Based on our experience with patient 2, we learned that, for patients in a severe preoperative general condition, IAT should not be performed at the same time that a TP is performed. We also know that lower purity of the grafts might be a cause of portal thrombus, even in IAT. To perform TP with IAT as safe, preoperative inflammatory control is of great importance. If an emergency operation is needed for severe inflammation, TP and islet isolation should be done first. Then, cultured islets are transplanted after the patient has recovered from his or her severe general condition. A short culture of the grafts may be beneficial not only for the recipient but also for the islets per se due to mitigation of the inflammatory status. In addition, intramuscular islet transplantation can be an alternative and safe choice to prevent portal thrombus (*5*).

In conclusion, AVM is appropriately treated with TP with IAT. We performed this treatment for three AVM patients and had good outcomes in two of the patients. Further optimizations based on a systematic evaluation of clinical experiences are needed to improve the outcome and safety of this promising approach.

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N.S. treated the patients, planned the research design, performed the data analysis, wrote the first draft, and designed the figure. M.G. performed the islet isolation, planned the research design, performed the data analysis, and revised the draft. F.M. performed all the surgeries, planned the research design, and revised the draft. H.H., K.N., and M.M. performed the surgeries and treated the patients. H.Ya. performed islet isolation and transplantation. Y.H., S.Y., and S.Saw. controlled the blood glucose with intensive insulin therapy. S.O., T.O., K.F., and H.Yo. treated the patients. T.I. performed islet isolation. M.H. screened all patients for the indication of IAT. Y.I., S.Se., T.R., Y.K., K.F., S.E., T.S., H.K., S.Sat., and M.U. checked the final version of the draft. T.R. performed surgery for complications in patient 2. All authors checked the article and contributed to improving the draft.
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Postoperative Cholestasis and Cholangitis After Total Pancreatectomy with Biliary Reconstruction Impair the Function of Autotransplanted Islets

Total pancreatectomy (TP) with islet autotransplantation (IAT) is a good therapeutic option for benign pancreatic diseases that require TP (*1*). In general, TP requires resection and reconstruction of the common bile duct by choledochojejunostomy. Therefore, postoperative cholangitis may occur after reconstruction of the biliary tract (*2*).

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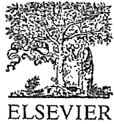

ELSEVIER

Effects of Glucagon-Like Peptide 1 Analogue on the Early Phase of Revascularization of Transplanted Pancreatic Islets in a Subcutaneous Site

R. Nishimura, A. Ushiyama, S. Sekiguchi, K. Fujimori, N. Ohuchi, S. Satomi, and M. Goto

ABSTRACT

Objective. The subcutaneous space is an ideal site for pancreatic islet transplantation. However, one of the main obstacles is poor revascularization. Recently, glucagon-like peptide 1 (GLP-1) analogues are emerging as a new treatment option for patients with type 2 diabetes, because they have been shown to decrease β-cell apoptosis. Therefore, we hypothesized that administration of a GLP-1 analogue in the early phase may facilitate revascularization of transplanted pancreatic islets by decreasing apoptotic changes of vascular endothelial cells within and without the graft. In this study, we evaluated the effects of GLP-1 analogue liraglutide on revascularization at a subcutaneous site with the use of a highly sensitive imaging system. We combined a dorsal skinfold chamber (DSC) technique with multiphoton laser-scanning microscopy (MPLSM).

Methods. Donor pancreatic islets isolated from C57BL/6-Tg (CAG-EGFP) mice were syngeneically transplanted into a dorsal skinfold chamber mounted on recipient mice. Male C57BL/6N mouse as recipients were divided into 3 groups: control, donor islet–treated, and recipient-treated groups. In the donor islet–treated group, the pancreatic islets were cultured with liraglutide (1 μmol/L) for 24 hours. The recipient-treated mice were injected with liraglutide (100 μg/kg subcutaneously) twice daily for 8 days. The time-dependent changes of newly formed vessels surrounding the islet grafts were imaged with MPLSM on days 1, 4, and 7. To evaluate islet graft revascularization, we measured vascular volume surrounding the islet with the Volocity system.

Results. In the first 4 days after pancreatic islet transplantation, no significant difference was detected in newly formed vessels among the 3 groups. Also, no significant difference was detected to increase rates at 7 days after transplantation.

Conclusions. In this study, administration of GLP-1 analogue liraglutide in the early phase after pancreatic islet transplantation did not promote revascularization of transplanted islet grafts.

Pancreatic islet transplantation is an attractive and promising therapy for type 1 diabetes. Most of clinical cases have been performed by infusion into the portal vein. However, the liver site is not necessarily the optimal one for pancreatic islet implantation, because it is associated with procedure-related complications, including hemorrhage and thrombosis. Furthermore, the strong innate immune response of the instant blood-mediated inflammatory reaction may be enhanced in the liver.[1] Therefore, many researchers have sought to optimize islet engraftment and function by using alternative sites of pancreatic islet transplantation.[2]

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The subcutaneous space is an ideal site owing to minimum invasiveness and easy access. However, one of the main obstacles to successful pancreatic islet transplantation at this site is poor revascularization. Recently, glucagon-like peptide 1 (GLP-1) analogues are emerging as new treatment options for patients with type 2 diabetes. They have been shown to decrease β-cell apoptosis.[3,4] Therefore, we hypothesized that administration of a GLP-1 analogue in the early phase may facilitate revascularization of transplanted pancreatic islets by decreasing apoptotic changes in vascular endothelial cells within and without the graft. In the present study, we evaluated the effects of a GLP-1 analogue liraglutide, on revascularization at a subcutaneous site with the use of a highly sensitive imaging system that combined a dorsal skinfold chamber (DSC) with multiphoton laser-scanning microscopy (MPLSM).

MATERIALS AND METHODS

Donor pancreatic islets isolated from C57BL/6-Tg (CAG-EGFP) mice (Japan SLC, Shizuoka, Japan) were syngeneically transplanted into nonmetallic dorsal skinfold chambers mounted on the backs[5] of male C57BL/6N mice (Japan SLC). The 3 groups included: controls, donor islet–treated, and recipient-treated groups. In the donor islets-treated group, pancreatic islets had been cultured with liraglutide (1 μmol/L) for 24 hours.[6] The recipient-treated mice were injected with liraglutide (100 μg/kg subcutaneously) twice daily for 8 days.[7] The time-dependent changes of pancreatic islets and newly formed vessels surrounding the islet grafts were imaged with the use of MPLSM (Fluo view FV1000MPE; Olympus) at days 1, 4, and 7.[8,9] Texas Red (0.1 mL of 10 mg/mL; Invitrogen, Leek, The Netherlands) was injected intravenously to visualize the blood vessels. To evaluate islet graft revascularization, we measured the vascular volume surrounding the islets with the Volocity system (Perkin Elmer, Waltham, Massachusetts).[10] Data are presented as mean ± SEM.

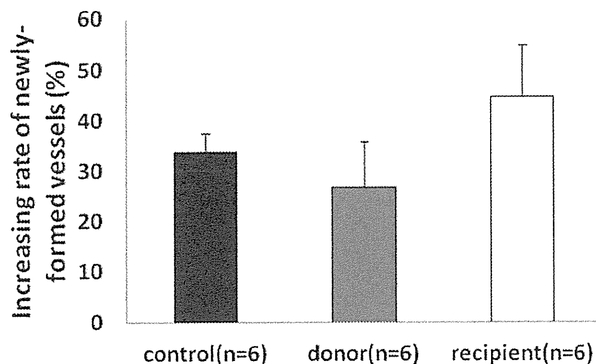

Fig 1. Increasing rate of newly formed vessels surrounding the transplanted pancreatic islets at day 4 after islet transplantation. Increasing rate = (vascular volume at day 4 − vascular volume at day 1)/(vascular volume at day 1). The *x*-axis indicates the days after pancreatic islet transplantation, and the *y*-axis indicates the increasing rate of newly formed vessels.

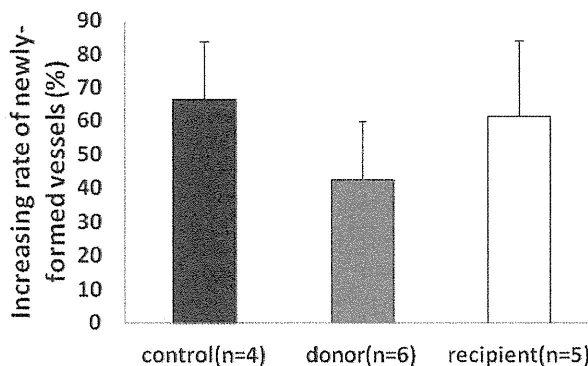

Fig 2. Increasing rate of newly formed vessels surrounding the transplanted pancreatic islets at day 7 after islet transplantation. Increasing rate = (vascular volume at day 7 − vascular volume at day 1)/(vascular volume at day 1). The *x*-axis indicates the days after pancreatic islet transplantation, and the *y*-axis indicates the increasing rate of newly-formed vessels.

RESULTS

In the first 4 days after pancreatic islet transplantation, no significant difference was detected in the rates of newly formed vessels among the 3 groups: control (n = 6); 33.8 ± 9.2%; donor islet–treated (n = 6): 26.9 ± 21.7%; and recipient-treated (n = 6): 44.7 ± 25.2%; (Fig 1). Also, no significant difference was detected at 7 days after transplantation controls (n = 4): 66.6 ± 34.2%; donor islet–treated (n = 6): 42.8 ± 42.4; and recipient-treated (n = 5): 61.6 ± 50.7%; (Fig 2).

DISCUSSION

To survive and function, transplanted pancreatic islets must be revascularized quickly, because their isolation procedure usually destroys arterial and venous connections from surrounding tissues. In this study, administration of the GLP-1 analogue liraglutide in the early phase after pancreatic islet transplantation showed no promoting effects on revascularization of transplanted islet grafts.

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Thioredoxin-1 Attenuates Early Graft Loss after Intraportal Islet Transplantation in Mice

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Abstract

Aims: Recent studies suggest that decreasing oxidative stress is crucial to achieve successful islet transplantation. Thioredoxin-1 (TRX), which is a multifunctional redox-active protein, has been reported to suppress oxidative stress. Furthermore, it also has anti-inflammatory and anti-apoptotic effects. In this study, we investigated the effects of TRX on early graft loss after islet transplantation.

Methods: Intraportal islet transplantation was performed for two groups of streptozotocin-induced diabetic mice: a control and a TRX group. In addition, TRX-transgenic (Tg) mice were alternately used as islet donors or recipients.

Results: The changes in blood glucose levels were significantly lower in the TRX group compared with the TRX-Tg donor and control groups ($p<0.01$). Glucose tolerance and the residual graft mass were considerably better in the TRX group. TRX significantly suppressed the serum levels of interleukin-1β ($p<0.05$), although neither anti-apoptotic nor anti-chemotactic effects were observed. Notably, no increase in the 8-hydroxy-2′-deoxyguanosine level was observed after islet infusion, irrespective of TRX administration.

Conclusions: The present study demonstrates that overexpression of TRX on the islet grafts is not sufficient to improve engraftment. In contrast, TRX administration to the recipients exerts protective effects on transplanted islet grafts by suppressing the serum levels of interleukin-1β. However, TRX alone appears to be insufficient to completely prevent early graft loss after islet transplantation. We therefore propose that a combination of TRX and other anti-inflammatory treatments represents a promising regimen for improving the efficacy of islet transplantation.

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Competing Interests: The authors have the following interests. Junji Yodoi is a board member and shareholder of Redox Bioscience Inc. who provided TRX to this study. There are no further patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

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Introduction

Since the development of the Edmonton protocol, islet transplantation has become an effective option for the clinical treatment of type 1 diabetic patients [1]. However, several donor pancreases are still needed to cure each diabetic patient. In order for islet transplantation to become widespread, the transplantation process must be improved.

