). All animals analyzed using IPGTT at 1 year posttransplant remained euglycemic, despite variations in AUC (data not shown).

zVAD-FMK therapy enhances intraportal islet graft function. In clinical islet transplantation, the graft is transplanted intraportally, and there are significant physiological differences in the portal system compared with the renal subcapsular space that may have a deleterious effect on islet survival. Indeed, it has been reported that intraportal transplantation of islet grafts results in focal areas of liver necrosis and the subsequent release of proinflammatory cytokines and induction of islet apoptosis (23). This finding may also account for the increased islet mass necessary to reverse diabetes after intraportal islet transplantation in mice, compared with the renal

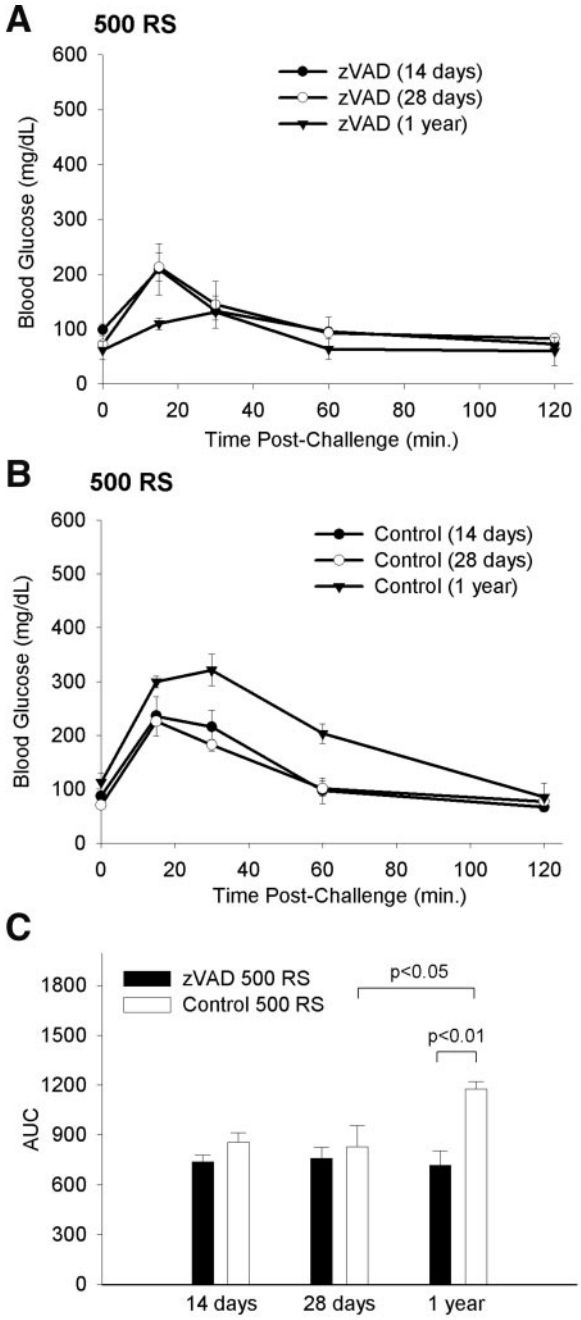

FIG. 2. A short course of zVAD-FMK therapy preserves nonmarginal mass islet graft function in the long term. To evaluate the function of islet grafts in zVAD-FMK–treated (*A*) and control (*B*) animals posttransplant, IPGTTs were performed at several time points posttransplant. Isolated islets were precultured in zVAD-FMK–containing medium (100 mmol/l) for 2 h before transplantation, and diabetic recipients received daily zVAD-FMK therapy (10 mg/kg s.c.) from days 0 to 5 posttransplant. Control islets were incubated in vehicle-containing medium, and recipients were injected with vehicle from days 0 to 5 posttransplant. As shown in *A*, there was no significant change in glucose tolerance test curves at 14 days, 28 days, or 1 year posttransplantation of a nonmarginal mass of 500 islets combined with zVAD-FMK therapy (renal subcapsular islet grafts). However, control animals receiving a nonmarginal islet mass of 500 exhibited a decay in graft function over time, as evidenced by the elevated peak in the glucose tolerance test curve at 1 year posttransplant. *C*: There was a significant decrease in AUC for zVAD-FMK–treated animals compared with control animals at 1 year posttransplant ($P < 0.01$). Data are representative of $n = 5$ animals per time point, and only euglycemic animals in each cohort were evaluated. RS, renal subcapsular islet grafts.

capsular site (24). To confirm that zVAD-FMK therapy could also improve islet survival in the clinical scenario, islets were transplanted intraportally (portal vein) using the same pretransplant zVAD-FMK incubation period and posttransplant dosing scheme outlined in our renal subcapsular study. After transplantation of a nonmarginal islet mass of 800 in the portal vein, both zVAD-FMK–treated and control animals rapidly achieved euglycemia (Fig. 4*A*). However, when a marginal islet mass of 500 was transplanted into the portal vein, 75% (6 of 8) of zVAD-FMK–treated animals became euglycemic, whereas all of the controls remained severely diabetic ($n = 7$, $P < 0.001$ by log rank test) (Fig. 4*B*). Further reduction in the transplanted islet mass to 150 in the portal vein resulted in 71% (5 of 7) diabetes reversal in zVAD-FMK–treated animals, with none of the controls establishing euglycemia (0 of 7, $P < 0.001$ by log rank test) (Fig. 4*C*). Compared with zVAD-FMK–treated animals receiving 800 or 500 islets in the portal vein, those animals receiving 150 islets in the portal vein required up to 12 days to become euglycemic, despite only receiving zVAD-FMK injections for 5 days posttransplant (Fig. 4*C*).

