The Caspase Selective Inhibitor EP1013 Augments Human Islet Graft Function and Longevity in Marginal Mass Islet Transplantation in Mice

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OBJECTIVE—Clinical islet transplantation can provide insulin independence in patients with type 1 diabetes, but chronic graft failure has been observed. This has been attributed in part to loss of ≥60% of the transplanted islets in the peritransplant period, resulting in a marginal implant mass. Strategies designed to maximize survival of the initial islet mass are likely to have major impact in enhancing long-term clinical outcomes. EP1013 (*N*-benzyloxycabonyl-Val Asp-fluoromethyl ketone [zVD-FMK]), is a broad-spectrum caspase selective inhibitor with no observed toxicity in rodents.

RESEARCH DESIGN AND METHODS—The therapeutic benefit of EP1013 was examined in a syngeneic rodent islet transplant model using deceased donor human islets to determine whether the amount of tissue required to restore euglycemia in diabetic animals could be reduced.

RESULTS—EP1013 (combined pretransplant islet culture for 2 h and in vivo treatment for days 0–5 posttransplant) significantly improved marginal islet mass function following syngeneic islet transplantation in mice, even at lower doses, compared with previous studies using the pan-caspase inhibitor *N*-benzyloxycabonyl-Val Ala-Asp-fluoromethyl ketone (zVAD-FMK). EP1013 supplementation in vitro improved human islet yields following prolonged culture and reversed diabetes following implantation of a marginal human islet mass (80–90% reduction) into mice.

CONCLUSIONS—Our data suggest that EP1013 therapy will markedly reduce the islet mass required in clinical islet transplantation, improving insulin independence rates following single-donor infusion. ***Diabetes*** **57:1556–1566, 2008**

The introduction of the Edmonton Protocol in 2000 renewed interest and belief in clinical islet transplantation as a therapeutic strategy to treat patients with brittle diabetes and hypoglycemic unawareness (1,2). More recently, it has become evident that many patients experience partial graft failure over time, characterized by a return to exogenous insulin therapy in >85% of transplanted patients despite persistent C-peptide levels (>0.5 nmol/l) and excellent A1C levels at 5 years posttransplant (1,2). The observation that most patients remain C-peptide positive suggests that nonimmunological mechanisms contribute to chronic loss of functional islet mass over time, particularly since a chronic decline in graft function has been observed in islet autograft recipients (3,4). One of the key features in the success of the Edmonton Protocol was a large islet implant mass of >13,000 islet equivalents (IE)/kg body wt, generally derived from two deceased organ donors (1). This substantial requirement for donor tissue is directly related to early posttransplant islet loss, since transplanted islets must passively engraft over a period of 2–3 weeks following portal infusion. During this time, damaging factors, including hypoxia, tissue factor secretion, inadequate revascularization, and cytokine exposure, contribute to loss of transplanted tissue (2,5,6). It is therefore not surprising that ≥60% of the transplanted β-cell mass is destroyed by apoptosis and necrosis in the early engraftment period, as estimated in both animal models and clinical islet transplantation (4,7–9). Therapeutic agents that can maximize islet survival in the early posttransplant engraftment period are likely to have a major impact in enhancing long-term clinical outcomes.

In an effort to reduce early engraftment loss of transplanted islets, we have explored a number of strategies to inhibit caspases, the key enzymes that regulate apoptosis (10–12). Since the majority of posttransplant β-cell apoptosis occurs in the days or weeks following portal infusion, therapeutic agents that prevent cell death would theoretically only be required for a short period. Also, given the role of apoptosis in other disease processes, therapeutic agents with titratable dosing and ease of withdrawal are particularly attractive. This has lead to the exploration of synthetic peptidyl caspase inhibitors as a means to preserve transplanted islets (13–15). We have shown that the pan-caspase inhibitor *N*-benzyloxycabonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-FMK) potently protects islets in syngeneic marginal-mass transplantation at a daily dose of 10 mg/kg s.c., administered for up to 5 days posttransplant (13). zVAD therapy significantly improved marginal islet mass function and resulted in preservation of graft insulin reserve beyond 1 year posttransplant in mice (13).

Despite the dramatic benefit of zVAD therapy in this model, its unsecured intellectual property rights and lack of selectivity (activity against the cysteine protease calpain I and others) inspired us to search for a potentially more clinically translatable compound (16–18). EP1013 (zVD-FMK), is a dipeptide caspase inhibitor that is selec-

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AUC, area under the curve; CMRL, Connaught Medical Research Laboratories; IE, islet equivalent; IPGTT: intraperitoneal glucose tolerance test; zVAD-FMK: *N*-benzyloxycabonyl-Val Ala-Asp-fluoromethyl ketone; zVD-FMK, *N*-benzyloxycabonyl-Val Asp-fluoromethyl ketone.

tive for caspases, including 1, 3, 6, 7, 8, and 9, and has no detectable activity against other proteases such as calpain I, cathepsin B, renin, or thrombin (19,20). More importantly, this compound has been shown to have markedly enhanced potency in vitro and in vivo when compared with zVAD and no observed toxicity in rodents (19,20). In the present study, the efficacy of EP1013 treatment was explored using syngeneic marginal-mass mouse islet transplantation and primary human islet transplantation into immunodeficient mice.

RESEARCH DESIGN AND METHODS

Animals. Immunodeficient B6-RAG$^{-/-}$ mice (B6.129S7-*Rag1*^{tm1Mom}/J) were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions. BALB/C mice were obtained from a colony maintained by the University of Alberta and housed under conventional conditions. All animals were cared for according to the guidelines of the Canadian Council on Animal Care, and ethical approval was obtained from the animal welfare committee at the University of Alberta.

Human islet isolation. Research cadaveric donor pancreata were removed with prior informed written consent and with human research ethics board approval at the University of Alberta. Organs were stored in chilled University of Wisconsin solution or histidine-tryptophan-ketoglutarate preservation solution before islet isolation. Islet isolation was performed as previously described (1,21,22). The islet yield and purity were assessed using dithizone, and viability was assessed with Syto green/ethidium bromide staining using established methods (Cedarlane Laboratories and Sigma-Aldrich, ON, Canada) (23,24). The islet preparations ranged from 50 to 75% purity and 80 to 85% viability. Islet counts were completed in triplicate with dithizone staining using a standard diameter of 150 μm. Connaught Medical Research Laboratories (CMRL) 1066 culture medium was used for all experiments, unless otherwise noted (11).

Caspase inhibitor therapy. EP1013 was obtained from Epicept (San Diego, CA), and zVAD-FMK was obtained from Bachem (Torrance, CA). Stock preparations of both EP1013 and zVAD-FMK were prepared in sterile DMSO to make the compounds water soluble. For in vitro and in vivo experiments, stock solutions were diluted into sterile saline to produce a final concentration of 1 mg/ml. For control experiments, a solution containing DMSO in sterile saline at the same concentration as caspase inhibitor stocks was used.

Glucose-stimulated insulin release assays. Triplicate aliquots containing 1,000 human IE were washed in low-glucose medium (CMRL containing 2.8 mmol/l D-glucose supplemented with 10% FCS). The media was then replaced with either low- or high-glucose medium (Connaught Medical Research Laboratories (CMRL) containing 20 mmol/l D-glucose supplemented with 10% FCS) and incubated for 1 h at 37°C, 5% CO_2. Aliquots from the supernatant were analyzed for human insulin content using a radioimmunoassay (Linco Research, St. Charles, MO). In each experimental condition, the fold stimulation was calculated by dividing the mean insulin released from islets cultured in the high-glucose medium by the mean insulin released from islets cultured in the low-glucose medium in parallel.