The instant blood-mediated inflammatory reaction (IBMIR) that occurs as an innate immune response, characterized by activation of both the coagulation and complement cascades, is a major cause of islet graft loss [2–4]. We and others have demonstrated that tissue factor (TF) and monocyte chemoattractant protein-1 (MCP-1) expressed on the grafted islets elicit the IBMIR [4–9]. Therefore, to achieve successful outcomes of islet transplantation from a single donor organ, avoidance of the IBMIR is necessary.

On the other hand, pancreatic β cells are also vulnerable to oxidative stress because of their low intrinsic level of antioxidant gene expression [10]. Furthermore, Sklavos et al. reported that redox modulation with catalytic antioxidant protects islets from antigen-independent ischemia-reperfusion injury and hinders the antigen-dependent alloimmune response [11]. Thus, protecting the islet grafts from oxidative stress appears to be another crucial factor to achieve successful islet transplantation.

Thioredoxin-1 (TRX) is a ubiquitously expressed, small (14 kDa) multifunctional protein that has a redox-active disulfide/dithiol within a conserved –Cys-Gly-Pro-Cys- sequence [12–14]. TRX protects cells against oxidative stress by scavenging reactive oxygen species, and exerts anti-inflammatory and anti-apoptotic effects by regulating cytokines, signaling molecules and

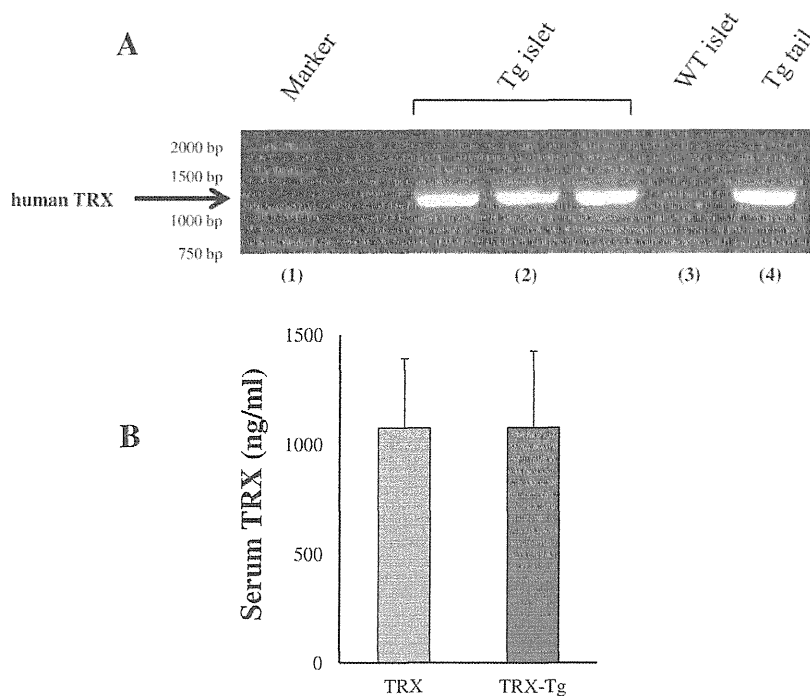

Figure 1. The TRX RNA expression on the islets isolated from TRX-Tg mice. The TRX expression on the islets isolated from TRX-Tg mice was confirmed by PCR (**A**). The TRX band is approximately 1,300 base pairs. (**1**): The size marker (Sigma-Aldrich, Inc), (**2**): Islet DNA from a TRX-Tg mouse, (**3**): Islet DNA from a C57BL/6 mouse, (**4**): Tail tip DNA from a TRX-Tg mouse. **B** The serum levels of TRX at 6 h after islet transplantation in the TRX (n = 5) and TRX-Tg recipient (n = 3) groups. No significant difference was detected between two groups (p = 0.87).
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transcription factors [15–18]. In addition, TRX regulates neutrophil activation and chemotaxis by acting directly on neutrophils [19–21].

In the present study, we investigated the effects of TRX on early graft loss after islet transplantation using a syngeneic mouse model which provides an innate inflammatory milieu without interference from the specific immune system. One of the advantages of islet transplantation, unlike whole organ transplantation, is the ability to modify the grafts during a culture period prior to transplantation. Therefore, if the overexpression of TRX on the isolated islets would improve the outcome of islet transplantation, this can be an ideal and feasible approach with only a limited risk of side effects of TRX. To investigate this possibility, TRX-transgenic (Tg) mice were used as either islet donors or recipients. Furthermore, continuous infusion of exogenous recombinant human TRX (rhTRX) was also performed for the recipients to mimic clinical conditions. Our findings shed light on the potential role of TRX in islet transplantation.

Methods

Ethics Statement

All the animals in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health [22]. The protocol was approved by the Committee on the Ethics of Animal Experiments of Tohoku University (Permit Number: 22 IIARE-Animal-24). All surgery was performed under isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan) anesthesia, and all efforts were made to minimize suffering.

Animals

Male wild type (WT) C57BL/6 mice were purchased from Japan SLR Inc. (Shizuoka, Japan) and male TRX-Tg C57BL/6 mice, in which human TRX complementary deoxyribonucleic acid (DNA) was inserted between the β-actin promoter and its terminator, were raised in our facility. The generation of TRX-Tg mice was described previously [23]. The presence of the human TRX transgene was confirmed by a polymerase chain reaction (PCR) analysis with mouse genomic DNA as a template and synthetic oligonucleotides as primers: forward primer, 5′-CAGATCGAGAGCAAGAC-3′; reverse primer, 5′-CAGGAAACAGCTATGAC-3′. The expression of TRX protein was shown in various tissues in our previous characterization [23].

Induction and diagnosis of diabetes in the recipients

Diabetes was induced by intravenous injection of 200 mg/kg streptozotocin (SIGMA-ALDRICH, Inc, MO, USA) 5 days before surgery. Mice whose nonfasting blood glucose levels were ≥400 mg/dL on two consecutive measurements were considered diabetic. Serial blood glucose levels were determined, and recipients whose nonfasting blood glucose levels were <200 mg/dL on two consecutive measurements were considered to be cured.

Islet isolation and transplantation

Before removal of the pancreas, the cannulated bile duct was injected with 4 mL of cold Hanks' balanced salt solution (HBSS) containing 1 g/L collagenase (Sigma type V; Sigma Chemicals, St. Louis, MO, USA). After addition of 4 mL HBSS, the pancreas was digested at 37°C for 12 min. Thereafter, density-gradient centrifugation was performed using Histopaque-1119 (Sigma Diagnostics, St. Louis, MO, USA) and Lymphoprep^{TM} (Nycomed

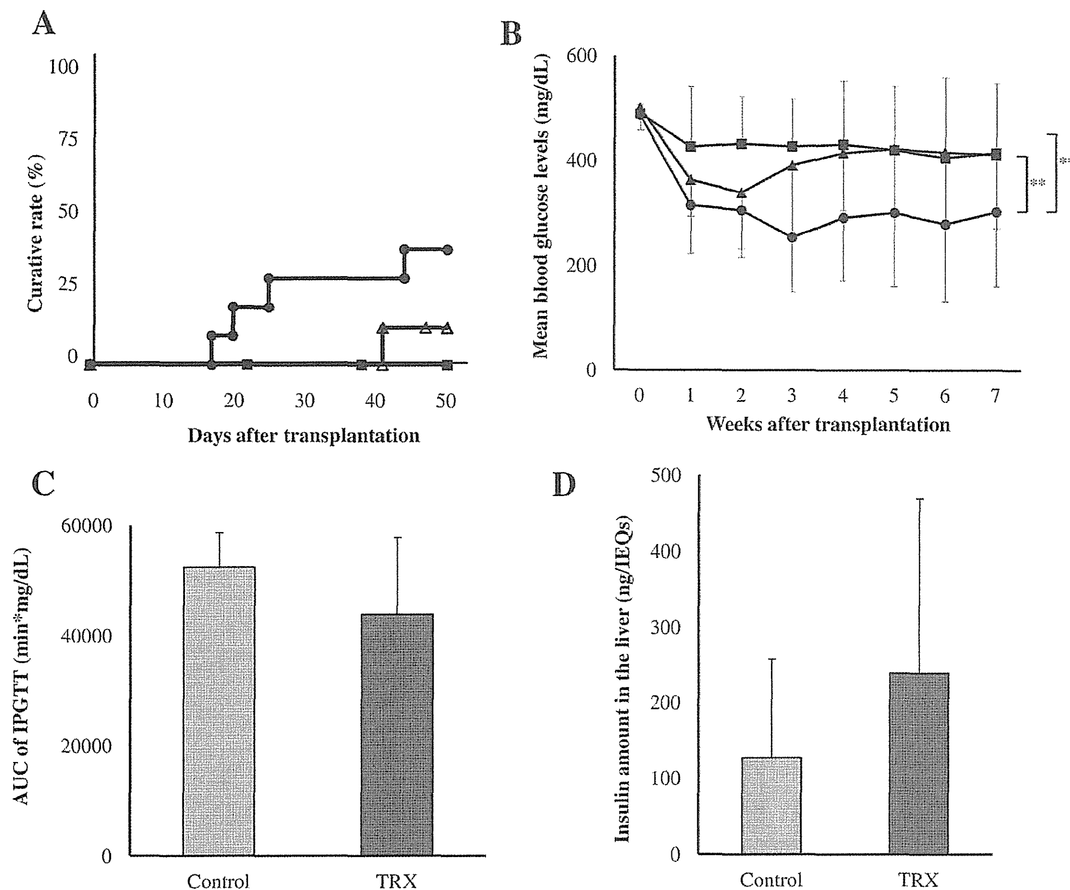

Figure 2. The effects of TRX on islet engraftment. Pancreatic islets isolated from WT or TRX-Tg C57BL/6 mice were transplanted into WT C57BL/6 mice that were rendered diabetic by streptozotocin injection (200 mg/kg intravenously). **A** Diabetic animals received 6 IEQs/g of WT islets with saline (square: Control group), 6 IEQs/g of WT islets with TRX (circle: TRX group), or 6 IEQs/g of TRX-Tg islets (triangle: TRX-Tg donor group). Serial blood glucose levels were measured. Recipients whose nonfasting blood glucose levels were <200 mg/dL on two consecutive measurements were considered to be cured (p = 0.08). **B** The changes in the mean blood glucose levels every week among the three groups were estimated (**p<0.01). **C** IPGTTs were performed 50 days after islet transplantation (n = 3). The results of the IPGTTs were compared using AUC. The glucose tolerance tended to be ameliorated in the TRX group compared to the control group (p = 0.10). Almost all mice in the TRX-Tg donor group were too feeble to undergo the IPGTT. **D** After the IPGTT, the recipient livers in the control and TRX groups were retrieved, and the amount of insulin in the livers of the recipients per transplanted islet was calculated (n = 3). There was a tendency for there to be higher insulin in the TRX group compared with the control group (p = 0.3).
doi:10.1371/journal.pone.0070259.g002

Pharma AS, Oslo, Norway) to isolate the pancreatic islets. The islets were cultured in RPMI-1640 containing 5.5 mmol/L glucose and 10% fetal bovine serum at 37°C in 5% CO_2 and humidified air before examination.

Diabetic C57BL/6 mice underwent intraportal islet transplantation under isoflurane anesthesia. Murine islets were infused in a total volume of 300 µL into the recipient liver through the portal vein using a 27-gauge Surshield (TERUMO, Inc, Japan).