A brief period of zVAD-FMK therapy posttransplant promotes long-term intraportal islet graft function. After intraportal transplantation, islets are exposed to the instant blood-mediated inflammatory reaction, which may lead to islet fibrosis and thus a more pronounced chronic decline in islet function after transplantation at this site (25–27). To examine the function of zVAD-FMK and control portal vein islet grafts long term, euglycemic animals were followed up to 1 year posttransplant with IPGTT. Because none of the control animals receiving marginal mass grafts containing 500 islets became euglycemic, only zVAD-FMK–treated animals in this cohort could be followed. As demonstrated in Fig. 5*A*, there was no difference in IPGTT profiles between control grafts of 800 islets, zVAD-FMK–treated grafts of 800 islets, or zVAD-FMK–treated grafts of 500 islets at 28 days posttransplant. However, after 1 year, the control animals with 800 islets in the portal vein exhibited a marked increase in peak blood glucose values (Fig. 5*B*). When AUC was analyzed, there was a significant increase in AUC from 28 days to 1 year posttransplant in the control animals with 800 islets transplanted in the portal vein ($P < 0.05$), compared with no increase in zVAD-FMK–treated animals with 800 islets (Fig. 5*C*). zVAD-FMK–treated animals with 500 islets transplanted in the portal vein exhibited a significant decline in AUC at 1 year compared with 28 days ($P < 0.01$), which is a similar finding to marginal mass islet grafts transplanted under the kidney capsule (Figs. 3*B* and 5*C*). Surprisingly, AUC values in zVAD-FMK–treated animals with 500 islets transplanted in the portal vein were significantly lower than control animals with 800 islets transplanted in the portal vein at 1 year, despite the higher transplanted islet mass in the controls ($P < 0.01$) (Fig. 5*C*).

A brief treatment period with zVAD-FMK preserves graft insulin content and prevents islet apoptosis posttransplant. Because our study involves transplantation of syngeneic islets into chemically diabetic recipients, the possibility exists that treatment with a caspase inhibitor could lead to endogenous β-cell preservation and/or hyperplasia after STZ treatment, which could result in improved blood glucose values, despite a minimal direct benefit to the transplanted islets. To investigate this hypothesis, a series of nonmarginal mass grafts containing 500 islets were transplanted under the renal capsule using

