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Table. Primers and probes used in this study.

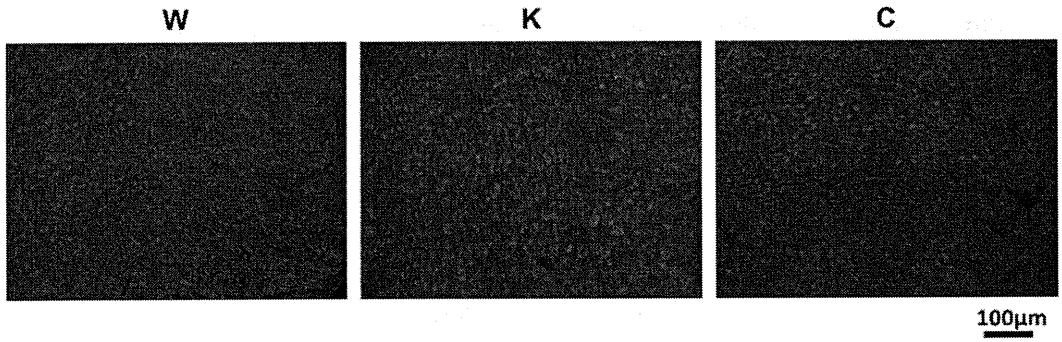
	Forward primer	Reverse Primer	Probe
GAPDH	GCATGGCCTTCCGTGTTC	GATGCCTGCTTCACCACCTT	CCGCCTGGAGAAACCTGCCAAGTATG
TNF-α	CCACCACGCTCTTCTGTCTACT	TTGGTGGTTTGCTACGACGT	CCCAGACCCTCACACTCAGATCATCTTC

Supplementary figure legends

Supplementary figure 1. Capillary formation in the iACS-treated heart. Four weeks after infarction and iACS transplantation, CD31 staining was performed to assess angiogenesis. Representative images from each group are shown. The density of CD31-positive capillaries was the same among the groups. Green, CD31; blue, nuclei.

Supplementary figure 2. Hemodynamic effects of WT-iACS transplantation in rat AMI. In the iACS-treated group, the dP/dt_{max} was significantly higher and the dP/dt_{min} significantly lower than in the control ($P<0.05$ v.s. sham-treated group, *unpaired t* test). The τ value was not significantly different between the groups, although it was smaller in the iACS-treated group ($P=0.12$ v.s. sham-treated group, *unpaired t* test). The ESPVR value was significantly higher in the iACS-treated group. tx, treatment.

Supplementary figure 1



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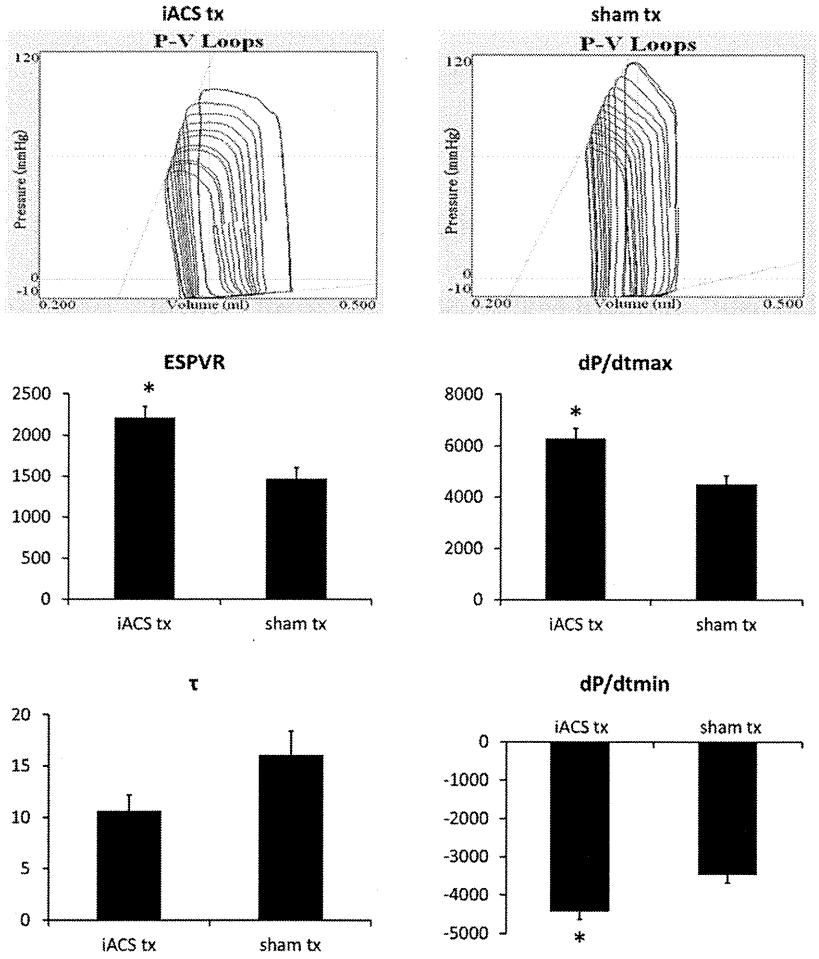
Supplementary figure 2