Experimental groups

Six islet equivalents (IEQs)/g of syngeneic WT islets were transplanted intraportally into two groups of streptozotocin-induced diabetic WT mice: controls (n = 6) and the TRX (Redox Bio Science, Inc., Kyoto, Japan) -treated group (n = 9). The TRX group was treated with a bolus of rhTRX (40 µg) followed by a continuous infusion (0.4 mg/kg/h) for 7 days. TRX concentration used in this study was in reference to the study of Ueda et al. [20]. Recipients were injected with equivalent amounts of saline as controls. In addition, TRX-Tg islets were transplanted intraportally into streptozotocin-induced diabetic WT mice (TRX-Tg donor group, n = 7), and WT islets were injected into streptozotocin-induced diabetic TRX-Tg mice (TRX-Tg recipient group, n = 7).

Intraperitoneal glucose tolerance testing

The intraperitoneal glucose tolerance test (IPGTT) was performed 50 days after islet transplantation in the control and TRX groups. D-glucose (2.0 g/kg) was infused intraperitoneally, and the blood glucose concentrations were determined before and at 5, 10, 20, 30, 60, 90 and 120 min after the glucose injection.

The amount of insulin in the livers of the recipients

Recipient livers were retrieved in 2–7 days after IPGTT and homogenized on ice. After adding deionized water up to 10 mL and 25 mL of 0.18M HCl in 96% ethanol, the homogenate was stored at 4°C for 24 h and was then centrifuged at 2,150 *g* for 10 min. The resulting supernatant was stored at −80°C. The insulin concentration in the supernatant was evaluated using a

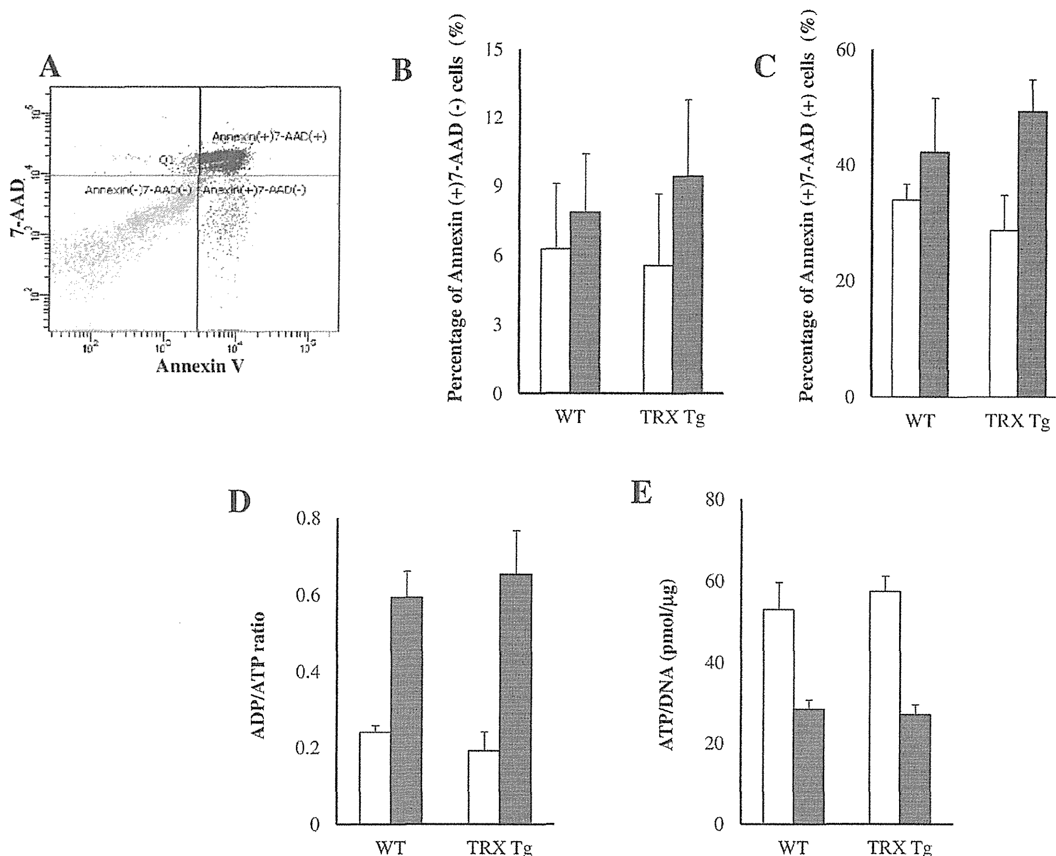

Figure 3. Evaluation of the islet viability under co-culture with inflammatory cytokines. Freshly isolated islets were cultured with (black bar) or without (white bar) human IL-1β, mouse TNF-α, or mouse IFN-γ at 37°C for 18 h, then the islets were dispersed into individual cells by treatment with Accutase at 37°C for 10 min. To assess the viability of these cells, the Annexin-V/7-AAD assay (n = 5, **A, B, C**), ADP/ATP test (n = 3, **D**), and the ATP/DNA test (n = 3, **E**) were conducted.
doi:10.1371/journal.pone.0070259.g003

commercial enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden).

Evaluation of islet viability under co-culture with inflammatory cytokines

Freshly isolated islets were cultured with or without 50 U/mL human interleukin (IL)-1β (Roche Diagnostics, Indianapolis, IN), 1000 U/mL mouse tumor necrosis factor (TNF)-α (Roche Diagnostics), and 1000 U/mL mouse interferon (IFN)-γ (Roche Diagnostics) at 37°C for 18 h, then islets were dispersed into individual β cells by treatment with Accutase (Innovative Cell Technologies, San Diego, CA) at 37°C for 10 min. To assess the viability of these cells, the Annexin-V (Becton Dickinson, Franklin Lakes, NJ)/7-Amino-Actinomycin D (7-AAD) assay (n = 5), adenosine diphosphate (ADP)/adenosine triphosphate (ATP) test (n = 3) [24], and ATP/DNA test (n = 3) [25] were conducted.

Blood analyses

For analyzing the blood samples, the clinically relevant graft dose (8 IEQs/g) was applied. Whole blood samples were collected before and at 6 h after islet infusion, and the samples were clotted for 0.5–1 h at room temperature, then centrifuged for 10 min at 2,200 g. The serum samples were frozen at −80°C. Serum levels of cytokines such IL-1β, IL-6, IFN-γ, MCP-1, and keratinocyte chemoattractant (KC) were determined using the Bio-Plex Suspension Array System (Bio-Rad, Hercules, CA). The serum levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a sensitive marker of oxidative stress [26], at 6 h after islet infusion were measured using a Highly Sensitive 8-OHdG Check ELISA (Nikken SEIL Co., Ltd., Shizuoka, Japan). For comparison, we also analyzed the serum levels of 8-OHdG in naive mice.

Flow cytometric analyses

Hepatic mononuclear cells of recipient mice were prepared as previously described [27]. In this assay, the clinically relevant graft dose (8 IEQs/g) was also applied. Single cells (1.5×10^5) suspended in phosphate-buffered saline (PBS) with 0.5% bovine serum albumin (BSA) were incubated with saturating concentrations of murine antibodies for 30 min at 4°C on ice in the dark, and subsequently washed and resuspended in PBS with 0.5% BSA. The cell-associated light scatter and fluorescence were determined with the BD FACSCanto™ II instrument (Becton Dickinson). A total of 10,000 viable cells were analyzed. The antibodies used for these analyses were as follows: FITC anti-mouse Ly-6G/Ly-6C (BioLegend, San Diego, CA), APC anti-mouse CD11b (Biolegend), FITC anti-mouse CD3ε (eBioscience, San Diego, CA), and PE anti-mouse αGalCer-CD1d complex (eBioscience). The expression of TF on Gr1$^+$ CD11b$^+$ cells in recipient livers was detected by a flow cytometric analysis using rabbit anti-mouse

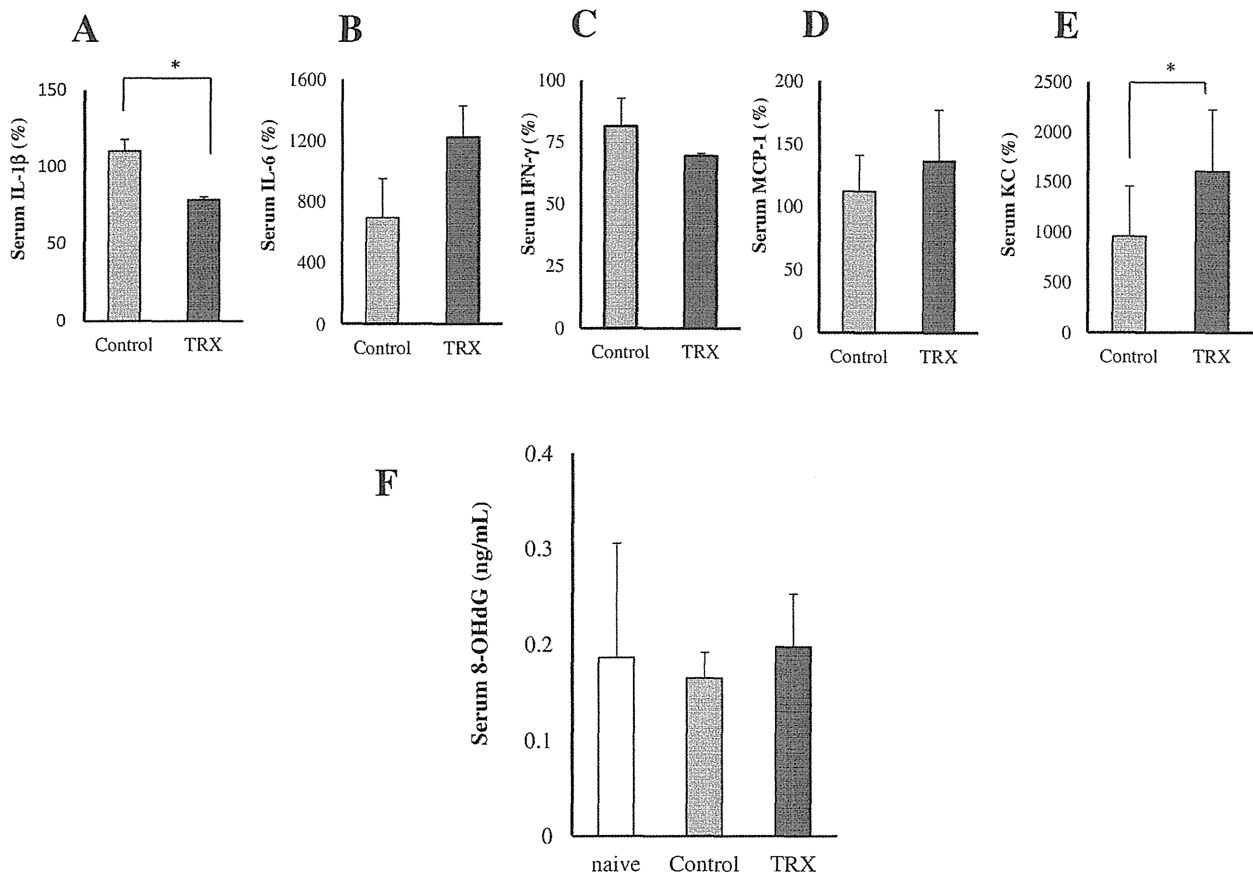

Figure 4. The results of the analyses of blood samples. A–E Inflammatory mediators in serum samples were measured before and at 6 h after islet transplantation (8 IEQs/g) (n = 3). All values were expressed as the percentage of the pre-transplant cytokine levels in the serum. Systemic administration of TRX significantly suppressed the serum levels of IL-1β (*p<0.05). In contrast, the serum KC levels in the TRX group were significantly increased compared to the control group (*p<0.05). **F** To evaluate the influence of oxidative stress on transplanted islets, the serum levels of 8-OHdG at 6 h after islet infusion were measured (n = 4). For comparison, we also analyzed the serum levels of 8-OHdG in naive mice (n = 5).
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tissue factor IgG (American Diagnostica, Stamford, CT), followed by secondary Alexa Fluor 647 donkey anti-rabbit IgG (Invitrogen).