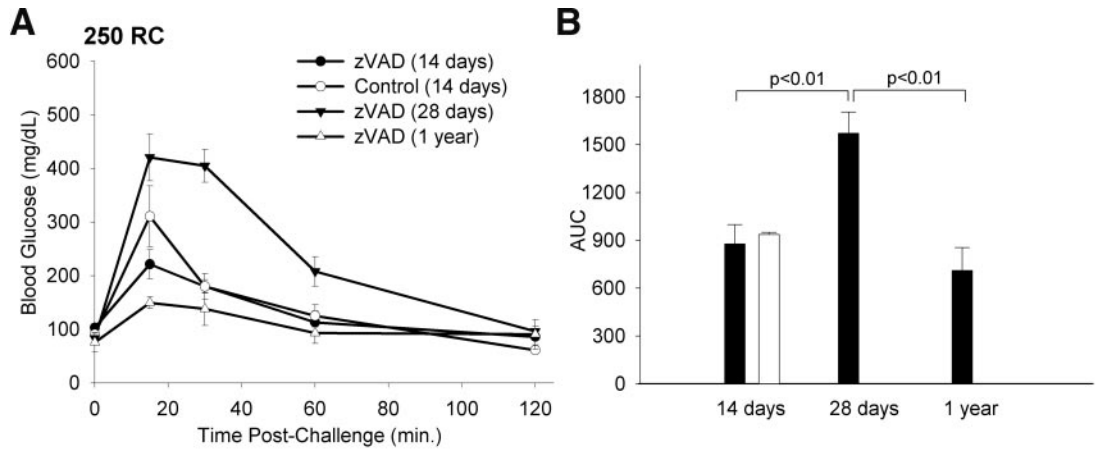

FIG. 3. A Short course of zVAD-FMK therapy preserves marginal mass islet graft function over time. To evaluate the function of marginal mass islet grafts in zVAD-FMK–treated and control animals posttransplant, IPGTTs were performed at several time points posttransplant. Very few control animals became euglycemic after transplantation of 250 islets, and thus long-term follow-up of these animals was not possible. Isolated islets were precultured in zVAD-FMK–containing medium (100 mmol/l) for 2 h before transplantation, and diabetic recipients received daily zVAD-FMK therapy (10 mg/kg s.c.) from days 0 to 5 posttransplant. Control islets were incubated in vehicle-containing medium, and recipients were injected with vehicle from days 0 to 5 posttransplant. *A*: After transplantation of a marginal mass under the kidney capsule (renal subcapsular), zVAD-FMK–treated animals exhibited good islet graft function at day 14, which appeared to decay at 28 days posttransplant (▼). Even though zVAD-FMK therapy was only administered for 5 days posttransplant, islet graft function in zVAD-FMK–treated animals returned to day 14 levels by 1 year posttransplant. *B*: A significant increase in AUC for zVAD-FMK–treated animals was observed between 14 and 28 days posttransplant, which disappeared by 1 year posttransplant ($P < 0.01$). Control animals were analyzed at 14 days posttransplant, and no significant difference was observed when compared with zVAD-FMK–treated animals at the same time point. Data are representative of $n = 5$ animals per time point (control at 14 days: $n = 3$), and only euglycemic animals in each cohort were evaluated. ■, 250 renal subcapsular islet grafts with zVAD; □, 250 control renal subcapsular islet grafts.

the same in vitro pretransplant incubation period and posttransplant injection schedules used in our previous studies. Islet grafts were then analyzed for total insulin content and apoptosis at the following time points: pretransplantation (an equivalent aliquot of 500 islets that had been cultured for 2 h) and 24 h, 14 days, and 28 days posttransplant. All of the animals were transplanted on the same day with the same islet preparation to prevent variability in islet preparations from affecting this study. Intraportal islet grafts were not used for this analysis because of the technical difficulties associated with measurement of total graft insulin content and quantification of apoptosis in intraportally transplanted islets. As shown in Fig. 6, there was no difference in insulin content between zVAD-FMK–treated and control islets before transplantation. A significant difference in insulin content was observed at 24 h posttransplant between zVAD-FMK–treated and control islets ($P < 0.0005$), but total insulin content in both cohorts was dramatically reduced, which is likely caused by massive β-cell degranulation associated with the immediate posttransplant period. However, at 14 days posttransplant, there was a significant reduction in graft insulin content in control animals that was not present in zVAD-FMK–treated recipients ($P < 0.0005$), a finding that was also observed at 28 days posttransplant (Fig. 6*A*). This progressive decline in graft insulin content in the control animals has been reported by other groups and supports the metabolic exhaustion theory (21,22,28,29). However, zVAD-FMK–treated animals did not exhibit a decline in graft insulin content; instead, graft insulin content levels remained steady from 14 to 28 days, despite the absence of zVAD-FMK therapy at that point posttransplant (Fig. 6*A*).

To prove that zVAD-FMK therapy reduced apoptosis in transplanted islets, islet grafts were harvested as outlined above and analyzed for apoptosis using TUNEL staining. Slides were blinded before analysis by an independent observer to prevent bias. Pretreatment with zVAD-FMK before transplantation dramatically reduced the percentage of apoptotic islet cells ($P < 0.0005$) (Fig. 6*B*), where ~40% of the control islet cells already exhibited signs of early apoptosis. At 24 h after transplantation, control islets continued to be highly apoptotic (~40% of cells), whereas zVAD-FMK–treated animals had significantly less apoptotic cells within the graft (Fig. 6*B*). By 14 and 28 days posttransplant, there was very little detectable apoptosis in either zVAD-FMK–treated or control animals. Islet grafts were harvested for this study using recovery nephrectomies, and all of the animals returned to hyperglycemia, which confirms that the transplanted islets were responsible for the maintenance of euglycemia (data not shown). These findings demonstrate that zVAD-FMK therapy directly benefits the islet graft.

No evidence of systemic toxicity after zVAD-FMK treatment. Because our data suggest that zVAD-FMK therapy could have a significant impact on marginal mass islet survival in the clinical setting, serological assessments were carried out after zVAD-FMK treatment to confirm that no systemic toxicity was present. STZ-administered animals were used as controls for zVAD-FMK–treated animals because zVAD-FMK–treated animals had also received STZ, and STZ is associated with a number of organ-specific toxicities, particularly the liver and kidney (30). As shown in Table 1, there was no evidence of renal dysfunction as measured by blood urea nitrogen and creatinine after zVAD-FMK treatment, compared with untreated or STZ-administered control animals. In fact, there was evidence that zVAD-FMK ameliorated STZ-induced nephrotoxicity in C57BL/6 animals, as indicated by their creatinine levels ($P = 0.015$). There was no significant change in liver function tests for zVAD-FMK–treated animals compared with STZ treatment alone. These data are supported by the fact that zVAD-FMK–treated islet recipients were followed for >1 year posttransplant with no evidence of chronic pathological changes or disease. It should also be noted that 10 days of zVAD-FMK injections