Enzymatic assays. Calpain 1 and 2 activity was measured using the fluorimetric kit QIA120, and cathepsin B activity was measured using the fluorimetric kit CBA001, according to the manufacturer's protocol (both from EMD Biosciences, Gibbstown, NJ). For each experimental condition, 1,000 human IE were analyzed in triplicate. The percent activity was determined by dividing the mean relative fluorescence units obtained for each experimental condition by that obtained for untreated islets.

Islet transplantation studies. Mouse islets were isolated using established methods (25). Streptozotocin (Sigma-Aldrich Canada, Mississauga, ON) was administered to recipient mice to induce diabetes (BALB/C: 250 mg/kg i.p. and B6-RAG$^{-/-}$: 180 mg/kg i.p.), and animals were considered to be diabetic after two consecutive blood glucose measurements ≥325 mg/dl using a OneTouch Ultra glucometer (Lifescan Canada, Burnaby, BC). Before transplantation, mouse islet preparations were incubated in medium containing caspase inhibitor (EP1013 or zVAD at 100 μmol/l, in DMEM with 10% FCS; Invitrogen Canada) or vehicle for 2 h, and 250 islets (renal subcapsular) or 500 islets (intraportal) were implanted. For human islet studies, islets were cultured for 72–96 h with EP1013 (100 μmol/l) or vehicle control at 37°C to allow time for recipient mice to become chemically diabetic. After this incubation step, islets were washed with PBS, counted using dithizone, and transplanted. Transplant recipients were given a single subcutaneous injection of either vehicle or caspase inhibitor on the day of transplant and for 5 days thereafter. Euglycemic animals (blood glucose <200 mg/dl) from each cohort were selected randomly (n = 3), and the graft-bearing kidney was removed to establish that the islet graft was functional, as determined by a return to hyperglycemia postnephrectomy.

Human C-peptide assays. Nonfasting serum was collected from mice via tail vein bleeds, and the serum human C-peptide levels were quantified in triplicate using human C-peptide enzyme-linked immunosorbent assay kits (Alpco Diagnostics, Windham, NH).

Glucose tolerance tests. Transplanted animals were fasted for 16–20 h and injected intraperitoneally with 50% dextrose in Ringer's solution at 2 g/kg body wt. Blood glucose levels were assessed 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. To determine the total graft insulin content, islet grafts were harvested from the kidney capsule and stored at −80°C until bulk analysis could be performed using an insulin radioimmunoassay (Linco Research), as reported previously (26).

Statistics. SigmaPlot 10 and SigmaStat 3.5 (SPSS) were utilized for all statistical analysis in this study, and results are expressed as means ± SE. Student's t tests or Mann-Whitney rank-sum tests for paired and unpaired data were used to compare results in each experimental condition, and an ANOVA was used to analyze multiple groups. Euglycemic Kaplan-Meier survival analyses were compared using the log-rank test.

RESULTS

EP1013 therapy enhances marginal mass function in syngeneic mouse islet transplantation. The first objective in this study was to determine whether EP1013 could replicate or improve marginal islet mass engraftment rates reported using the pan-caspase inhibitor zVAD (13). In the present study, the efficacy of EP1013 at a similar dose (10 mg/kg) as well as two reduced doses (3 and 1 mg/kg) was examined and compared with the known effective dose of zVAD (10 mg/kg) (13). This dose titration was relevant, since EP1013 has been shown to have enhanced potency compared with zVAD (20). As shown in Fig. 1*A*, only 24% of control animals achieved euglycemia with 250 islets (n = 2/9), while systemic treatment with EP1013 for 6 days significantly improved marginal islet mass function at 10 mg/kg (82%, n = 9/11), 3 mg/kg (100%, n = 10/10), and 1 mg/kg (100% n = 9/9), which was comparable with results obtained with zVAD at 10 mg/kg (100%, n = 8/8). Two animals in the 10 mg/kg EP1013 treatment group exhibited primary islet graft nonfunction, but the diabetes reversal rate for this group was not significantly different from the 3 mg/kg EP1013, 1 mg/kg EP1013, or 10 mg/kg zVAD groups. Mean blood glucose levels in both EP1013- and zVAD-treated animals were tightly controlled by the islet graft (Fig. 1*B*). zVAD-treated animals had lower mean blood glucose levels in the first week posttransplant, but the difference was not significant when compared with EP1013-treated animals.

Once the protective benefit of EP1013 therapy had been confirmed using renal subcapsular marginal-mass islet transplantation, the experiments were extended to include the clinically relevant intraportal transplant site. A higher implant mass is required to reverse diabetes at this site compared with subrenal capsular transplantation, which has been linked to nonspecific inflammatory response postinfusion secondary to islet embolization and resulting liver ischemia in mice (27,28). The benefit of EP1013 was even more pronounced than what was observed using zVAD following intraportal transplantation, with 100% of the EP1013-treated animals achieving euglycemia with 500 islets at both 10 mg/kg (n = 5/5) and 3 mg/kg (n = 5/5), while only 62.5% of zVAD-treated animals at 10 mg/kg (n = 5/8) and 0% of the controls (n = 0/7) established euglycemia (P = 0.045 for EP1013 at 10 mg/kg versus zVAD by log-rank) (Fig. 2*A*). Mean blood glucose levels for EP1013-treated animals with intraportal grafts (Fig. 2*B*) were similar to those observed in renal subcapsular grafts (Fig.