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Significant Improvement in Islet Yield and Survival with Modified ET-Kyoto Solution (ET-Kyoto/Neutrophil Elastase Inhibitor)

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Running head: Beneficial effect of sivelestatin islet transplantation

Key words: Type 1 diabetes, islet transplantation, neutrophil, neutrophil elastase

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ABSTRACT

Although islet transplantation can achieve insulin independence in patients with type 1 diabetes, sufficient number of islets derived from two or more donors is usually required to achieve normoglycemia. Activated neutrophils and neutrophil elastase (NE), which is released from these neutrophils, can directly cause injury in islet grafts. We hypothesized that inhibition of NE improves islet isolation and islet allograft survival. We tested our hypothesis by examining the effects of modified ET-Kyoto solution supplemented with sivelestat, a NE inhibitor (S-Kyoto solution), on islet yield and viability in islet isolation and the effect of intraperitoneally injected sivelestat on islet graft survival in a mouse allotransplant model. NE and proinflammatory cytokines such as Tumor Necrosis Factor (TNF)-α and Interleukin (IL)-6 increased markedly at the end of warm digestion during islet isolation and exhibited direct cytotoxic activity against the islets causing their apoptosis. The use of S-Kyoto solution significantly improved islet yield and viability. Furthermore, treatment with sivelestat resulted in significant prolongation of islet allograft survival in recipient mice. Furthermore, serum levels of IL-6 and TNF-α at 1 and 2 weeks posttransplantation were significantly higher in islet recipients than before transplantation. Our results indicated that NE released from activated neutrophils negatively affects islet survival and that its suppression both *in vitro* and *in vivo*

improved islet yield and prolonged islet graft survival. The results suggest that inhibition of NE activity could be potentially useful in islet transplantation for patients with type1 diabetes mellitus.

INTRODUCTION

Since the reporting of the Edmonton protocol, islet transplantation has become one of the treatment options for patients with type 1diabetes mellitus (8,41,43-48). Islet transplantation is a minimal invasive approach for β-cell replacement compared with pancreas transplantation (18,44,47). However, a sufficient number of islets derived from two or more donor pancreas are usually required to achieve insulin independence, since a substantial number of transplanted islets fail to engraft into the recipient liver for a variety of reasons such as apoptosis, inflammation and ischemia (1,3,27,29,37,41,42,47,58,59). Furthermore, research into islet transplantation has been hindered by the inability to isolate a sufficient number of islets from a single donor pancreas (4,15,16,45,47). Thus, there is a need for novel strategies that increase islet yield, maintain high islet quality, and protect transplanted islet grafts.

Indeed, the islet isolation procedure itself can lead to tissue destruction and activation of cellular and non-cellular components of the pancreas, including resident neutrophils, macrophages and T cells, which probably play an important role in impairment of islet survival (1,4,31,37,42). In the present study, we focused on the role of neutrophils, in particular neutrophil elastase (NE), against islets during islet isolation. The NE is a 29-kDa (kilodalton) glycoprotein chymotrypsin-like serine protease stored in azurophil granules in its inactive form until it is released after

neutrophil exposure to inflammatory stimuli (17,49,55). Once released, NE is fully active, and the excessive release of NE degrades elastin, collagens, laminins and other extracellular matrix components, thereby leading to subsequent tissue damage through endothelial cell injury (12,17,49,54,55).