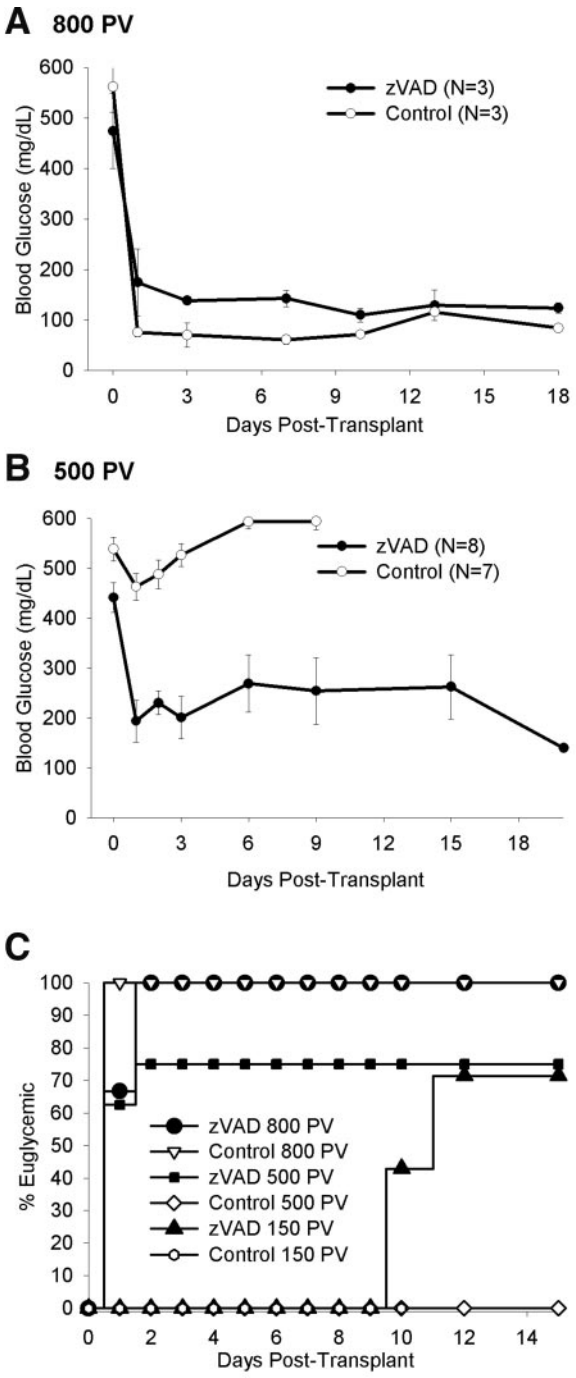

FIG. 4. zVAD-FMK therapy promotes survival of intraportal marginal mass islet grafts. *A*: Syngeneic nonmarginal mass islet grafts containing 800 islets that had been precultured for 2 h in zVAD-FMK–containing medium (100 μmol/l) (●) or control medium (○) were transplanted into the portal circulation (PV) in diabetic recipients. Recipients received subcutaneous injections of zVAD-FMK (10 mg/kg) or vehicle at least 2 h before transplantation and daily thereafter until day 5 posttransplant. There was no significant difference in mean blood glucose values between zVAD-FMK and control animals after transplantation of 800 islets intraportally ($n = 3$ per cohort). *B*: Marginal mass islet grafts containing 500 islets were transplanted intraportally in syngeneic diabetic BALB/C recipients using the same zVAD-FMK in vitro culture and in vivo therapeutic regimen outlined in *A*. zVAD-FMK–treated animals ($n = 8$) maintained significantly lower blood glucose values as compared with vehicle controls ($n = 7$, $P < 0.001$). Control animals were euthanized at day 9 posttransplant because of severe hyperglycemia. *C*: Return to euglycemia posttransplant was achieved in 100% of zVAD-FMK–treated and control animals receiving nonmarginal mass islet grafts containing 800 islets. After transplantation of a marginal mass of 500 islets, 75% zVAD-FMK–treated animals returned to euglycemia ($n = 8$), whereas none of the control animals became euglycemic ($n = 7$, $P < 0.001$). A further reduction in the islet mass to 150 islets resulted in euglycemia in 71% of zVAD-FMK–treated animals ($n = 7$), compared with 0% of the controls ($n = 7$, $P < 0.001$).

in STZ-administered animals did not improve blood glucose levels (mean 457.2 ± 136.8 mg/dl on day 10), confirming that zVAD-FMK therapy does not result in dramatic β-cell recovery or functional improvement in STZ-administered animals. There was also no difference in insulin staining of pancreas sections prepared from untreated or zVAD-FMK–treated STZ-diabetic animals (data not shown).