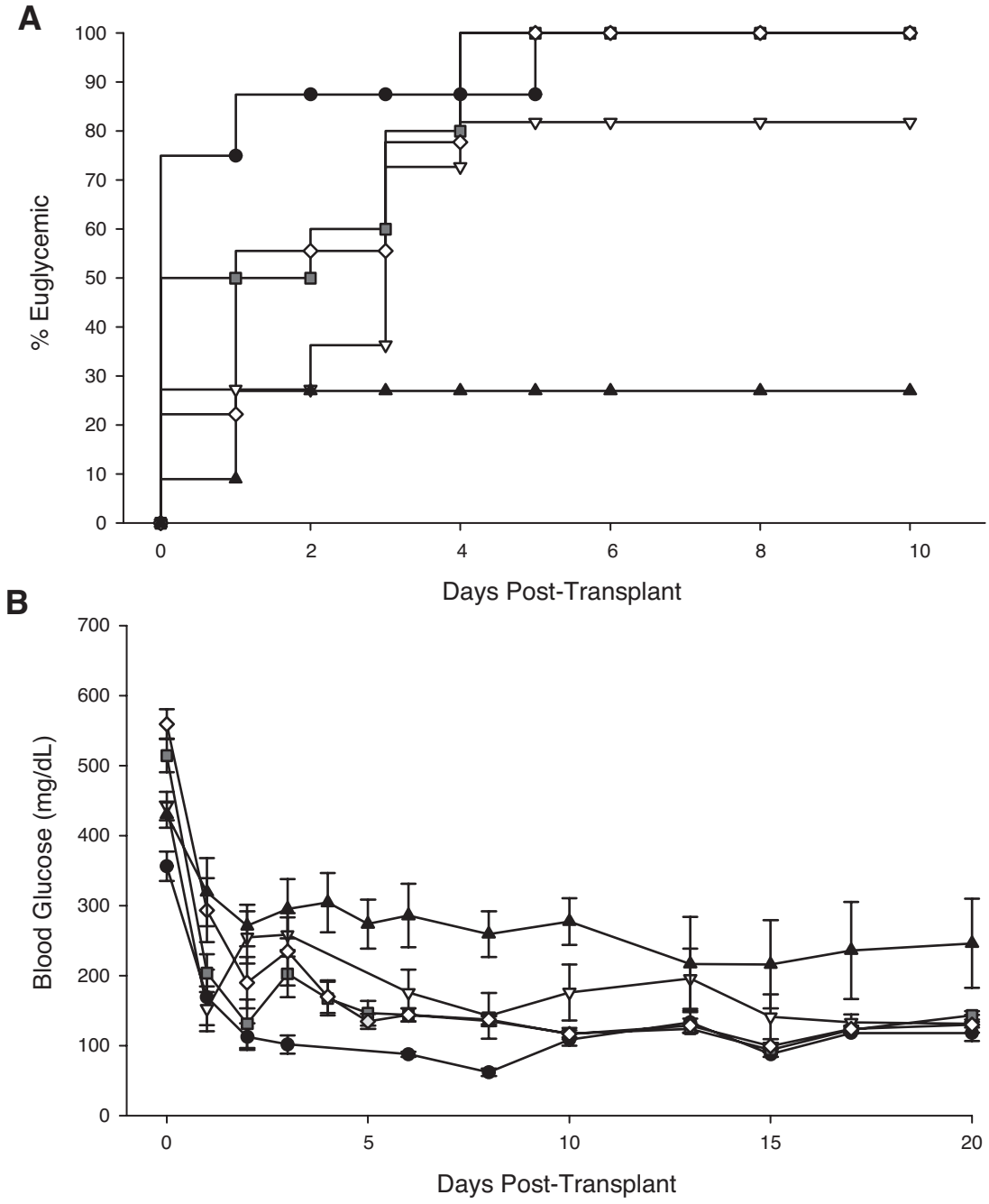

FIG. 1. EP1013 therapy promotes survival of syngeneic renal subcapsular marginal mass islet grafts and has equal efficacy at reduced doses as compared with zVAD. Marginal mass islet grafts containing 250 syngeneic islets that had been cultured for 2 h in EP1013 or zVAD at 100 μmol/l or control medium were transplanted under the kidney capsule in chemically diabetic recipients. Recipients received EP1013 (10, 3, or 1 mg/kg s.c.), zVAD (10 mg/kg s.c.), or vehicle from days 0 to 5 posttransplant. *A*: While vehicle control animals only became euglycemic 28% of the time, 100% of zVAD-treated (10 mg/kg) and EP1013-treated (3 or 1 mg/kg) animals became normoglycemic with a marginal islet mass ($P < 0.001$ for all treated groups vs. control). There were two animals in the 10 mg/kg EP1013 treatment group that had primary nonfunction, but the overall rate of reversal was not significantly different from the 3 or 1 mg/kg EP1013 cohorts. ●, zVAD (10 mg/kg, $n = 8/8$); ▽, EP1013 (10 mg/kg, $n = 9/11$); ▨, EP1013 (3 mg/kg, $n = 10/10$); ◇, EP1013 (1 mg/kg, $n = 9/9$); ▲, vehicle control ($n = 2/9$). *B*: EP1013 therapy resulted in rapid reversal of hyperglycemia and tight blood glucose control following transplantation of a marginal islet mass. There was no difference in blood glucose levels or stability as measured by SE for animals treated with either 10, 3, or 1 mg/kg EP1013, although there was a brief period where zVAD-treated animals exhibited lower average blood glucose levels. Data are presented as means ± SE. Recovery nephrectomies performed in randomly selected treated or control animals at >180 days posttransplant resulted in 100% return to hyperglycemia (data not shown). ●, zVAD (10 mg/kg); ▽, EP1013 (10 mg/kg); ▨, EP1013 (3 mg/kg); ◇, EP1013 (1 mg/kg); ▲, vehicle control.

1*B*) and demonstrate the early posttransplant instability of intraportally transplanted islets in euglycemic animals (EP1013 at 10 mg/kg; ▨), even in the presence of caspase inhibitor therapy. The low dose of EP1013 at 1 mg/kg was not explored in this model due to the delayed graft function observed using 3 mg/kg.

EP1013 therapy enhances functional syngeneic islet mass and promotes longevity of islet graft function. As an indicator of functional islet mass, graft-bearing kidneys were removed from transplanted animals 2 weeks posttransplant and analyzed for total insulin content. As shown in Fig. 3*A*, total graft insulin content was similar in