Sivelestat (ONO-5046) is a low molecular weight synthetic specific and competitive inhibitor of NE activity (12,17,21,30,49,50,54,55,60). This agent has been employed clinically in Japan and shown to attenuate acute lung injury associated with systemic inflammation response, which is sometimes seen after infection, surgical intervention, traumatic or burn injury (11,49,50,60). In addition, sivelestat exhibits potent cytoprotective properties in animal models of liver and lung transplantation (30,54), hepatectomy (17,21) and ischemia/reperfusion injury (17,54,55).

The objectives of the present study were to determine whether the addition of sivelestat to the islet isolation solution improves islet yield and viability. We also investigated the cytoprotective effects of sivelestat in islet recipients. The results suggested that NE inhibition using sivelestat is an attractive new therapeutic option in islet isolation and transplantation and could have a significant impact on patients with type 1 diabetes by allowing successful one-donor to one-recipient.

MATERIAL AND METHODS

Drugs and reagents

Sivelestat (ONO-5046) is a newly synthesized agent known to selectively inhibit NE. Sivelestat was a generous gift from Ono Pharmaceutical Co., Ltd. (Osaka, Japan). A stock solution was prepared by dissolving 200 mg of sivelestat at room temperature in 20 ml of phosphate-buffered saline (PBS) with 24.5 mg of sodium carbonate and stored at 4 °C until use.

Preservation solutions

University of Wisconsin (UW, Bristol-Myers Squibb Company, Princeton, NJ) and ET-Kyoto (Otsuka Pharmaceutical, Tokyo, Japan) solution were prepared. Stock solutions of 20 μM sivelestat in UW and ET-Kyoto were prepared as S-UW, S-Kyoto, each. Sivelestat did not change the density of islets or other tissue components of the pancreas.

Mice

Male C57BL/6J mice and Balb c/A mice, 10-12-week-old, weighting 20-30 g, were purchased from CLEA Japan, Inc. (Tokyo). All experiments were approved by the International Animal Care and Use Committee (IACUC) of Osaka University Medical School.

Islet isolation and assessment

Briefly, after clamping the distal common bile duct under anesthesia, the common bile duct was cannulated. Then, the pancreatic tissue was distended by using 3 ml of isolation solution containing 1 mg/ml of collagenase VIII (Sigma-Aldrich). The distended pancreas was excised and incubated in 37 °C warm shaker for 15 min. The digested pancreas was washed with appropriate isolation solution three times by centrifugation (270 x *g*, 2 min,4°C), then purified with a discontinuous density gradient (1.111, 1.104, 1.097, 1.072 g/ml) in isolation solution containing iodixianol (Optiplep®, Axis-Shild, Oslo, Norway). The purified islets were collected and cultured with Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) supplemented with 10% Fetal bovine serum (FBS) (Sigma-Aldrich), 100 U/ml penicillin, 100µg/ml streptomycin and 0.1 mM non-essential amino acids (Invitrogen, Carlsbad, CA) under 5% CO_2 atmosphere at 37°C (26).

To evaluate the isolated islets, islet count, islet equivalents (IEQ), distribution of islet size, and islet purity were determined as described previously (35,40). Islet yield and distribution of islet size were determined by measuring islets after dithizone staining (Wako, Osaka) using VH analyzer (Keyence, Osaka). The purification recovery rate was defined as the percentage of IEQ recovered after purification compared to the IEQ before purification (34). Islet purity was assessed by four independent investigators.

In vitro cytotoxicity assay

The cytotoxic activity of NE against isolated islets was assessed by the Lactate Dehydrogenase (LDH) assay kit (Roche Applied Science, Mannheim, Germany).