DISCUSSION

The success of zVAD-FMK treatment in this study provides compelling evidence that pan-caspase inhibitor therapy is beneficial in promoting early marginal mass islet engraftment posttransplant. These data also demonstrate that a brief treatment period of 5 days with zVAD-FMK enhanced marginal mass islet graft function in the long term (>1 year) for both renal subcapsular and intraportal grafts. Initially, the impact of zVAD-FMK therapy was investigated using a nonmarginal mass of 500 islets transplanted under the kidney capsule, which we anticipated would be a control experiment to confirm that zVAD-FMK therapy was not toxic in islet transplantation. The observation that zVAD-FMK therapy could significantly improve survival with a nonmarginal mass islet graft was unexpected, but analysis using IPGTTs confirmed this result (Figs. 1*C* and 2*C*). The finding that zVAD-FMK therapy significantly improved graft function with the marginal mass of 250 islets transplanted under the kidney capsule supported the results obtained using 500 islets. Based on these successful results, the more challenging intraportal islet transplantation model was investigated. Although no detectable benefit with a nonmarginal islet mass of 800 was observed in these studies, a modest reduction to 500 islets in the portal vein resulted in a dramatic reduction in graft function in control animals (0%), whereas the majority of zVAD-FMK–treated animals became euglycemic (75%) (Fig. 4). The further reduction to 150 islets in the portal vein was initially thought to be a failure in the zVAD-FMK–treated group, but after 10–12 days posttransplant, the majority of these animals became euglycemic, whereas none of the control-treated mice reversed in this group. Although zVAD-FMK was only given for 5 days, it may be that prevention of the majority of posttransplant apoptosis during those first few days allowed these severely marginal mass grafts to begin to exert detectable function once they had revascularized.

The exact cause of chronic graft failure in islet transplantation has been extremely difficult to assess in clinical patients because intraportal delivery of transplanted islets prevents routine biopsy to determine rates of rejection or fibrosis and amyloid deposition over time. When isolated islets are infused into the liver, they must travel through the portal circulation until they become lodged in sinusoids. At this point the transplanted islets will only receive oxygen through passive diffusion until revascularization occurs, which generally requires up to 2 weeks (31,32). However, even in optimized animal models, transplanted islets only achieve at best 50% of the vasculature and perfusion present in the native pancreas (31,32). Thus, most of the transplanted islets fail to engraft, and the remaining marginal islet mass must function maximally to