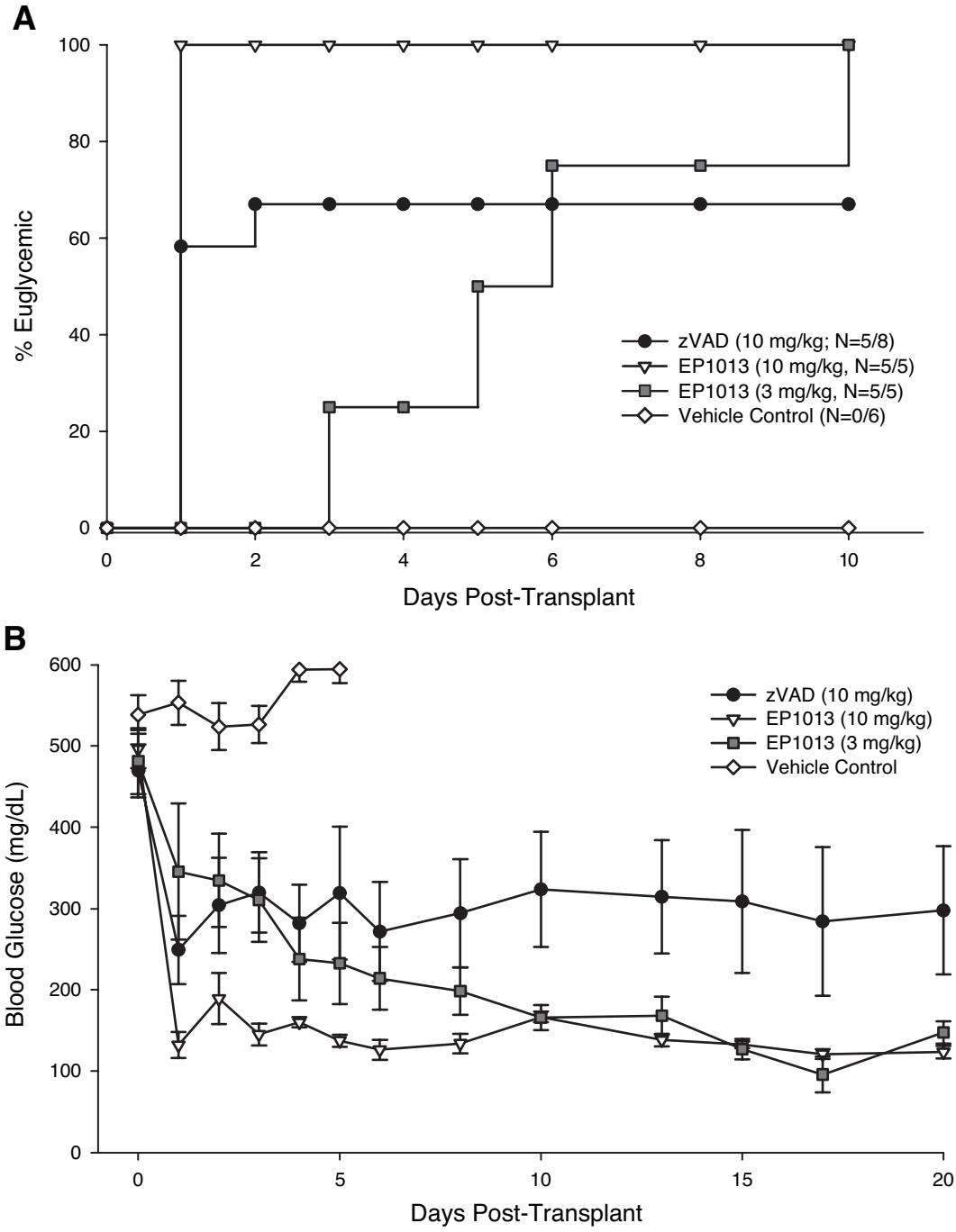

FIG. 2. EP1013 therapy enhances survival of syngeneic intraportal marginal mass islet grafts and has greater potency than zVAD, even at reduced doses. Marginal mass islet grafts containing 500 syngeneic islets that had been cultured for 2 h in EP1013- or zVAD-supplemented (100 μmol/l) or control medium were transplanted into the portal vein of chemically diabetic recipients. Recipients received EP1013 (10 or 3 mg/kg s.c.), zVAD (10 mg/kg s.c.), or vehicle from days 0 to 5 posttransplant. *A*: While vehicle control animals remained severely hyperglycemic following implantation of 500 islets into the portal circulation, 100% of EP1013-treated (10 or 3 mg/kg) animals became normoglycemic ($P < 0.001$ for both EP1013 treated groups vs. controls). EP1013 therapy at 10 and 3 mg/kg resulted in significantly more diabetes reversal even when compared with zVAD treatment (10 mg/kg), which resulted in a 63% return to normoglycemia ($P < 0.05$ for both EP1013 treated groups vs. zVAD). It should be noted that EP1013 treatment at 3 mg/kg resulted in a longer time to reverse diabetes in transplanted animals as compared with animals receiving a higher dose (10 mg/kg). *B*: EP1013 therapy resulted in excellent glycemic control following transplantation of a marginal islet mass, as measured by mean nonfasting blood glucose levels. Data are presented as means ± SE.

animals that received EP1013 injections at 10, 3, or 1 mg/kg, and each of these treatment groups was significantly higher than vehicle-treated animals ($P < 0.001$ for each dose vs. control). The mean insulin content was similar to that previously observed in zVAD-treated animals (13). Graft insulin content for islets transplanted into the portal vein was not determined due to difficultly in insulin extraction at this site, particularly with a marginal implant mass.

To further understand the efficacy of each EP1013 dose in preventing chronic dysfunction of marginal-mass islet grafts, intraperitoneal glucose tolerance tests (IPGTTs) were performed sequentially posttransplant (Fig. 3*B* and *C*). Since very few vehicle-treated animals became eugly-

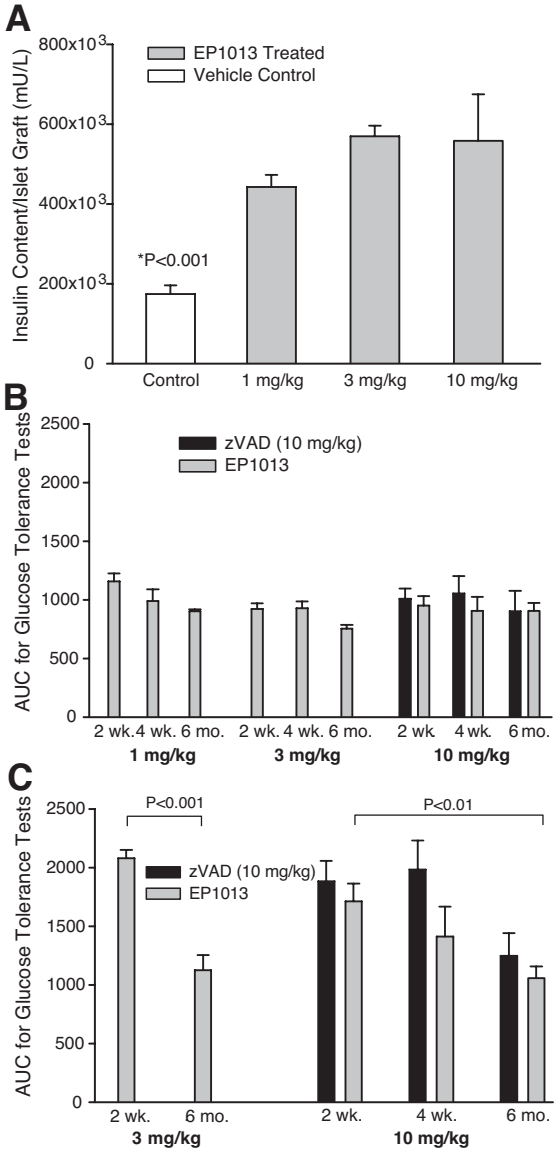

FIG. 3. EP1013 therapy in the early posttransplant period increases the functional syngeneic islet mass and preserves graft function over time. *A*: To assess the functional islet mass in transplanted animals, graft bearing kidneys were harvested from normoglycemic EP1013-treated and control animals, and the islet graft was removed for measurement of total insulin content. There was no difference in graft insulin content in animals that had received 10, 3, or 1 mg/kg EP1013, and all three treatment groups had significantly more insulin content than vehicle control animals ($P < 0.001$ for each drug dose). To evaluate the function of marginal mass islet grafts in EP1013-treated animals, IPGTTs were performed at several time points posttransplant in euglycemic animals. AUC was calculated as an indicator of graft stability. Very few control animals became normoglycemic following transplantation of a marginal islet mass; thus, long-term follow-up of these animals was not possible. ▨, EP1013 treated; □, vehicle control. *B*: Following transplantation of a marginal mass under the kidney capsule, EP1013 treatment at 10, 3, and 1 mg/kg (▨) resulted in stable IPGTT AUC through 6 months posttransplant, which was comparable with that obtained using zVAD (10 mg/kg) (■) as previously reported (13). *C*: Following transplantation of a marginal mass into the portal circulation, EP1013 treatment at 10 mg/kg (▨) resulted in IPGTT AUC that was comparable with that obtained using zVAD (10 mg/kg) (■). Intraportal grafts in both treatment groups demonstrated an increased functional reserve over time, as indicated by a decrease in AUC, but it should be noted that the AUC at 6 months following intraportal islet transplantation was comparable with that observed in renal subcapsular grafts (*B*). A reduced dose of EP1013 (3 mg/kg) resulted in IPGTT AUC that was comparable with those observed in both zVAD (10 mg/kg) and EP1013 (10 mg/kg) groups. Data are representative of at least $n = 4$ animals per time point, and only euglycemic animals in each cohort were evaluated.

cemic with a marginal implant mass, these animals could not be analyzed. Data in Fig. 3*B* demonstrate that EP1013 treatment at 10, 3, and 1 mg/kg results in stable and robust marginal mass renal subcapsular islet graft function, with no difference in AUC at 2 weeks, 4 weeks, and 6 months posttransplant between dosing groups or zVAD-treated animals. Further analysis of 3 and 1 mg/kg EP1013 treatment groups at >1 year posttransplant exhibited similar AUC values (13- to 15-month AUC was 1,147.9 ± 140.6 for the 3 mg/kg group and 1,093.3 ± 75.4 for the 1 mg/kg group). As shown in Fig. 2, intraportal marginal-mass islet grafts require a longer engraftment period, and as such these grafts had elevated AUC for IPGTTs up to 1 month posttransplant (Fig. 3*C*). However, graft function improved over time in both the 10 and 3 mg/kg EP1013 treatment groups, resulting in AUC values that were similar to those in renal subcapsular islet transplantation by 6 months posttransplant (Fig. 3*B* and *C*).

While a theoretical risk for cancer development exists when caspase inhibitors are given systemically, our studies do not indicate any significant increase in cancer rates in BALB/C mice followed for >1 year. Among animals that had received EP1013 for 5 days posttransplant, 10 of 14 animals were still healthy >14 months posttransplant. Four animals died unexpectedly with postmortem diagnoses, including cirrhosis (63 weeks), peritoneal infection (54 weeks), epithelial tumor (renal origin, 72 weeks), and lymphosarcoma (73 weeks). Only five animals in the vehicle control group were followed >1 year posttransplant, since most of these animals remained diabetic following implantation of a marginal islet mass, but of these two died unexpectedly with postmortem diagnosis of lymphosarcoma (54 and 55 weeks). There was no difference in cancer development between EP1013-treated and control animals (2 of 14 [14.3%] in the EP1013 treated group versus 2 of 5 [40%] in the vehicle control group, $P = 0.174$ by log-rank). It is more likely that the development of tumors in this model system can be linked to streptozotocin treatment, which has been shown to result in malignancy in 30% of BALB/c mice even after one 200 mg/kg injection (29).