Histological analyses of transplanted livers

The clinically relevant graft dose (8 IEQs/g) was applied to increase the possibility of finding the grafts in the host livers. The recipient livers with islet grafts were retrieved 24 h after islet infusion and fixed, embedded in paraffin, cut into blocks at regular intervals, and sliced into 4 μm sections. Deparaffinized sections were incubated with a polyclonal guinea pig anti-insulin antibody (Dako, Denmark), then with a goat anti-rabbit EnVision kit (Dako). To detect apoptosis in transplanted islets, TdT-mediated dUTP nick end labeling (TUNEL) staining was performed using a TACS 2TdT-DAB In Situ Apoptosis Detection Kit (Trevigen, Inc.,USA). The number of TUNEL positive islets was counted by double-blind evaluations.

Statistical analyses

All data are expressed as the means ± SD. Statistical significance was determined using Student's *t* test, the Mann-Whitney U test, or a one- and two-factor analysis of variance with Bonferroni post hoc test. *P* values <0.05 were considered to be significant. An analysis of euglycemic conversion was performed by the Kaplan- Meier method with a log-rank test.

Results

The effects of TRX on islet engraftment

The TRX RNA expression on the islets isolated from TRX-Tg mice was confirmed (Fig. 1A). No significant difference was detected in the serum levels of TRX at 6 h after islet transplantation between the TRX (n = 5) and TRX-Tg recipient group (n = 3, Fig. 1B). All mice used in the present study were severely hyperglycemic (>400 mg/dL) before transplantation. Although none of the 6 mice transplanted with 6 IEQs/g became normoglycemic in the control group, 4 of 9 mice (44.4%) transplanted with the same amount of islets in the TRX group became normoglycemic, and 1 of 7 mice (14.3%) that received the same amount of TRX-Tg islets in the TRX-Tg donor group became normoglycemic during the 7 week study period (Fig. 2A). The mean blood glucose levels were significantly lower in the TRX group compared with the other groups (p<0.01, two-factor analysis of variance, Fig. 2B).

The glucose tolerance showed a tendency to be ameliorated in the TRX group compared to the control group (area under the curve (AUC): 43,901±13953 (n = 5) vs. 52,381±6307 min*mg/

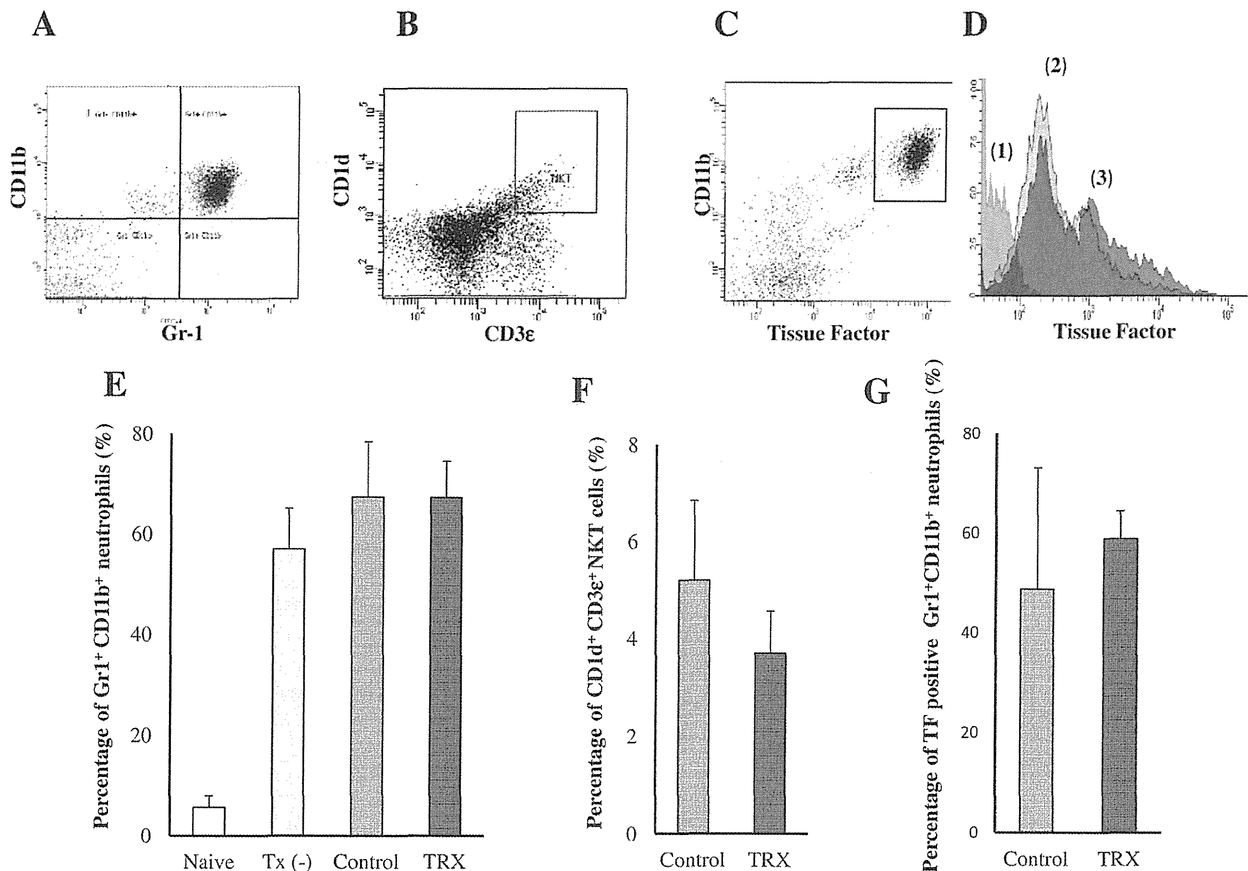

Figure 5. The results of the flow cytometric analyses. We examined whether systemic administration of TRX had any effects on the accumulation of $Gr1^+$ $CD11b^+$ (n = 3; **A, E**), $CD1d^+$ $CD3\varepsilon^+$ (n = 4; **B, F**), and tissue factor (TF)-positive $Gr1^+$ $CD11b^+$ (n = 3; **C, D, G**) mononuclear cells in the livers of mice receiving islets in the control and TRX groups. **D** Histogram of TF positive $Gr1^+$ $CD11b^+$ cells ((**1**); isotype control, (**2**); control, (**3**); TRX). No differences were detected between the TRX and control groups regarding the accumulation of these cells in the transplanted livers.
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dL (n = 3), p = 0.10; the Mann-Whitney U test, Fig. 2C). Furthermore, the amount of insulin in the liver of the recipients also tended to be higher in the TRX group compared with the control group (239.3±230.0 (n = 5) vs. 127.5±130.3 ng/IEQs (n = 3), p = 0.30; the Mann-Whitney U test, Fig. 2D). Almost all of the mice in the TRX-Tg donor group were too feeble to undergo the IPGTT and measurement of insulin in the liver. And all of the 7 diabetic TRX-Tg mice transplanted with WT islets (6 IEQs/g) remained hyperglycemic and died during 9~45 days (mean 21.7) after transplantation.

TRX-Tg islets did not protect against injury induced by inflammatory cytokines

We evaluated whether TRX-Tg islets had tolerance toward injury induced by inflammatory cytokines in an in vitro assay. In the Annexin-V/7-AAD assay, there were no differences in the percentage of Annexin-V positive, 7-AAD negative cells (namely apoptotic β cells, 9.42±3.35 vs. 7.86±3.10, n = 5; one-factor analysis of variance, Fig. 3A, B), or Annexin-V positive, 7-AAD positive cells (namely dead β cells, 49.26±5.43 vs. 42.20±9.33, n = 5, Fig. 3C) between the TRX-Tg and WT islets co-cultured with inflammatory cytokines. Similarly, no differences were detected between the TRX-Tg and WT islets with regard to the ADP/ATP ratio (0.65±0.11 vs. 0.59±0.07, n = 3; one-factor analysis of variance, Fig. 3D), and ATP/DNA ratio (27.1±2.37 vs. 28.4±2.33, n = 3; one-factor analysis of variance, Fig. 3E).

The analyses of blood samples

Inflammatory mediators in serum samples were measured before and at 6 h after islet transplantation (n = 3). All values were expressed as the percentage of the pre-transplant cytokine levels in the serum. Systemic administration of TRX significantly suppressed the serum levels of IL-1β ($p<0.05$, Student's *t* test, Fig. 4A). In contrast, the serum KC levels in the TRX group were significantly increased compared to the control group ($p<0.05$, Student's *t* test, Fig. 4E). No significant differences in other cytokines such as IL-6, IFN-γ and MCP-1 were observed (Student's *t* test, Figs. 4B–D).

To evaluate the influence of oxidative stress on the transplanted islets, the serum levels of 8-OHdG at 6 h after islet infusion were measured (n = 4). No differences were seen in these groups, but no increase of 8-OHdG was observed after islet infusion in either group (with one-factor analysis of variance, Fig. 4F).

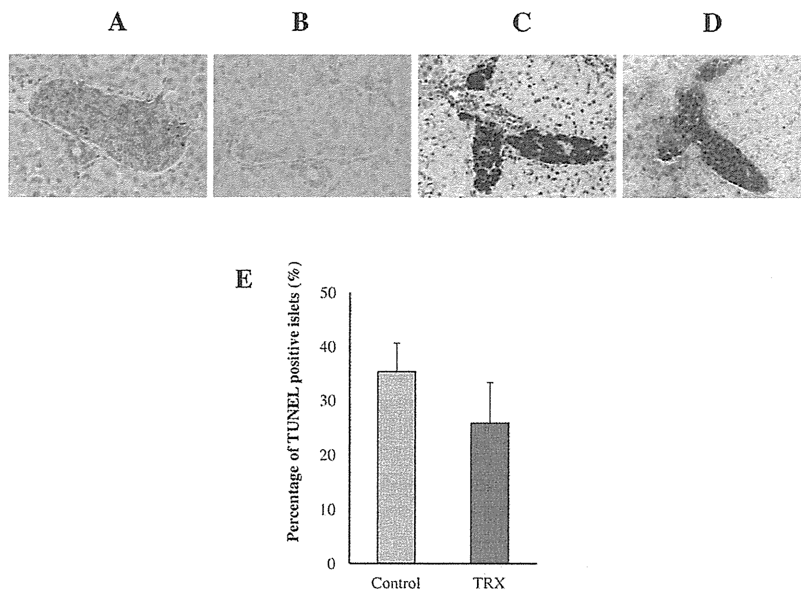

Figure 6. TdT-mediated dUTP nick end labeling (TUNEL) staining of transplanted islets. To examine the anti-apoptotic effect of TRX on transplanted islets, TUNEL staining was performed for the transplanted livers at 24 h after islet transplantation in the TRX and control groups (n = 3). The same islets were stained with both insulin (**A, C**) and TUNEL (**B, D**), respectively. **B** A TUNEL negative islet, **D** A TUNEL positive islet., **E** The rate of TUNEL positive islets was lower in the TRX group than in the control group, but the difference did not reach statistical significance (p = 0.28).
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TRX was unable to regulate the accumulation of Gr1$^+$ CD11b$^+$, CD1d$^+$ CD3ε$^+$, and TF positive Gr1$^+$ CD11b$^+$ cells in the livers of mice receiving islets

We examined whether the systemic administration of TRX had any effect on the accumulation of Gr1$^+$ CD11b$^+$ (mainly neutrophils), CD1d$^+$ CD3ε$^+$ (natural killer T (NKT) cells), and TF-positive Gr1$^+$ CD11b$^+$ cells in the livers of mice receiving islets, which are thought to be essential components of early graft loss after islet transplantation [27–29]. No differences were detected between the TRX and control groups regarding the accumulation of Gr1$^+$ CD11b$^+$ cells (Fig. 5E), CD1d$^+$ CD3ε$^+$ cells (Fig. 5F), or TF- positive Gr1$^+$ CD11b$^+$ cells (Fig. 5G), with one-factor analysis of variance.