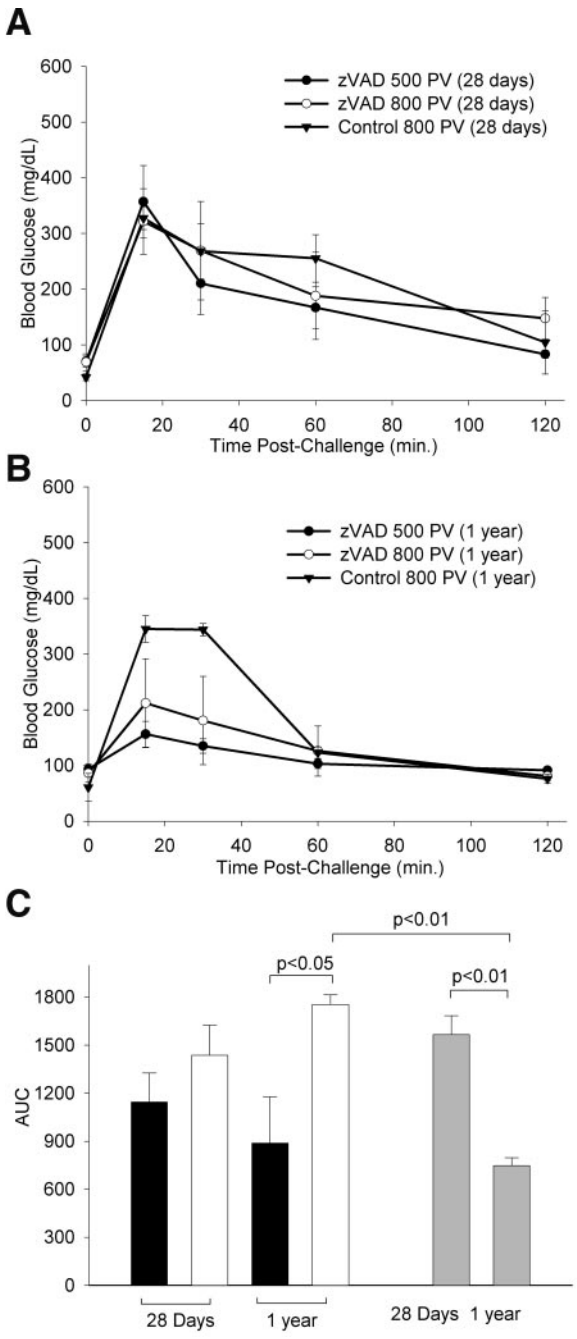

FIG. 5. A short course of zVAD-FMK therapy preserves marginal mass intraportal islet graft function in the long term. To evaluate the function of intraportal (PV) marginal mass islet grafts in zVAD-FMK–treated and control animals posttransplant, IPGTTs were performed at 1 month and 1 year posttransplant. None of the control animals became euglycemic after transplantation of 500 islets, and thus long-term follow-up could not be completed in this cohort. Isolated islets were precultured in zVAD-FMK–containing medium (100 mmol/l) for 2 h before transplantation, and diabetic recipients received daily zVAD-FMK therapy (10 mg/kg s.c.) from days 0 to 5 posttransplant. Control islets were incubated in vehicle-containing medium, and recipients were injected with vehicle from days 0 to 5 posttransplant. *A*: After intraportal transplantation of a nonmarginal mass of 800 islets, no difference was observed between zVAD-FMK–treated and control animals at 28 days posttransplant. Despite a reduced islet mass, zVAD-FMK–treated animals transplanted with 500 islets (●) exhibited similar glucose tolerance as animals that received 800 islets (both control and zVAD-FMK–treated). *B*: Despite a brief treatment period with zVAD-FMK for 5 days posttransplant, islet graft function in zVAD-FMK–treated animals persisted for 1 year posttransplant, whereas a decay was observed in control animals that received an islet mass of 800 (▼). *C*: A significant difference in AUC was observed between zVAD-FMK–treated and control animals that were transplanted with 800 islets by 1 year posttransplant ($P < 0.05$). There was also a significant decrease in AUC in zVAD-FMK–treated animals with an islet mass of 500 from 28 days posttransplant to 1 year posttransplant (▨). Despite the reduced islet mass, zVAD-FMK–treated animals that received 500 islets had a significantly lower AUC at 1 year posttransplant compared with control animals that received 800 islets ($P < 0.01$). Data are representative of $n = 5$ animals per time point, and only euglycemic animals in each cohort were analyzed. ■, 800 intraportal islet grafts with zVAD; □, 800 control intraportal islet grafts; ▨, 500 intraportal islet grafts with zVAD.

maintain euglycemia, which may eventually lead to metabolic dysfunction and exhaustion (22). To determine whether a brief treatment period with a caspase inhibitor could protect islet function in the long term, euglycemic recipients of marginal mass islet grafts were followed over time using IPGTTs. This type of testing may not be ideal in euglycemic islet recipients because a recipient with a significantly larger islet mass may possess the same peak blood glucose value and curve profile as a recipient with a much smaller islet mass. In this study, it was not until 1 year posttransplant that a significant change in AUC for IPGTT was observed, primarily in control animals. Because these animals were all still euglycemic, this finding supports the metabolic exhaustion theory, where a slow progressive loss in β-cell function occurs in the absence of immune rejection. The transient increase in AUC in zVAD-FMK–treated marginal mass recipients suggests that there was some degree of β-cell dysfunction during the latter engraftment period, which eventually resolved once the graft adjusted to the metabolic demands of the recipient (Figs. 3 and 5).

Given the limitations of IPGTTs in islet transplantation, graft insulin reserve and rates of apoptosis were measured posttransplant in zVAD-FMK–treated animals (Fig. 6). These data clearly demonstrate that zVAD-FMK treatment for only a few days significantly preserves graft insulin reserve over time, which supports the data obtained using IPGTTs, where there was no change in AUC at 1 year. Conversely, control animals lost a significant portion of the graft insulin reserve, and this explains the progressive, slow loss of β-cell mass over time, as evidenced by the increase in AUC at 1 year posttransplant (Figs. 2 and 5). Although pretreatment with zVAD-FMK in vitro in the absence of systemic therapy resulted in a significant reduction in TUNEL-positive cells at the time of transplant (Fig. 6*B*), this did not translate into a detectable benefit after transplantation of a marginal islet mass (Fig. 1*D*). Our data suggest that pretreatment with a caspase inhibitor can potently prevent isolation-induced apoptosis, but the stressful transplant procedure and hypoxic environment within the implant site lead to a high level of caspase activation, such that any residual zVAD-FMK present is rapidly consumed and thus of limited benefit in the early posttransplant period. The discrepancy between graft insulin content and apoptosis rates at time 0 and 24 h supports this concept (Fig. 6*A*). Whereas both time points have similar rates of apoptosis, massive β-cell degranulation was only observed at 24 h, suggesting that the dominant injury is ischemia in the early phase posttransplant (Fig. 6). The requirement for zVAD-FMK systemic therapy in the posttransplant period supports this hypothesis.

In the clinical context, improvement of marginal mass islet function could have a broad impact. From a supply standpoint, single-donor infusion could become routine with zVAD-FMK treatment, and low-yield but high-quality