EP1013 improves human islet yields and viability following prolonged culture and enhances marginal-mass human islet graft function. Based on the encouraging findings using EP1013 in syngeneic marginal-mass islet transplantation in mice, the benefit of caspase inhibitor therapy was examined using deceased donor human islets. The dose of 3 mg/kg EP1013 was used for all human islet studies based on the data shown in Fig. 3. For these studies, the human islets were maintained in culture with or without EP1013 (100 μmol/l) for 72–96 h until recipient animals were confirmed to be diabetic. As shown in Fig. 4, incubation with EP1013 consistently resulted in higher islet yields following prolonged culture (Fig. 4*A*), and these islets had a significantly increased viability (Fig. 4*B* and *C*). Following renal subcapsular transplantation into mice, nonmarginal implantation masses of 2,500 IE (80 IE/g body wt) and 1,500 IE (50 IE/g body wt) resulted in a 100% reversal of diabetes in both EP1013-treated and vehicle-treated animals, although the reduced mass of 1,500 IE required approximately twice as long to reverse in control animals (15.8 ± 1.9 days) versus EP1013-treated animals (8.2 ± 3.6 days) (Fig. 5*A*). To test the efficacy of EP1013 in marginal-mass human islet grafts, 150–300 IE were transplanted (5–10 IE/g body wt; 80–90% mass reduction). As shown in Fig. 5*B*, 70% of EP1013-treated

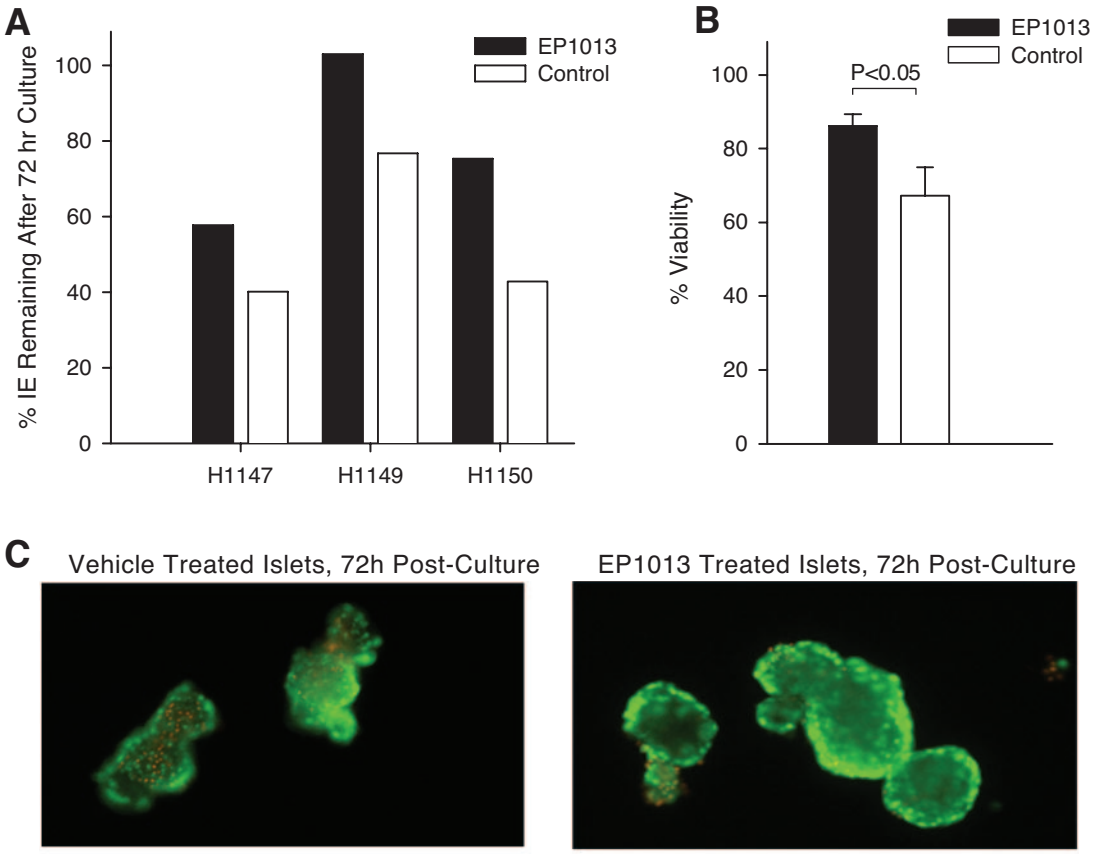

FIG. 4. Addition of EP1013 to culture medium improves human islet yields and viability following prolonged culture periods. Human islets were maintained in culture for 72–96 h with EP1013 (100 μmol/l) or vehicle-supplemented medium while recipient mice were rendered chemically diabetic using streptozotocin. Human islet preparations were obtained 4–24 h following isolation and ranged from 50 to 75% purity and 80 to 85% viability at the time of receipt. *A*: Following 72 h culture in the presence of EP1013, every human islet preparation received had a higher yield of IE (58–100% of initial quantity) when compared with vehicle control–treated islets (40–76% of initial quantity). ■, EP1013; □, control. *B*: Following 72 h of culture, human islets maintained in the presence of EP1013 had a significantly higher viability (86.12 ± 3.22%) compared with vehicle control (67.24 ± 7.67%), as measured by Syto green/ethidium bromide staining. ■, EP1013; □, control. Representative ×100 images of viability staining are shown in *C*, with viable cells staining green and dead cells staining red. The data presented are representative of three independent human islet preparations.

animals returned to normoglycemia by 3 weeks posttransplant, while all of the vehicle-treated animals remained severely diabetic ($P < 0.005$ by log-rank). Interestingly, despite the dramatically reduced islet mass, EP1013-treated animals that received 150–300 IE became euglycemic 15.8 ± 2.5 days posttransplant, which was comparable with the time observed in control animals receiving 1,500 IE. Mean blood glucose levels demonstrate that early posttransplant graft function was quite variable, which can likely be attributed to the variations among the human islet tissue used in this study (Fig. 5*C* and *D*).

EP1013 therapy preserves human islet mass and promotes longevity of human islet graft function. To confirm that EP1013 promotes marginal mass human islet function by preserving implanted islets during engraftment, islet grafts were harvested following nephrectomy and subjected to insulin extraction. Despite a similar functional outcome for nonmarginal mass islet grafts of 1,500–2,500 IE in EP1013-treated and control animals, there was a significantly higher graft insulin content in EP1013-treated animals (Fig. 6*A*). Following further reduction in implantation mass from 1,000 to 150–300 IE, the protective benefit of EP1013 treatment was even more evident ($P < 0.005$ for 1,000 IE and $P < 0.001$ for 150–300 IE). As an additional indicator of functional human mass posttransplant, nonfasting serum human C-peptide levels were measured 28 days following implantation. Data in Fig. 6*B* illustrates that EP1013-treated animals had significantly higher human C-peptide levels at every islet mass tested compared with controls, even when 100% of both the EP1013-treated and vehicle-treated animals returned to normoglycemia, as shown in Fig. 5. To characterize the functional reserve of marginal-mass human islet grafts in EP1013-treated animals posttransplant, IPGTTs were performed in euglycemic animals at 30 days posttransplant, and AUC was calculated as an indicator of graft stability. While the difference did not reach statistical significance for AUC between EP1013- and vehicle-treated animals receiving 2,500, 1,500, or 1,000 IE, the mean combined AUC for all three cohorts was significantly less in EP1013-treated animals ($P < 0.02$) (Fig. 6*C*). Since no control animals became euglycemic following transplantation of 150–300 IE, comparison of EP1013-treated and control animals in this group could not be determined.