TUNEL staining of transplanted islets

To confirm the anti-apoptotic effects of TRX on transplanted islets, TUNEL staining was performed for the transplanted livers at 24 h after islet transplantation in the TRX and control groups (n = 3). The rate of TUNEL positive islets was lower in the TRX group compared with the control group, but the difference did not reach significance (25.9±7.42 vs. 35.3±5.37, p = 0.28; the Mann-Whitney U test, Fig. 6E).

Discussion

Several recent studies suggested that oxidative stress, as well as the IBMIR, appears to be one of the crucial factors affecting successful islet transplantation [30,31]. TRX has been reported to effectively suppress oxidative stress [32]. Moreover, it also exhibited anti-inflammatory and anti-apoptotic effects in various experimental models [15–18]. Therefore, we examined the effects of TRX on early graft loss after islet transplantation.

Unexpectedly, the TRX locally expressed on islet grafts was insufficient to protect them from the innate immune system, although systemic administration of TRX to the recipients appeared to be effective. Our present *in vivo* data were also supported by the *in vitro* study using TRX-Tg islets and the assessment of inflammatory cytokines including IL-1β, TNF-α, and IFN-γ (Fig. 3). These findings suggest that a substantial amount of TRX is required to protect the grafts after islet transplantation. On the other hand, Chou FC et al. previously reported that overexpression of TRX did prolong graft survival against inflammatory insults [32]. One possible explanation for this discrepancy is that the expression level of TRX by lentivirus in their study may be far higher than that of the TRX-Tg mice. The dosage effect of the localized overexpression of TRX should be further examined.

In the present study, we found that the serum levels of IL-1β were significantly suppressed in the TRX group. Corroborating our findings, Yoshioka et al. and Billiet et al. have reported that TRX could regulate the activation of NF-κB, followed by the suppression of IL-1β production [17,33]. It has been reported that pancreatic islets are highly sensitive to proinflammatory cytokines such as IL-1β [34–36]. Montolio et al. [37] reported that IL-1β mRNA in the transplanted islets was upregulated immediately after islet infusion, and that IL-1β played a crucial role in the extensive β-cell death found in the initial days after islet transplantation. Taken together, the suppression of IL-1β observed in the present study may contribute to the protection of the transplanted islet grafts by TRX.

In contrast, the serum level of KC, the murine homologue of human IL-8, was increased in the TRX group compared with the control group. Consistent with this finding, Bertini et al. [38] reported that TRX *per se* was chemotactic for monocytes, polymorphonuclear leukocytes, and T lymphocytes. This may partially explain why no beneficial effects of TRX were seen in some of the TRX-treated mice. In view of its potential clinical applications, further investigations of the independent effects of KC on islet engraftment are needed.

The serum levels of 8-OHdG were measured to assess the involvement of oxidative stress in the transplanted islets. No increase in the 8-OHdG levels was observed after islet infusion even in the control group, suggesting that oxidative stress, at least in this model, has a minimal effect on islet transplantation. These results imply that the upregulated inflammatory mediators, rather than oxidative stress, may have stronger impact on the outcome of islet transplantation.

To determine the significance of the systemic distribution of TRX in the recipients, streptozotocin-induced diabetic TRX-Tg mice were also used as the recipients. Notably, the TRX-Tg mice were especially sensitive to streptozotocin, and this toxicity became pronounced following surgical stress, irrespective of the islet infusion, and resulted in both liver and renal damage, though it was previously reported that histological analysis of the pancreas revealed similar extent of beta cell destruction between wild type and TRX-Tg mice after streptozotocin treatment [39]. The detailed mechanism underlying this effect remains uncertain, but likely reflects the multifunctional roles of TRX. Subsequent analyses using this model were not performed in the present study.

In the FACS analysis, unlike previous reports in which lipopolysaccharide (LPS)-induced neutrophil infiltration was effectively suppressed by rhTRX [19,20], we were unable to confirm the role of TRX in the regulation of neutrophil chemotaxis to the liver at 6 h after islet transplantation. One possible explanation for this discrepancy is the cause of the neutrophil infiltration; TRX may inhibit the specific signaling pathway activated by LPS. Of particular interest, the accumulation of $Gr1^{+}$ $CD11b^{+}$ cells in the liver was significantly increased by infusion of transplant medium itself (without any islets) to the liver. This indicates that neutrophil chemotaxis after intraportal islet transplantation can be attributed to not only islet grafts, but also to the transplant procedure itself. In this FACS analysis, we also evaluated the TF expression on leukocytes in the liver, since NF-κB suppression is known to cause downregulation of TF [40,41]. However, the accumulation of TF-positive neutrophils was not affected by TRX, suggesting that TRX is not related to the inhibition of the IBMIR.

One of the strong points of TRX treatment is its safety. TRX is expected to be easily approved for testing in clinical trials, since it is currently in a phase II clinical trial for the treatment of acute respiratory distress syndrome.

In summary, the present study demonstrates that localized overexpression of TRX on the islet grafts is not sufficient to improve their engraftment. In contrast, exogenous TRX administration to the recipients exerts protective effects on transplanted islet grafts by suppressing the serum levels of IL-1β. However, TRX alone appears to be insufficient to completely prevent early graft loss after islet transplantation. We therefore propose that the combination of TRX and an anti-IBMIR treatment, such as C5a inhibitory peptide [27], represents a promising regimen for improving the efficacy of islet transplantation, although further optimization will be required in a clinical setting.

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Author Contributions

Conceived and designed the experiments: KA MG. Performed the experiments: KA AI TI MG. Analyzed the data: KA S. Sekiguchi KF S. Satomi NO MG. Contributed reagents/materials/analysis tools: JY. Wrote the paper: KA JY S. Satomi HM MG.

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A comparison of the main structures of *N*-glycans of porcine islets with those from humans

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After producing α1-3-galactosyltransferase knockout (GKO) pigs, most of the organs of these pigs showed less antigenicity to the human body. However, wild-type adult pig islets (API) that originally contained negligible levels of α-galactosidase now showed a clear antigenicity to human serum. In this study, *N*-glycans were isolated from both APIs and human islets. Their structures were then analyzed by a mapping technique based on their high-performance liquid chromatography elution positions and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometric data. Both preparations contained substantial amounts of high-mannose structures. The *N*-glycans from human islets were separated into 17 neutral, 8 mono-sialyl and 4 di-sialyl glycans, and the API glycans were comprised of 11 neutral, 8 mono-sialyl, 3 di-sialyl, 2 mono-sulfated, 3 mono-sialyl-mono-sulfated and 1 di-sulfated glycans. Among them, the API preparation contained one neutral, five mono-sialyl glycans and six sulfated glycans that were not detected in human islets. The structures of 9 of these 12 could be clearly determined. In addition, a study of the sulfate-depleted API suggests that sulfate residues could be antigenic to humans. The data herein will be helpful for future studies of the antigenicity associated with API.

Keywords: N-glycan / pig islets / sulfated glycan / xenotransplantation

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Introduction

The increasing challenges associated with the worldwide shortage of donor organs have led to a renewed interest in xenotransplantation. The pig pancreas is considered to be the most suitable source of islets for clinical xenotransplantation. Some clinical trials have resumed in New Zealand, Russia, etc., using islets from a wild-type pig via the use of an immuno-isolation technique (Elliott 2011). In addition, based on data collected from the "Inventory of human xenotransplantation practices" (http://www.humanxenotransplant.org/index.html), many clinical trials appear to be ongoing.

On the other hand, after producing α1-3-galactosyltransferase knockout (GKO) pigs (Dai et al. 2002; Takahagi et al. 2005), most of the organs from these pigs were found to show less antigenicity to the human body. However, wild-type adult pig islets (API) that originally contained negligible levels of α-galactosidase (α-Gal) (Galα1-3Gal) (Galili et al. 1987) showed a clear antigenicity to human serum (Komoda et al. 2004), and this fact represents a significant obstacle to successful xenotransplantation (Thompson et al. 2011).

Concerning the so-called non-Gal epitopes, many studies related to glycoproteins and glycolopids are on-going in attempts to identify them. However, our knowledge of non-Gal glycoantigens is still incomplete. That is, previous analyses of *N*-glycans from pigs included the use of additional tissues, in addition to islets. However, besides α-Gal and Hanganutziu-Deicher (Varki et al. 2009; Yamamoto et al. 2013) antigen expression, the glycosylation of API remains relatively unclear (Breimer 2011; Byrne et al. 2011; Miyagawa et al. 2012).

We wish to report herein on the analysis of the glycosylation of the *N*-linked sugars of API, compared with the corresponding values for human islets, using a high-performance liquid chromatography (HPLC) technique, which is capable of providing reliable data. The collected data will be of use in future research concerning non-Gal antigens and promises to provide us with clues for producing new types of immuno-modified pigs with less antigenicity than GKO pigs.

Results

Isolation of N*-glycans of the porcine and human islets*

N-glycans derived from porcine (11.9 mg of protein) and human islets (12.47 mg of protein) were separated into five peaks, based on increasing acidity using a diethylaminoethyl (DEAE) column. The following peaks were produced: Neutral

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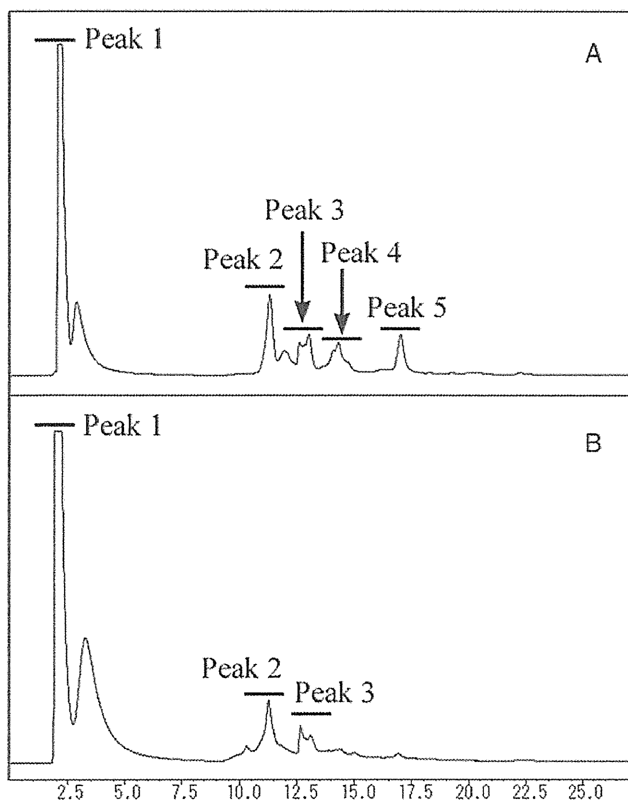

Fig. 1. Anion exchange DEAE elution profiles of PA-glycans derived from porcine islets (**A**) and the human islets (**B**). The PA-glycans were fractionated according to their sialic acid content and sulfate residues as neutral (Peak 1), mono-sialyl (Peak 2), di-sialyl or mono-sulfate (Peak 3), mono-sialyl-mono-sulfate (Peak 4) and di-sulfate (Peak 5) oligosaccharide fractions as indicated.