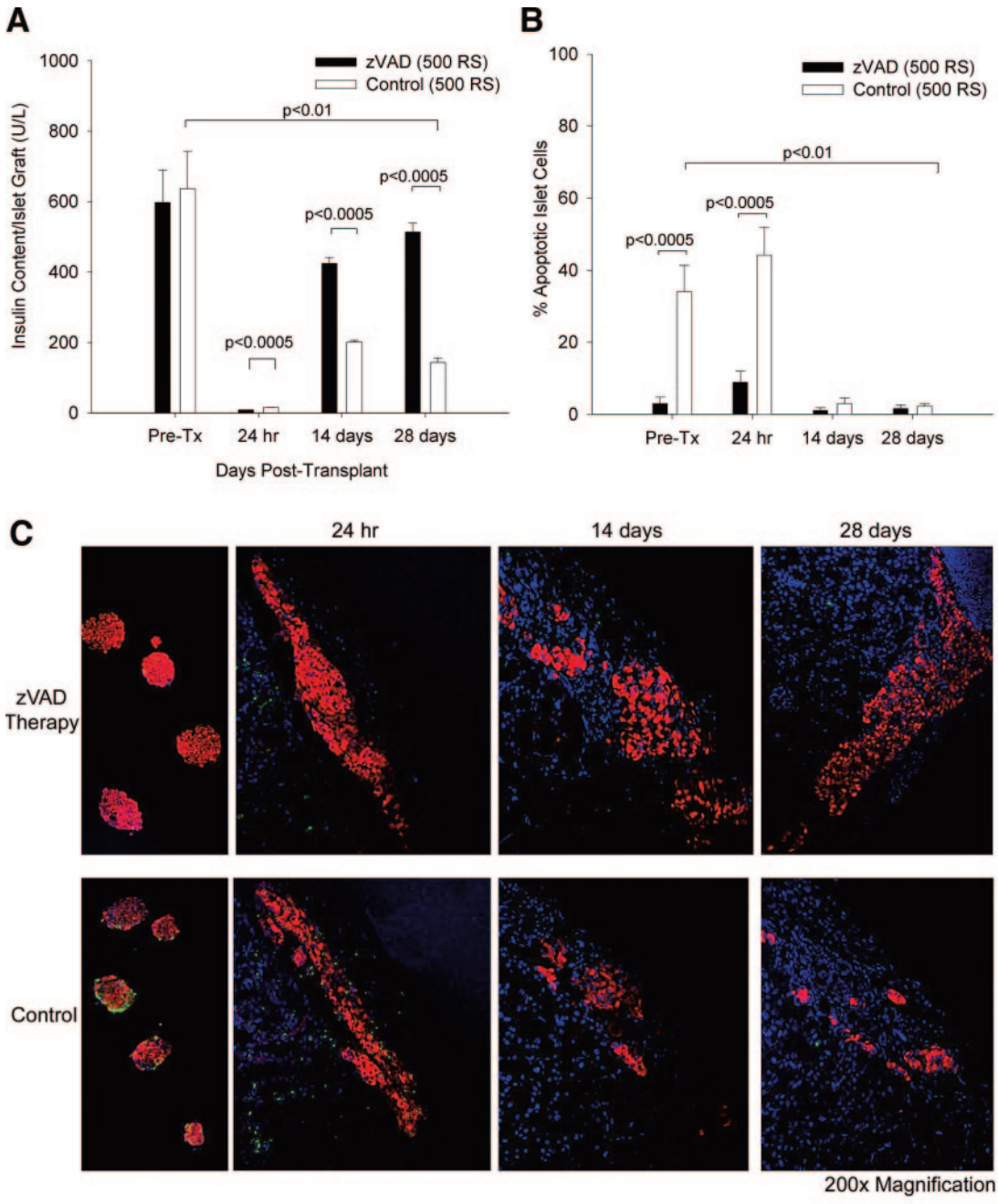

FIG. 6. A short course of zVAD-FMK therapy preserves islet graft insulin content and decreases posttransplant β-cell apoptosis. Islet grafts (500 renal subcapsular) were harvested from control and zVAD-FMK–treated animals at 24 h, 14 days, and 28 days posttransplant and analyzed for graft insulin content and β-cell apoptosis using TUNEL analysis. Before transplantation, isolated islets were precultured in zVAD-FMK–containing medium (100 mmol/l) for 2 h, and diabetic recipients received daily zVAD-FMK therapy (10 mg/kg s.c.) from days 0 to 5 posttransplant. An equivalent aliquot of 500 islets was collected from the same islet preparation and analyzed as well (Pre-Tx). *A*: There was no difference in graft insulin content between zVAD-FMK–treated and control islets before transplantation. At 24 h posttransplant, a severe reduction in graft insulin content was observed in both zVAD-FMK–treated and control animals, which is likely a representation of massive β-cell degranulation during the stressful posttransplant period. At 7 days posttransplant, zVAD-FMK–treated animals had a significantly higher graft insulin content compared with control animals, a finding that persisted through 28 days posttransplant. Control animals demonstrated a progressive decline in graft insulin content posttransplant compared with pretransplant values ($P < 0.01$), whereas no significant change in graft insulin content in zVAD-FMK–treated animals was observed at any time posttransplant compared with pretransplant levels. *B*: zVAD-FMK treatment in vitro significantly reduced the number of TUNEL-positive apoptotic β-cells in isolated islets compared with control islets. There was very little evidence of β-cell apoptosis in zVAD-FMK–treated animals at 24 h posttransplant, whereas control animals exhibited a marked increase in the percentage of apoptotic β-cells ($P < 0.0005$). At either 14 or 28 days posttransplant, there was very little evidence of apoptosis in either zVAD-FMK–treated or control islet grafts. *C*: Representative immunofluorescence sections are shown at 200× magnification for each time point in zVAD-FMK–treated and vehicle control–treated mice. Insulin staining is shown in red, TUNEL staining in green, and all nuclei present in blue. These data are representative of $n = 3$ animals per time point, all transplanted with the same islet preparation on the same day. RS, renal subcapsular islet grafts.