zVAD impairs insulin secretion in human islets. To determine whether the enhanced benefit of EP1013 over zVAD was related to its selectivity for caspases compared with zVAD, which has some activity against other cysteine proteases including calpains and cathepsins, further in vitro experiments were carried out. Following 24 h incubation with zVAD, a significant decrease in calpain 1 and 2 activity (zVAD-treated islets had 17.3 ± 4.9% of control

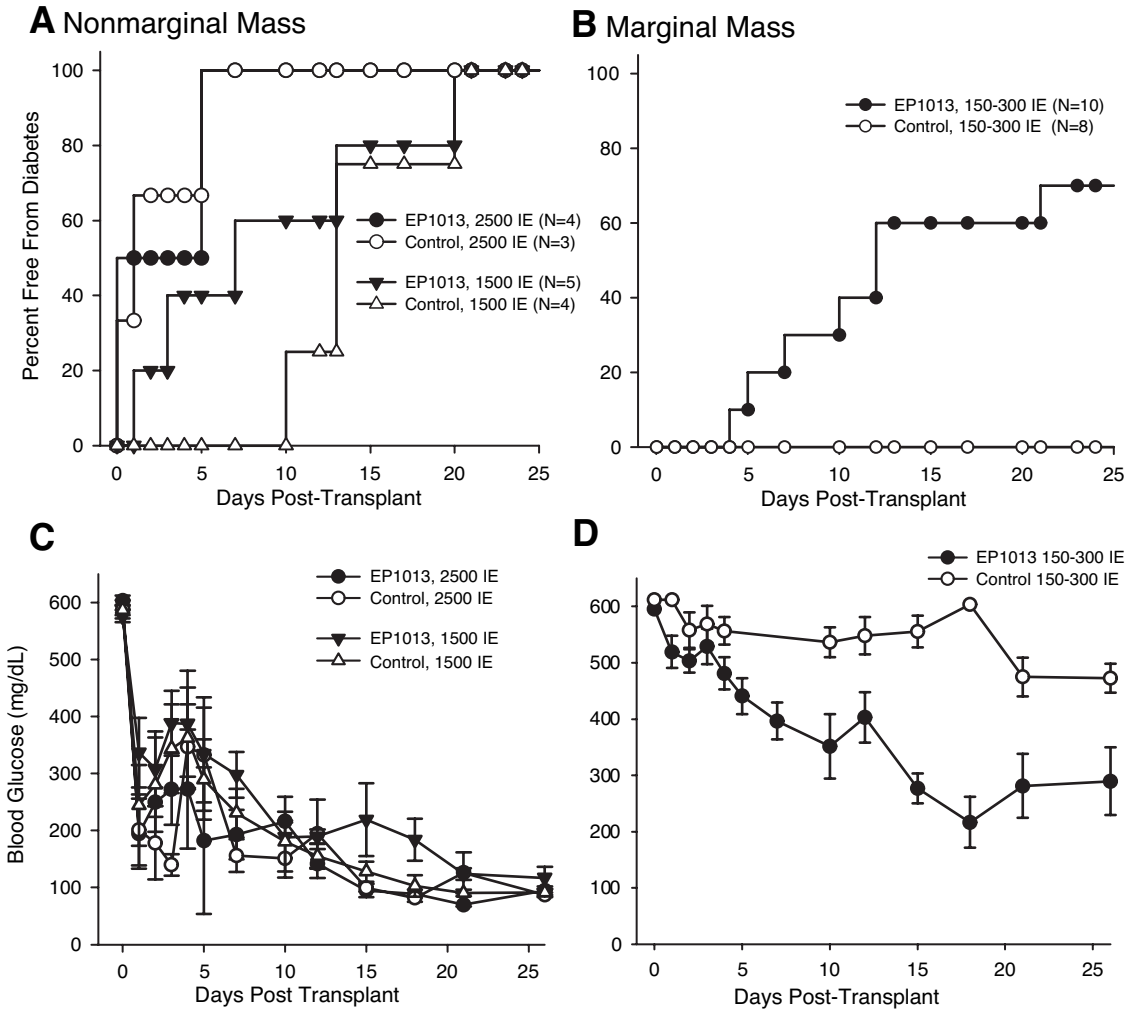

FIG. 5. EP1013 therapy preserves marginal-mass human islet graft function, reducing the number of islets required to reverse diabetes in immunodeficient mice. Human islets were maintained for 72–96 h in either EP1013 or vehicle-supplemented culture medium, while recipient mice were rendered chemically diabetic using streptozotocin. Human islet grafts containing 2,500, 1,500, or 150–300 IE were transplanted under the kidney capsule of confirmed diabetic B6-RAG$^{-/-}$ recipients (combined data from three independent preps). *A*: Implantation of a nonmarginal mass of 2,500 or 1,500 IE resulted in 100% return to normoglycemia in both EP1013- and vehicle-treated animals. However, the reduced mass of 1,500 IE required approximately twice as long to reverse in control animals (15.8 ± 1.9 days) versus EP1013-treated animals (8.2 ± 3.6 days). ●, EP1013, 2,500 IE (n = 4); ○, control, 2,500 IE (n = 3); ▼, EP1013, 1,500 IE (n = 5); △, control, 1,500 IE (n = 4). *B*: Transplantation of an 80–90% reduced islet mass of 150–300 IE resulted in 0% return to normoglycemia in control animals, whereas 70% of EP1013-treated animals returned to normoglycemia. EP1013-treated animals that received 150–300 IE became euglycemic on 15.8 ± 2.5 days posttransplant, which was comparable with the time observed in control animals receiving 1,500 IE. ●, EP1013, 150–300 IE (n = 10); ○, control, 150–300 IE (n = 8). *C* and *D*: Mean blood glucose levels for animals transplanted with human islets are shown. Early posttransplant graft function was quite variable, which can likely be attributed to the variations among the human islet tissue used in this study. Three independent human islet preparations were used for transplantation in these studies. *C*: ●, EP1013, 2,500 IE; ○, control, 2,500 IE; ▼, EP1013, 1,500 IE; △, control, 1,500 IE. *D*: ●, EP1013, 150–300 IE; ○, control, 150–300 IE.

islet activity; $P < 0.001$ vs. EP1013-treated islets and vehicle control islets) (Fig. 7*A*) and to a lesser extent cathepsin B was observed (zVAD-treated islets had 38.9 ± 4.7% of control islet activity; $P < 0.001$ vs. EP1013-treated islets and vehicle control islets) (Fig. 7*B*). Since the calpain enzymes have been implicated in insulin secretion pathways, glucose-stimulated insulin secretion assays were carried out after the 24-h incubation period (18). As shown in Fig. 7*C*, zVAD inhibited insulin secretion in the presence of a high-glucose medium (3,055.3 ± 65.7 mU/l), but this difference was not significant when compared with untreated control islets (4,009.6 ± 116.9 mU/l; P = 0.07). However, when the stimulation index (S_I) was calculated, zVAD-treated islets had a significantly lower S_I, demonstrating that zVAD may impair β-cell function (1.24 ± 0.05, *$P < 0.01$ vs. EP1013- and vehicle-treated islets).