Briefly, the harvested islets were plated at 30 islets/well in 96-well round plate.NE (Calbiochem, San Diego, CA) was then added to the wells at various concentrations and the plates were incubated for 24 h at 37 °C. Next, 100 µl of the culture supernatant was transferred into the wells of 96-well flat plate. The reaction mixture was added to each well and then absorbance was measured at 490 nm. Moreover, to examine the cytoprotective effects of sivelestat on NE cytotoxicity, 2, 20 or 200 µM of sivelestat was simultaneously added to the NE-containing wells and the plates were incubated for 24 h at 37 °C followed by measurement of absorbance at 490 nm.

Staining for naphthol AS-D chloroacetate esterase

To assess the accumulation of activated neutrophils in the pancreas during islet isolation, the tissue was stained by naphthol AS-D chloroacetate esterase (Sigma-Aldrich)as described previously(14,17). Briefly, the pancreas specimens obtained before and at the end of warm digestion were fixed in 10% formalin and embedded in paraffin. Tissue sections (2-µm thick) were stained with naphthol AS-D chloroacetate esterase. In addition, the specimens were counterstained with hematoxylin. Activated neutrophils were positively stained red-brown and counted under the microscope at a magnification of ×100.

Neutrophil elastase activity assay

NE enzyme activity was measured in the supernatant at each step of islet isolation, including before warm digestion, at the end of warm digestion and after purification, using the method described previously (11,12,17,55,59). For this

purpose, 20µl sample was incubated with1 mM of *N*-methoxysuccinyl-Ala-Ala-Pro-Val- *p*-nitroanilide (*p*- NA) (Sigma-Aldrich), which is a highly specific synthetic substrate for NE, in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5M NaCl at 37°C for 24 h. The incubated samples were plated onto a 96-well plate and then absorbance was measured at 405 nm to detect free *p*-NA.

Assessment of apoptosis of isolated islets

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed to detect apoptotic cells during islet isolation, using Apop Tag® Peroxidase in Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA) as described previously (53-55). The negative control was prepared by omission of the terminal transferase. Positive control was generated by treatment with deoxyribonuclease (DNase) I. Peroxidase activity was visualized with diaminobenzidine (DAB) substrate, which yielded a brown oxidation product and 0.5% methyl green was used for counterstaining. TUNEL-positive cells were counted under the microscope at × 400 magnification.

Scanning electron microscopy (SEM)

Morphological analysis of the isolated islets was carried out by SEM as described previously (31). The isolated islets were fixed with 2.5% PBS (0.1M, pH 7.4) glutaraldehyde (Tokyo Chemical Industry, Tokyo) solution at 4 °C for 24 h. After washing, they were post-fixed with 1% OsO_4/PBS for 2 h at 4 °C. Subsequently, the fixed islets were incubated in 1% tannin acid solution at 4 °C overnight and then

dehydrated. They were transferred to isoamyl acetate and dried in a critical-point dryer using liquid CO_2. They were mounted on the stage and observed under a scanning electron microscope (S-800, Hitachi, Tokyo). The morphology was assessed by four independent investigators.

Islet viability assay

The viability of isolated islets was assessed by tetramethyl rhodamine ethyl ester (TMRE; Molecular Probes, Eugene, OR) assay and 7-aminoactinomycin D (7-AAD; Molecular Probes) assay, as described previously (9). TMRE is an indicator of the mitochondria membrane potential (MMP) and used as a marker for live cells(9), while 7-AAD is used as a marker for dead cells or apoptotic cells(9). Briefly, the isolated islets were cultured for 24 h and then incubated in 1 ml of TrypLE Express (Invitrogen) for 15 min at 37 °C to prepare single islet cells and then dispersed. The single islet cells were incubated with 100 ng/ml of TMRE for 30 min at 4 °C. Subsequently, the fluorescence intensity of TMRE was analyzed with a FACS Calibur flow cytometer (BD Immunocytometry, San Jose, CA). In a similar fashion, the isolated islets were dispersed into single cells and then incubated with 1 μg/ml of 7-AAD.Subsequently, the fluorescence intensity of 7-AAD was analyzed with a FACS Calibur flow cytometer.