(N), Peak 1; mono-sialyl (M), Peak 2; di-sialyl (D) or mono-sulfate (S1), Peak 3; mono-sialyl-mono-sulfate (MS2), Peak 4 and di-sulfate (S2), Peak 5; glycan fractions with molar ratios (peak areas) of 84.4, 3.6, 2.6, 2.2 and 7.2% from APIs, and 94.0, 4.0, 2.0, 0 and 0% from the human islets, respectively (Figure 1).

Concerning API, when an octa decyl silyl (ODS) column was used, it was possible to separate the neutral fraction into fractions N1–N9, the mono-sialyl fraction into fractions M1–M6, the di-sialyl or mono-sulfate fraction into fractions D1–D3 and S1, the mono-sialyl-mono-sulfate fraction into fractions MS1–MS3 and the di-sulfate fraction into fraction S2. On the other hand, in the case of human islets, the neutral fraction was separated into N1–N13, the mono-sialyl into M1–M6 and the di-sialyl into D1–D4, as shown in Figure 2A–H.

Further analysis with GALAXY database

These ODS fractions were individually fractionated on an amide column and further subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometric (MALDI-TOF-MS) analysis. The porcine N2, N6, M2, M3 and S1 and the human N2, N5, N6, N12, M2 and M4 fractions were found to contain two kinds of *N*-glycans (Figures 3 and 4).

The coordinates of all of the *N*-glycans coincided with those for known references in the glycoanalysis by the three axes of MS and chromatography (GALAXY) database except for several *N*-glycan fractions including human fractions N5-1, N5-2, N6-1, N6-2, M1, M2-2 and porcine S1-1, MS1and MS3. Most of the *N*-glycan structures were then identified by the mapping technique on the basis of their HPLC elution positions and MALDI-TOF-MS data.

Structural analysis of each sample

Pyridylamino (PA)-glycans, which did not correspond to any of the *N*-glycans so far registered in GALAXY, were trimmed by treatment with an exoglycosidase, which produced known glycans (Yagi et al. 2005).

In the case of S1-2, no reactivity was detected by β-acethyl-hexiominidase. Next, a methanolysis treatment induced the conversion of S1-2 into S1-2a, but additional treatment with β-galactosidase resulted in no change to S1-2a. Moreover, the β-*N*-acethylhexosaminidase treatment converted S1-2a into S1-2b, and S1-2b was proved to be the same structure as M4.1 in GALAXY, as evidenced by the observation that samples of S1-2b and M4.1 co-chromatographed (Figure 5).

The MS2 sample was analyzed following a similar procedure. The sample did not serve as a substrate for β-galactosidase and α2,3-sialydase, but was converted into MS2a by treatment with α-sialydase. Further methanolysis and β-galactosidase converted MS2a into MS2b and MS2c, respectively. MS2b was next verified to be 210.4a in GALAXY by the co-chromatography of both samples. On the other hand, MS2c, when treated with β-*N*-acethylhexosaminidase and methanolysis, was converted into MS2d and MS2e, respectively. MS2e was also proved to be 110.4a in GALAXY by the co-chromatography of both samples (Figure 6).

Concerning S2, the sample was unchanged as a result of a β-*N*-acethylhexosaminidase treatment. On the other hand, a methanolysis treatment cleaved two sulfate residues from S2 and produced S2a, which was shown to be 210.4b in GALAXY by the co-chromatography of both samples (Figure 7).

In the analyses, a total of 28 and 29 *N*-glycan structures of API and human islets, respectively, were identified and the findings are summarized in Tables I–VI (Supplementary data, Figure S1).

Sodium chlorate treatment on pig islets

The effect of removal of the sulfate structures of pig islets on the antigenicity to human serum was investigated. The use of a sodium chlorate and a sulfate-free medium led to a significant reduction in antigenicity to human serum, suggesting that the sulfate structures in adult islets are targets for human natural antibodies (Figure 8).

Discussion

Twenty-eight kinds of *N*-linked glycans were identified in the case of the API glycans and 29 were identified from human islets, based on their HPLC elution peaks. While the human preparation contained neutral, mono-sialyl, di-sialyl *N*-linked glycans, the API sample contained not only these three types, but mono-sulfate, MS2 and di-sulfate types of *N*-linked glycans

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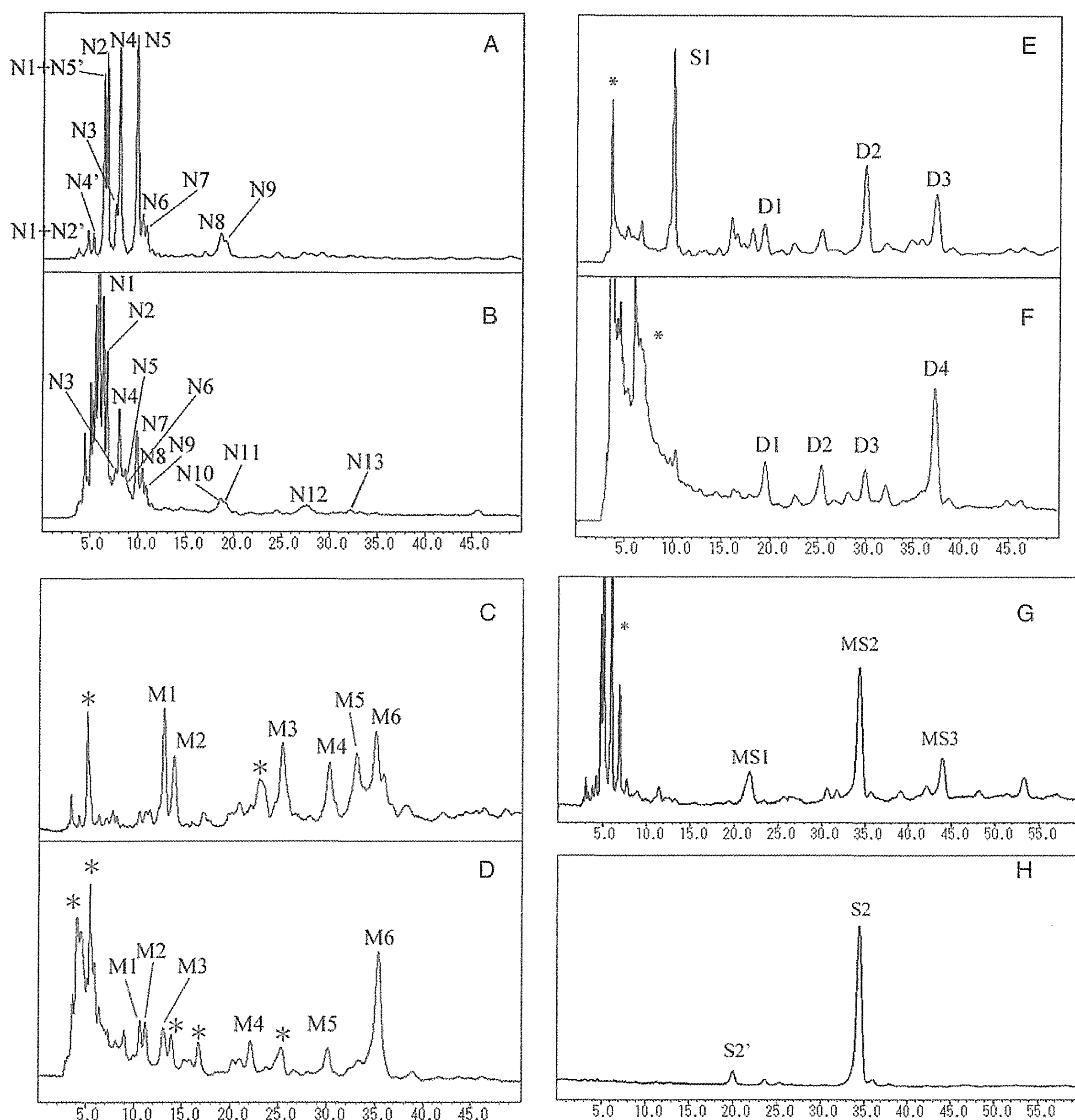

Fig. 2. Reverse-phase ODS elution profiles of PA-glycans obtained from each different fraction separated on the DEAE column. The neutral, mono-sialyl, di-sialyl or mono-sulfate, mono-sialyl-mono-sulfate and di-sulfate fractions were individually applied to the ODS column and gave elution profiles according to their hydrophobicity. (**A**) pig Peak 1, (**B**) human Peak 1, (**C**) pig Peak 2, (**D**) human Peak 2, (**E**) pig Peak 3, (**F**) human Peak 3, (**G**) pig Peak 4 and (**H**) pig Peak 5. N2′: Epimerization of N2; N4′: Epimerization of N4; N5′: Epimerization of N5; S2′: Epimerization of S2. Asterisks indicate the fractions containing no detectable PA-oligosaccharides.

as well. Among them, one neutral, five mono-sialyl and six sulfates of *N*-linked glycans in the API preparation were not detected in human islets. The structures of 9 of these 12 glycans were clearly identified in this study.

Concerning the characteristics of the *N*-glycans identified in the API preparation, the neutral glycans contained relatively high levels (%) of high-mannose type glycans. In comparison with the *N*-glycans from human islets, the high-mannose type of *N*-glycan found in API contains high levels (5 or 6) of mannoses. In addition, glycans with structures of fractions N6-2 were not detected in human islets. On the other hand, in the case of API, the relative content of sulfated *N*-glycans approached 10%. In addition, the di-sulfate type glycans represented 7% of the relative quantity, indicating that sulfated *N*-glycans are a common structure in *N*-glycans of API but do not appear to be produced by human islets. In addition, all the sulfates are attached to a β-linked *N*-acetylgalactosamine (GalNAc).