DISCUSSION

The present study demonstrates that the broad-spectrum caspase selective inhibitor EP1013 potently enhances marginal-mass islet graft function posttransplant, as confirmed by significantly higher graft insulin content and stable graft function using both murine syngeneic islets and human islets transplanted into immunodeficient mice. These data also indicate that EP1013 is more effective than the pan-caspase inhibitor zVAD, particularly following transplantation within the portal circulation, the clinical implantation site. Dose titration studies demonstrate that reduced EP1013 doses of 1–3 mg/kg are sufficient to preserve transplanted islets and graft functional reserve over time, confirming previous data that EP1013 is many-fold more potent than zVAD (20). More importantly, the amount of human islet tissue required to reverse diabetes

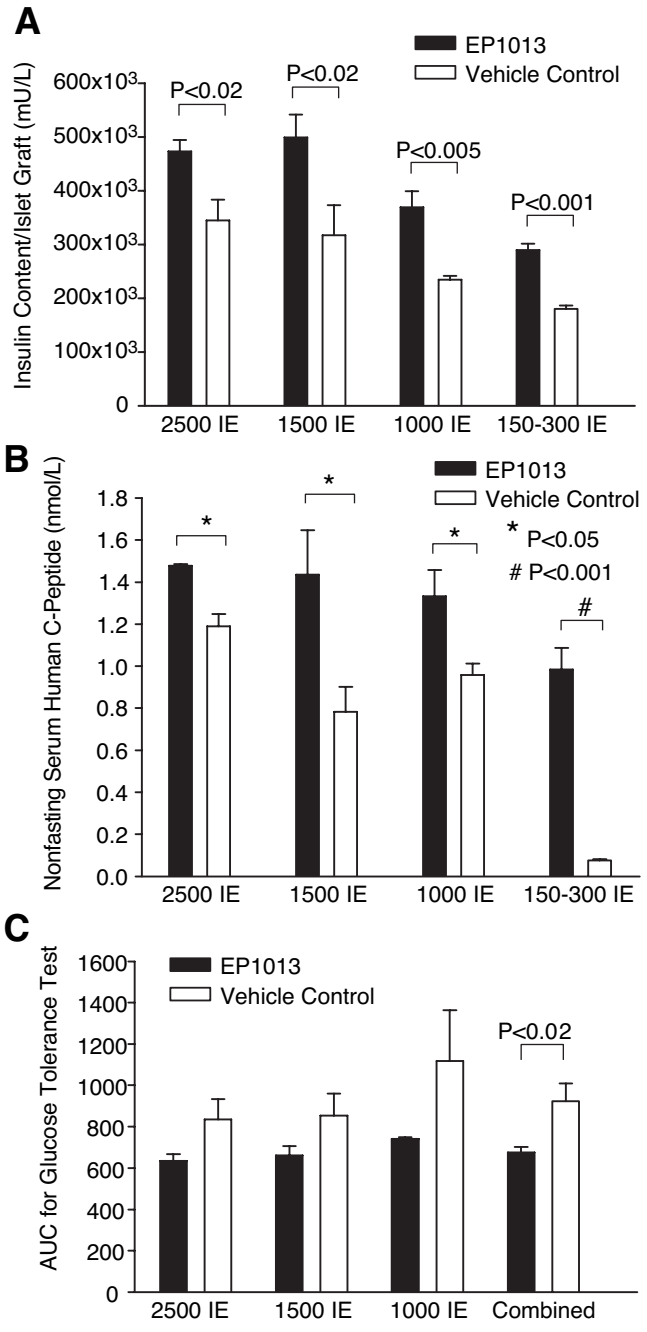

FIG. 6. EP1013 therapy preserves human islet mass and improves graft function. *A*: To assess the functional human islet mass in transplanted animals, graft-bearing kidneys were harvested from normoglycemic EP1013-treated and control animals at 30 days posttransplant, and the islet graft was removed for insulin extraction. Even though nonmarginal mass human grafts containing 2,500 or 1,500 IE reversed diabetes in 100% of both EP1013-treated and control animals (Fig. 5), graft insulin content was significantly higher in EP1013-treated animals. When the islet mass was decreased further to 1,000 or 150–300 IE, the total graft insulin content was also significantly higher in EP1013-treated animals as compared with controls. Human islet grafts containing 150–300 IE from EP1013-treated animals had insulin content comparable with that observed in control animals with 1,500 IE. *B*: Nonfasting serum human C-peptide levels were measured as a surrogate marker of islet graft function. EP1013-treated animals had significantly higher human C-peptide levels at every islet mass tested as compared with controls, even when 100% of both the EP1013-treated and vehicle-treated animals returned to normoglycemia as shown in Fig. 5. Human C-peptide levels in EP1013-treated animals with 150–300 IE were comparable with levels observed in control animals with 1,500 IE. *C*: To evaluate the function of marginal mass human islet grafts in EP1013-treated animals posttransplant, glucose tolerance tests (intraperitoneally) were performed in euglycemic animals at 30 days posttransplant. AUC was calculated as an indicator of graft stability. While there was no statistical difference for AUC between EP1013- and vehicle-treated animals receiving 2,500, 1,500, or 1,000 IE, the mean combined AUC for all three cohorts was significantly less in EP1013-treated animals. Since no control animals became euglycemic following transplantation of 150–300 IE, comparison of EP1013-treated animals and control animals in this group could not be determined. Data are representative of at least three animals per group, and only euglycemic animals in each cohort were evaluated. *A–C*: ■, EP1013; □, control.

in mice was reduced by 80–90% with the inclusion of a brief treatment period with EP1013, resulting in graft insulin content and nonfasting C-peptide levels that were similar to control animals with a 5- to 10-fold larger implant mass of 1,500 IE. Despite having a 10-fold–increased implant mass, animals with 1,500 to 2,500 IE grafts had only ~50% more total insulin content as compared with 150–300 IE grafts. This observation is likely related to the relatively high packed-cell volume required for higher islet equivalent grafts, which must be transplanted at several subcapsular sites and places the islets in close proximity to contaminating exocrine tissue, which likely causes enzymatic islet lysis and necrosis and thus an apoptosis-independent loss of functional β-cell mass in these grafts (30,31). While the effect of EP1013 in vitro pretreatment in the absence of continued posttransplant dosing was not explored in this study, the fact that human islet grafts required up to 3 weeks to reverse diabetes supports the necessity for posttransplant caspase inhibitor therapy, since a marginal implant mass will not function maximally until it has fully engrafted (32). This has been confirmed in previous studies in which pretransplant incubation with caspase inhibitor alone had no significant effect on marginal mass islet graft function (13,14). Our data also shows that using the caspase-selective inhibitor EP1013 with islets is ideal, as inhibition of other cysteine proteases including calpains and cathepsin B by zVAD can interfere with β-cell function.

These results suggest that inclusion of an early course of EP1013 therapy in the clinical setting could dramatically reduce the number of islets required to achieve insulin independence, which could have an immediate impact on the procedure at several stages. First, transplantation of high-quality islet preparations of low yield (<300,000 IE) could be explored. Recently published data has shown that 75% of 251 human pancreata processed at our center resulted in yields <300,000 IE, and these preparations were not transplanted since the implant mass would be marginal by current standards (<5,000 IE/kg) (33). Second, insulin independence following single-donor infusion has been elusive for most programs, with the exception of a cohort of patients at the University of Minnesota (34,35). Despite the seemingly large target combined implant mass of >10,000 IE/kg in clinical islet transplantation, it is very likely that the resulting postengraftment functional islet mass is quite marginal, perhaps in the range of 10–20% when compared with the islet mass in an intact healthy pancreas (36). As a result, this mass must function maximally to cross the threshold of insulin independence, and any trigger, including metabolic exhaustion, recurrent autoimmunity, subclinical allograft rejection, and/or chronic exposure to immunosuppressive agents within the portal circulation, can trigger the return to supportive doses of insulin in patients with partially functioning marginal-mass islet grafts (4,37). Our data suggest that preservation of transplanted islets with EP1013 therapy in