islet preparations might be considered useable for transplantation where they would be considered too minimal at present. Routine use of single-donor infusions would improve the safety of islet transplantation by reducing the risk of bleeding and portal vein thrombosis posttransplant because less tissue would be infused during a single procedure. Also, improvement of marginal mass islet transplantation would alleviate many of the concerns

TABLE 1
No evidence for liver, kidney, or heart toxicity following up to 10 days of treatment

	BL/6 (no treatment)	BL/6 + STZ	BL/6 + STZ + zVAD (10 days' treatment)	BALB (no treatment)	BALB + STZ	BALB/C + STZ + zVAD (5 days' treatment)
Kidney function						
Blood urea nitroge (mmol/l)	14.73 ± 3.59	12.30 ± 0.53	11.33 ± 0.12	8.03 ± 0.09	6.65 ± 0.15	5.90 ± 1.9
Creatinine (umol/l)	27.67 ± 3.18	12.33 ± 1.45	19.33 ± 0.88	13.33 ± 2.40	—	—
Liver function						
Alkaline phophatase (IU/l)	138.00 ± 18.82	200.00 ± 5.03	192.67 ± 2.03	132.00 ± 4.04	124.00 ± 14.00	119.00 ± 20.0
Alanine aminotransferase (IU/l)	51.00 ± 8.51	51.00 ± 2.31	49.00 ± 0.00	41.33 ± 1.45	79.50 ± 1.50	72.00 ± 1.0
γ-Glutamyltransferase (IU/l)	12.00 ± 3.61	9.00 ± 1.73	6.67 ± 0.88	6.33 ± 1.20	9.50 ± 0.50	6.00 ± 6.0
Cardiac function						
Creatine phosphokinase (IU/l)	334.33 ± 87.23	285.33 ± 9.82	187.67 ± 13.37	253.67 ± 10.84	308.50 ± 33.50	195.50 ± 73.5

Sera was collected from two strains of mice (BALB/C and C57BL/6) and analyzed after no treatment, streptozotocin administration, and streptozototin administration followed by zVAD treatment for up to 10 days. Renal function was evaluated using blood urea nitrogen and creatinine. Liver function was evaluated using alkaline phophatase, alanine aminotransferase, and γ-glutamyltransferase. Creatine phosphokinase was used as an indicator of cardiac muscle damage. Creatinine levels were significantly higher in zVAD-treated STZ-C57BL/6 mice compared with STZ-C57BL/6 control animals, suggesting that zVAD can prevent nephrotoxicity ($P = 0.015$). No significant difference was observed for any other values after zVAD treatment in either strain, compared with streptozotocin alone or no treatment. These data are representative of $n = 5$ animals per cohort.

surrounding living donor islet transplantation, where critics argue that the expected decline in long-term function of a marginal living donor islet mass cannot justify the risk to the donor. In cadaveric islet transplantation with a nonmarginal mass, caspase inhibitor therapy might result in tightly controlled blood glucose values, as observed by the animals in our study that received a nonmarginal mass and zVAD-FMK treatment (Fig. 1*A*). This could have a substantial impact on insulin independence rates because many patients return to a small dose of insulin to support their islet graft as it undergoes metabolic decay. The fact that zVAD-FMK therapy is only required transiently in the early posttransplant period suggests that this approach could be rapidly implemented because there would be few (if any) chronic exposure effects to fear. In fact, serological analysis revealed that zVAD-FMK may prevent renal damage caused by known nephrotoxic agents, such as calcineurin inhibitors. Our study suggests that no long-term toxicity exists after a short zVAD-FMK regimen; however, this would need to be confirmed in a large animal model before clinical trials because caspase inhibition, even transiently, may increase the risk for tumorigenicity in the long term. Even if toxicity was found with zVAD-FMK, the development of alternative pan-caspase inhibitors is currently underway, particularly for use in neurodegenerative diseases. As these are developed, our data suggest that they would have significant benefit in islet transplantation.

In summary, these data support the use of pan-caspase inhibitor therapy to broaden the availability of clinical islet transplantation. Enhancing islet engraftment posttransplant should prolong graft longevity, resulting in a more quiescent immunological state and thereby enhancing long-term rates of insulin independence. Inhibition of islet apoptosis in the immediate posttransplant period may reduce the amount and intensity of antirejection therapy, accelerating "accommodation" and drug minimization. If this could be achieved, or if stable immunological tolerance was enhanced through zVAD-FMK treatment, islet transplantation would be potentially safer and therefore more available to a broader spectrum of patients with type 1 diabetes.

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