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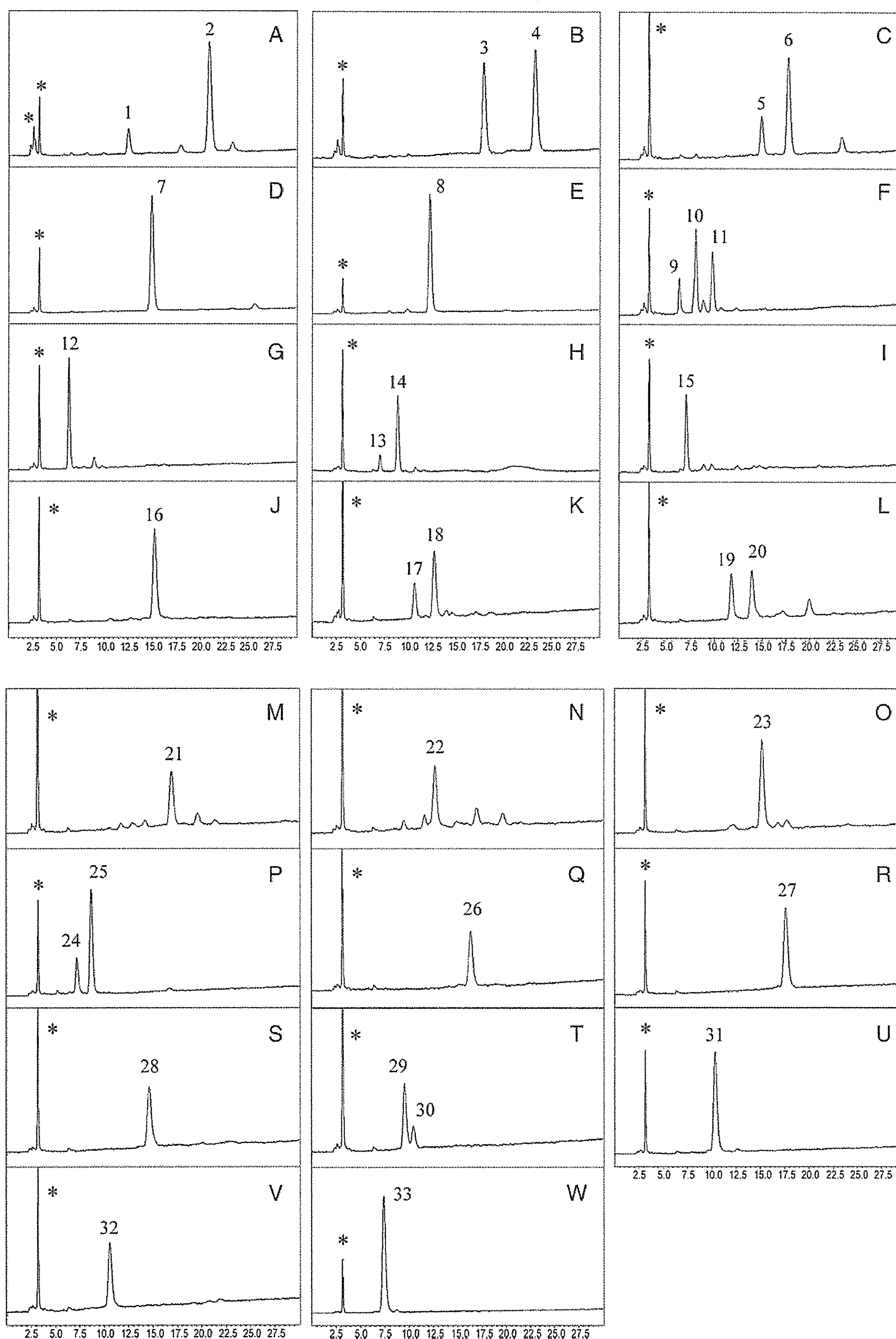

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Fig. 3. Amide column elution profiles of PA-glycans of pig islets from each different fraction separated on the ODS column. **(A)** ODS peak-N1 + N5′. Peak 1 is the epimerization of the ODS peak-N5. Peak 2 was then settled as N1. **(B)** ODS peak-N2. Peaks 3 and 4 correspond to N2-1 and N2-2, respectively. **(C)** ODS peak-N3. Peak 5 was contamination of the ODS peak-N4. Peak 6 corresponds to N3. **(D)** ODS peak-N4. Peak 7 corresponds to N4. **(E)** ODS peak-N5. Peak 8 corresponds to N5. **(F)** ODS peak-N6. Peak 9 was contamination of ODS peak-N7. Peaks 10 and 11 correspond to N6-1 and N6-2, respectively. **(G)** ODS peak-N7. Peak 12 corresponds to N7. **(H)** ODS peak-N8. Peak 13 was contamination of ODS peak-N9. Peak 14 corresponds to N8. **(I)** ODS peak-N9. Peak 15 corresponds to N9. **(J)** ODS peak-M1.

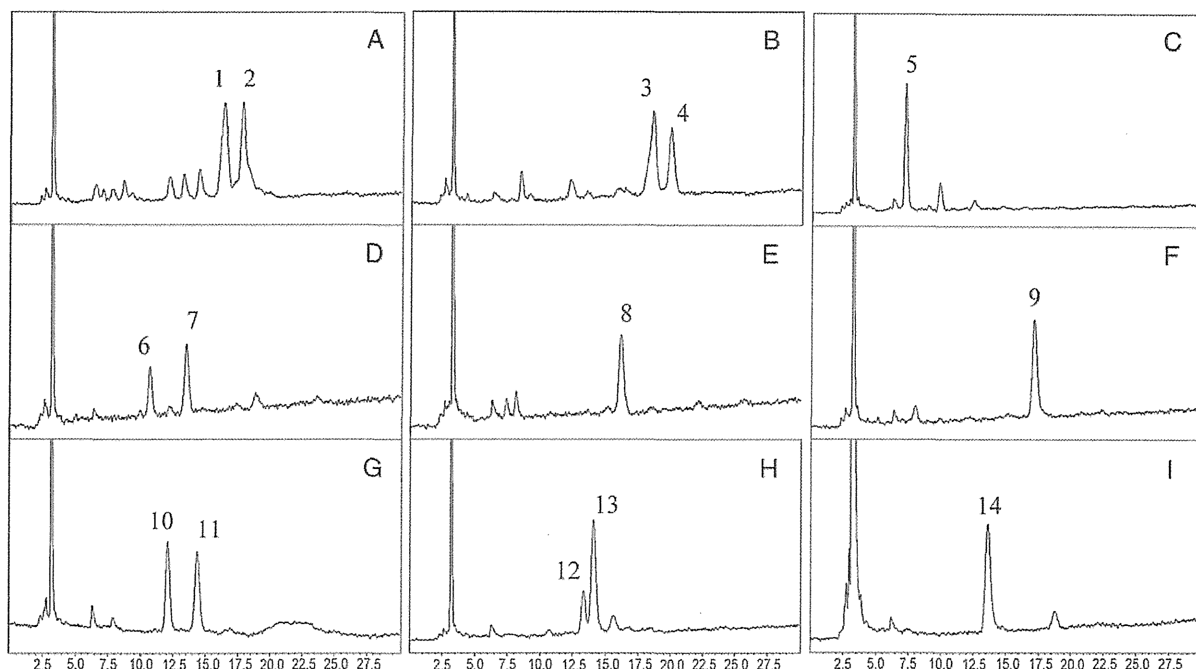

Fig. 4. Amide column elution profiles of PA-glycans from each fraction separated on the ODS column of human islets. **(A)** ODS peak-N5. Peaks 1 and 2 correspond to N5-1 and N5-2, respectively. **(B)** ODS peak-N6. Peaks 3 and 4 correspond to N6-1 and N6-2, respectively. **(C)** ODS peak-N11. Peak 5 corresponds to N11. **(D)** ODS peak-N12. Peaks 6 and 7 correspond to N12-1 and N12-2, respectively. **(E)** ODS peak-N13. Peak 8 corresponds to N13. **(F)** ODS peak-M1. Peak 9 corresponds to M1. **(G)** ODS peak-M2. Peaks 10 and 11 correspond to M2-1 and M2-2, respectively. **(H)** ODS peak-M4. Peaks 12 and 13 correspond to M4-1 and M4-2, respectively. **(I)** ODS peak-D2. Peak 14 corresponds to D2.

No terminal fucose was detected in the *N*-glycans from either type of islets in this study.

Previous studies reported by other groups concluded that many kinds of *N*-glycans are found in API, using MALDI-TOF/MS and MS/MS (Kim, Gil et al. 2008; Kim, Gil et al. 2009; Kim, Harvey et al. 2009). The difference in the number of detected *N*-glycans in this study can be attributed to the sensitivity of the MS method and HPLC. It, thus, appears that the accuracy of the data presented here using HPLC mapping in conjunction with a MALDI-TOF technique provided much more detailed information. That is, MS data are sensitive and can be rapidly obtained, but indicate only a glycan structure based on the calculated molecular weight. Therefore, discriminating between isomeric structures becomes difficult (Wheeler and Harvey 2001). In addition, except for N-glycolylneuraminic acid (NeuGc), it does not indicate the specific structure of sialyl acids present. On the other hand, the data reported herein can be used to identify the representative features of each *N*-glycan in the API preparation. However, the possibility that several glycans, such as pN6-2, pM2-1, pM2-2, pM3-1, pM3-2 and pM5, that were not detected in humans islets as major *N*-glycans are expressed in human islets at very low levels cannot be completely excluded. In addition, concerning the sulfated *N*-glycans such as S1-1, S1-2, S2, MS1 and MS3, the accuracy in identifying the position of the SOH3 attached to β1-4GalNAc was not clear in this study, and it is possible that these sulfated glycans also may be produced in human islets or other tissues, because humans produce several sulfotransferase enzymes that can catalyze the attachment of a sulfate to GalNAc (Boregowda et al. 2005).

Chlorate is a selective inhibitor of adenosine triphosphate sulfate adenylyltransferase, the first enzyme in the sulfate activation pathway (Girard et al. 1998). It inhibits all sulfotransferases. Therefore, although API had a diminished antigenicity to human serum, especially IgM, as a result of the presence of sodium chlorate treatment, a structural analysis of the changes on the sulfated *N*-glycans and other nonsulfated glycans of the API after the treatment might be needed to assess antigenicity issues. On the other hand, it was not possible to determine the binding site of the sulfate residue to GalNAc using this method. However, the possibility that the sulfate residue is one of the non-Gal antigens in pig islets cannot be excluded based on the data presented herein. Further study will be needed to analyze the non-Gal antigen in pig islets, especially to sulfotransferase enzymes.

In comparison with a report concerning the pig lung and trachea, using exactly the same HPLC mapping in conjunction with the MALDI-TOF technique, Sriwilaijaroen et al. (2011) reported a relatively small percent of high-mannose type

Peak 16 corresponds to M1. **(K)** ODS peak-M2. Peaks 17 and 18 correspond to M2-1 and M2-2, respectively. **(L)** ODS peak-M3. Peaks 19 and 20 correspond to M3-1 and M3-2, respectively. **(M)** ODS peak-M4. Peak 21 corresponds to M4. **N:** ODS peak-M5. Peak 22 corresponds to M5. **(O)** ODS peak-M6. Peak 23 corresponds to M6. **(P)** ODS peak-S1. Peaks 24 and 25 were identified as S1-1 and S1-2, respectively. **(Q)**: ODS peak-D1. Peak 26 corresponds to D1. **(R)** ODS peak-D2. Peak 27 corresponds to D2. **(S)** ODS peak-D3. Peak 28 corresponds to D3. **(T)** ODS peak-MS1. Peak 29 corresponds to MS1. Peak 30 is the epimerization of ODS peak-MS2. **(U)** ODS peak-MS2. Peak 31 corresponds to MS2. **(V)** ODS peak-MS3. Peak 32 corresponds to MS3. **(W)** ODS peak-S2. Peak 33 corresponds to S2. *Not a sugar.