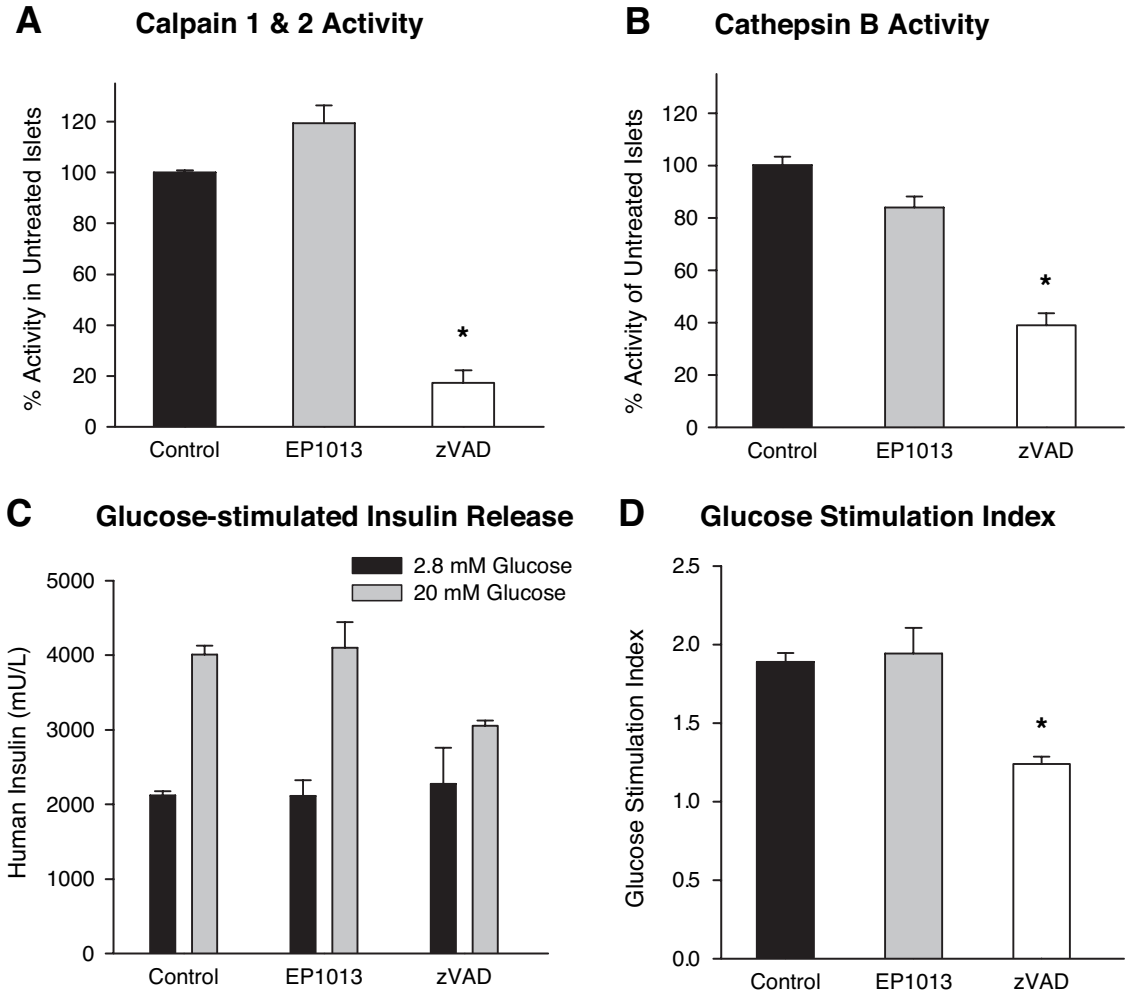

FIG. 7. zVAD inhibits noncaspase cysteine proteases including cathepsin B and calpain 1 and 2, resulting in impaired insulin secretion in human islets. To determine whether the enhanced benefit of EP1013 over zVAD was related to its selectivity, in vitro experiments were carried out following 24 h incubation with vehicle (■), EP1013 (100 μmol/l) (▨), or zVAD (100 μmol/l) (□). zVAD significantly inhibited calpain 1 and 2 (*P < 0.001 vs. EP1013-treated islets and vehicle control islets) (*A*) and to a lesser extent cathepsin B (*P < 0.001 vs. EP1013-treated islets and vehicle control islets) (*B*). Since the calpain enzymes have been implicated in insulin secretion pathways, glucose-stimulated insulin secretion assays were carried out after the 24-h incubation period. *C*: zVAD-inhibited insulin secretion in the presence of high glucose medium, but this result was not significant versus control islets (P = 0.07). However, when the stimulation index (S_I) was determined, zVAD treated islets had a significantly lower S_I (*P < 0.01 vs. EP1013- and vehicle-treated islets). All assays were performed in triplicate, and data are presented as means ± SE.

the early posttransplant engraftment period will enhance graft longevity, which could translate to improved insulin independence rates over time. Moreover, it is likely that EP1013 therapy will reduce early immune stimulation by dying islet tissue posttransplant, which could improve the efficacy of current immunosuppressive regimens, thereby improving long-term outcomes. Furthermore, avoidance of tissue death in the posttransplant period may be critical to the success of tolerance induction protocols. EP1013 therapy would also likely reduce procedural complications and sensitization by removing the risk for sequential infusions from multiple donors (38).

While a theoretical risk for malignant transformation exists when caspase inhibitor therapy is given systemically, data in the present study suggest that there is no increased incidence of cancer observed in mice beyond 1 year posttransplant. It should be noted that our study required the use of streptozotocin, a known carcinogen in mice, and as such detailed analysis of the added oncogenic potential of EP1013 therapy could not be evaluated, given the limited power analysis (29). However, our data suggest that a brief and carefully titrated EP1013 treatment period will not substantially increase cancer risk in islet transplantation. Additionally, a very similar structural analog of EP1013 was negative in mutagenicity testing by the Ames reverse-mutation assay with or without liver extract incubation (B. Tseng, unpublished data). Indeed, clinical trials of systemic caspase inhibitor therapy using IDN-6556, a pan-caspase inhibitor, have been carried out (39–41). One of these studies (40) was conducted in the setting of liver transplantation, under the cover of immunosuppression, and no cancers have been reported thus far in these patients. IDN-6556 has been shown to have a similar in vivo potency in mice compared with zVAD, and it has been used clinically at a dose similar to EP1013 in this study (3–10 mg/kg) with very few side effects (injection site inflammation and phlebitis) (39–42). Although oral administration of EP1013 was not examined in the present study, oral dosing of IDN-6556 was found to have a significant first-pass effect in the portal circulation (>4 h), suggesting that oral caspase inhibitor therapy could be of particular benefit in clinical islet transplantation (43). The clinical use of IDN-6556 in transplant patients suggests that EP1013 therapy can be explored in clinical islet transplan-

tation, provided that toxicity data in a large animal model is favorable. In fact, EP1013 may have an equal or even enhanced potency compared with IDN-6556 in humans, since data in the present study shows that EP1013 is more effective than the pan-caspase inhibitor zVAD in vivo, even at a 60–90% reduced dose, a result that has not been reported using IDN-6556 (42). Additionally, use of broad-spectrum caspase selective inhibitors like EP1013 may have additional non–apoptosis-related beneficial effects, as caspase-1 has been implicated in the generation of inflammatory processes including interleukin-1β production (44).

In summary, these data support the use of EP1013 therapy to inhibit engraftment-phase islet cell death and thus improve marginal-mass graft function in clinical islet transplantation. This would have substantial impact on the procedure by increasing the utility of suboptimal yield islet preparations and potentially increase insulin independence rates and long-term durability following single-donor infusion. The success of EP1013 therapy in this study using human islets suggests that caspase inhibitor therapy will be of great benefit to clinical islet transplantation, potentially increasing the number of patients that can be treated and improving insulin independence rates.

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