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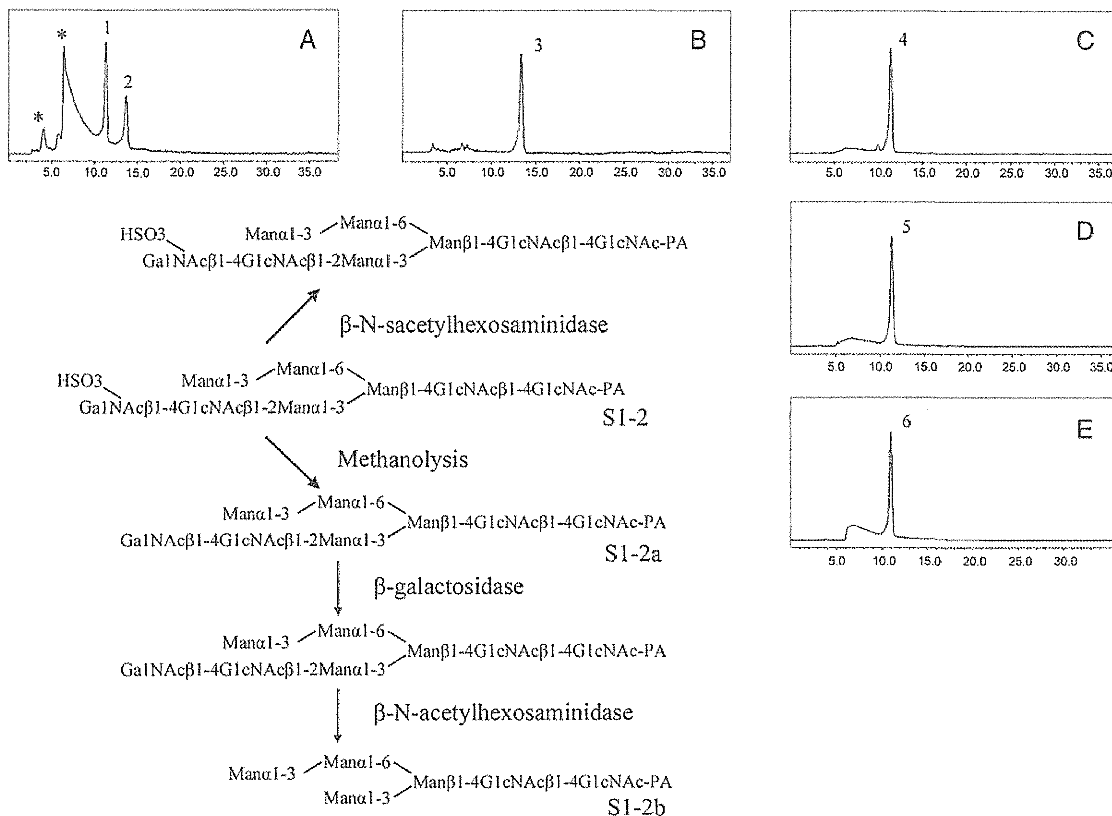

Fig. 5. Structural analysis of S1-2. (**A**) ODS peak after methanolysis treatment of S1-2. Peak 1 is the nonreacted sample, S1-2 (7.5 GU and 1641 Da). Peak 2 corresponds to S1-2a (8.3 GU and 1557 Da). (**B**) ODS peak after β-galactosidase treatment to S1-2a. Peak 3 is identical to S1-2a in ODS (GU) and molecular weight. (**C**) ODS peak after β-*N*-acetylhexosaminidase treatment of S1-2a. Peak 4 corresponds to S1-2b (7.5 GU and 1151 Da). (**D**) ODS peak after co-chromatography of S1-2b and M4.1. S1-2b was then proved to be the same structure as M4.1 in GALAXY. (**E**) ODS peak after β-galactosidase treatment to S1-2. Peak 6 is just the same as S1-2 in GU and molecular weight. * Not a sugar.

N-glycans. However, in this study, pig islets contain a relatively large percent of *N*-glycans, 81%, and human islets also contain 76.7%. Therefore, this evidence related to high-mannose types was assumed to be a typical feature of islets. It is noteworthy that in this pig islets study no evidence was found for the presence of α-Gal and NeuGc structures, while the pig lung and trachea clearly produce both antigens. Concerning α-Gal, as has been indicated in many reports, pig islets express very low levels of α-Gal. On the other hand, concerning NeuGc, our previous study reported that NeuGc is expressed on the *N*-glycans of API (Komoda et al. 2004). Therefore, pig islets must contain NeuGc in relatively minor amounts and, as a result, were not detected in this study, because pig lung and trachea contain relatively minor levels of NeuGc structures.

In addition, NeuGc-Gal-GlcNAc and Galα1-3 Lewis x (Lew^x) were recently reported as novel antigens, as evidenced by a structural analysis of *N*-glycans from the miniature pig kidney (Kim et al. 2006). However, neither of these antigens was detected in this study.

Blixt et al. (2009) reported on the carbohydrate specificities of sera obtained from clinical patients in whom neonatal bone pig islet-like cell clusters (NPCC) had been intraportally injected, using a printed covalent glycan array with 200 structurally defined glycans. Besides α-Gal and NeuGc, the patients had Abs against terminal α-linked GalNAc, β3-linked Gal especially Galβ1,3GlcNAc even if terminally sulfated or sialylated, β-GlcNAc except for β1,3-linked, oligomannosyl compounds, some neuranimic acid (NeuAc) and Galα1-3Lew^x. Compared with the data reported here, pM5 has β-GlcNAc, might be applicable for the target structure of the patients. In addition, N6-2, pM2-2 and pM3-2, which contain Manα1-3Manα1-6Man structures, are also potential target antigens. However, the antigenicity of NPCC may slightly be different from that for API.

As the other non-Gal antigens, the Forssman, the terminal GalNAc related to the Tn-antigen (GalNAcα-*O*-Ser/Thr), T-antigen (Thomsen-Friedenreich; Galβ13GalNAcα-*O*-Ser/Thr) and sialyl-Tn antigen (NeuAcα2,6GalNAcα-*O*-Ser/Thr) are also reported to be important (Ezzelarab et al. 2005). However, these glycans are related to *O*-glycans and glicolipids (Diswall et al. 2011).

In summary, as a feature, pig islets are rich in high-mannose type *N*-glycans, especially relatively low amounts of mannose. Several API structures, such as N6-2, pM2-1, 2-2, 3-1, 3-2, and pM5, and the sulfate structure, β-linked GalNAc-SOH3, were not detected in human islets. In addition, it is possible that the sulfated glycans of API are involved in the observed antigenicity to human serum. The data herein provide important information that can be useful to future clinical xenotransplantation studies.

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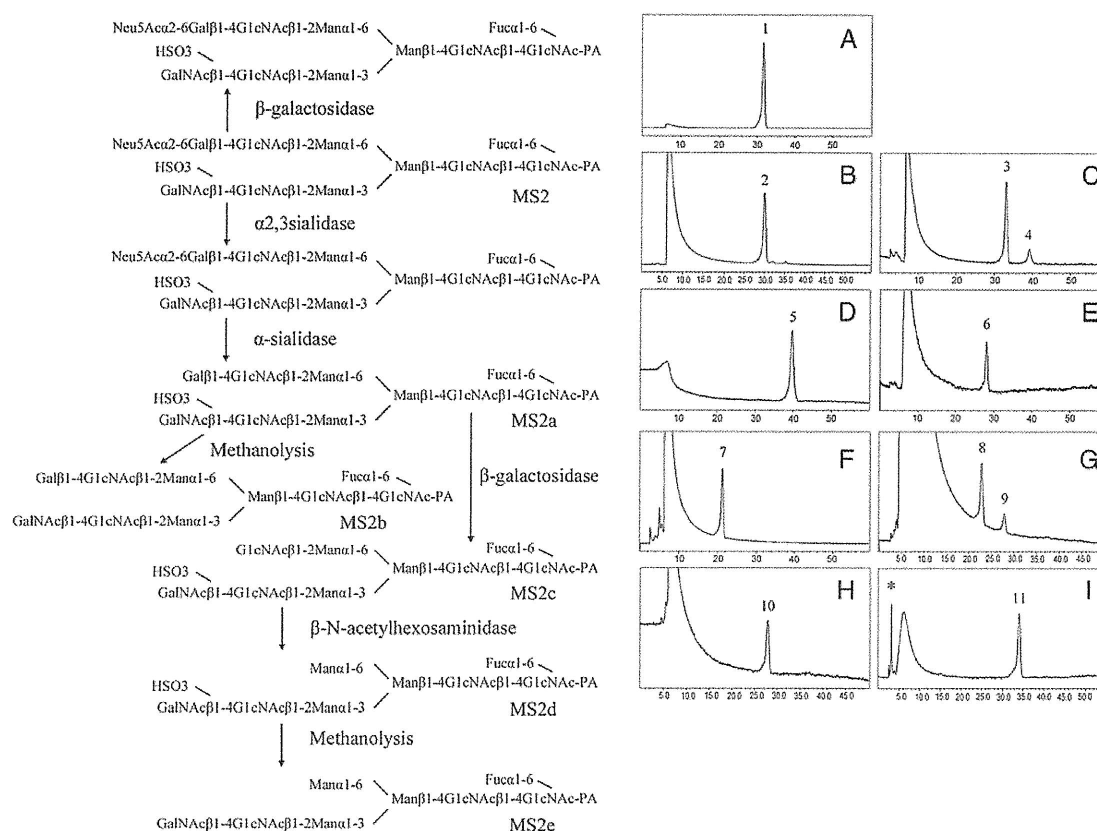

Fig. 6. Structural analysis of MS2. (**A**) ODS peak after α2,3-sialidase treatment to MS2. Peak 1 was just the same as MS2 in GU and molecular weight. (**B**) ODS peak after α-sialidase treatment to MS2. Peak 2 corresponds to MS2a (12.1 GU and 1988 Da). (**C**) ODS peak after methanolysis treatment to MS2a. Peak 3 is the nonreacted sample. Peak 4 lacked one sulfate residue from MS2a and corresponds to MS2b (13.9 GU and 1907 Da). (**D**) ODS peak after co-chromatography of MS2b and 210.4a in GALAXY. MS2b was proved to be the same structure as the 210.4a in GALAXY. (**E**) ODS peak after β-galactisidase treatment to MS2a. Peak 6 lacked one galactose from MS2a and corresponds to MS2c (11.4 GU and 1826 Da). (**F**) ODS peak after β-*N*-acetylhexosaminidase treatment to MS2c. Peak 7 corresponds to MS2d (9.7 GU and 1622 Da). (**G**) ODS peak after methanolysis treatment of MS2d. Peak 8 was the nonreacted sample. Peak 9 lacked one sulfate residue from MS2d and corresponds to MS2e (11.0 GU and 1541 Da). (**H**) ODS peak after co-chromatography of MS2e and 110.4a in GALAXY. MS2e was proved to be the same structure as the 110.4a in GALAXY. (**I**) ODS peak after β-galactisidase treatment to MS2. Peak 11 is identical to MS2 in GU and molecular weight.
* Not a sugar.

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Materials and methods

Pig islet isolation

Pancreatic glands were removed from several pigs at a slaughterhouse that handles young market weight pigs (Large White/ Landrace x Duroc, 6 months old, ~100 kg). Isolation of porcine islets was performed using the Islet Isolation Technique (Goto et al. 2004), with minor modifications. Purified islet fractions were pooled and cultured at 37°C in a humidified atmosphere with 5% CO_2 in CMRL1066 medium (Biochrom, Berlin, Germany) supplemented with 20% heat inactivated porcine serum, 2 mm *N*-acetyl-L-alanyl-L-glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N*1-2-ethanesulfonic acid, 100 IU/mL penicillin, 100 μg/mL streptomycin (Biochrom) and 20 μg/mL ciprofloxacin (Bayer, Leverkusen, Germany).

Human islet isolation

The method used to isolate islets has been reported previously (Matsumoto et al. 2002). In brief, the pancreas was distended with a cold enzyme solution through the pancreatic duct using a pressure-controlled pump system. In all cases, the distended pancreata were digested using the semi-automated method (Matsumoto et al. 2006). All centrifuged pellets were collected in cold storage/purification stock solution (Mediatech, Inc., Manassas, VA).

Islet isolations were conducted based on the Edmonton protocol with our modifications. The results of the isolations were evaluated based on the Edmonton protocol. Islets were purified with a COBE 2991 cell processor (CaridianBCT, Inc., Lakewood, CO) using density-adjusted iodixanol-based continuous density gradient. The final preparation of islets was assessed using dithizone staining (Sigma Chemical Co., St. Louis, MO) for islet yield and purity. Islet yield was converted into a standard number of islet equivalents (diameter standardizing to 150 μm). Islet viability was evaluated with fluorescein diacetate (10 μmol/L) and propidium iodide (15 μmol/L) staining. All procedures were done at the Baylor Research Institute, TX.