To further determine the islets viability, the colorimetric methyl tetrazolium salt (MTS) assay was performed as described previously (56). The viability of freshly isolated islets or cultured islets over either 24 or 48 h was evaluated by monitoring metabolic activity with MTS assay using the cell Titer 96 Aqueous One reagent

(Promega, Madison, WI). The colorimetric reagent was added to each well and incubated for 2 h and the absorbance values were read at 490 nm.

Glucose-stimulated insulin release in vitro

To evaluate the *in vitro* insulin function of islets, static glucose change was measured as described previously (33,35). Twenty islets were cultured overnight at 37 °C and then pre-incubated in low glucose culture medium (2.8 mM glucose) for 60 min at 37 °C. After pre-incubation, the islets were incubated in low glucose culture medium (2.8mM glucose) at 37 °C for 60 min. Subsequently, the supernatant of the culture medium was collected and the islets were incubated in high-glucose culture medium (20 mM glucose) at 37 °C for 60 min. Similarly, the supernatant was collected and insulin concentration measured by the mouse-insulin Enzyme-Linked ImmunoSorbent Assay (ELISA) kit (Mercodia, Uppsala, Sweden). Glucose-stimulated insulin concentration was expressed as the stimulation index (SI), calculated as the ratio of insulin released during exposure to high glucose over the insulin released during low glucose incubation.

Islet transplant experiments

The recipient 10-12-week-old male Balbc/A mice were divided at random into two experimental groups (Figure 6A, n=5/group) to receive allogeneic islets isolated from C57BL/6J mice by the use of either isolation solution (i.e., ET-Kyoto and S-Kyoto solutions). The recipient mice were rendered diabetic by a single injection of streptozotocin (STZ) (Nacalai tesque, Kyoto, Japan) at a dose of 180 mg/kg

intraperitoneally. Hyperglycemia was defined as glucose level of >400 mg/dl measured twice consecutively after STZ injection. Then, 500 of freshly isolated islets were transplanted under the kidney capsule. After transplantation, non-fasting blood glucose level was monitored using samples from tail blood by Glutest PRO (Sanwa kagaku, Nagoya, Japan). Normoglycemia after transplantation was defined as two consecutive blood glucose levels below 200 mg/dl. Islet rejection after transplantation was defined when two consecutive blood glucose levels exceeded 200 mg/dl. At one week post-transplantation, the engrafted kidneys were excised to assess the survival of islet grafts by immunohistochemistry. Moreover, the beneficial effects of monotherapy with intraperitoneal sivelestatin recipient mice were assessed. Sivelestat was administered at 100 mg/kg/day/for one day before transplantation and every day until 14 days after transplantation, as described previously (32). The recipient Balbc/A mice treated with sivelestat were divided at random into two experimental groups (n=5/group) to receive allogeneic islets isolated from C57BL/6J mice using ET-Kyoto or S-Kyoto solution.

Intraperitoneal glucose tolerance test (IPGTT)

The IPGTT was performed at one week post-transplantation, using the method described previously (2,9,58). Mice (n=3/group) were fasted overnight and then injected intraperitoneally (ip) with 2 g glucose in saline/kg body weight. Untreated diabetic mice and non-diabetic wild-type mice were transplanted with saline as a control. Blood glucose levels were measured before injection and at 15, 60, and 120 min after injection.

Immunohistochemical analysis

Immunohistochemical analysis was performed using the method described previously (36). The engrafted kidneys were excised, fixed in formalin and embedded in paraffin. Tissue sections (2-μm thick) were placed in 0.3% H_2O_2/methanol to quench endogenous peroxidase activity, and incubated with 5% bovine serum albumin (BSA)-PBS to block non-specific reaction. The slides were incubated with rabbit anti-insulin polyclonal antibody (pAb, Santa Cruz Biotechnology, CA) to detect the transplanted islets. The sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Bethyl Laboratories Inc., Montgomery, TX) and then immunostaining was visualized with 0.02% DAB (Sigma-Aldrich) as the chromogen. After washing, the sections were counterstained with hematoxylin. Control tissue sections were prepared in a similar fashion except no primary antibody was used.

Measurement of proinflammatory cytokines

The supernatant was collected after each step of islet isolation, including before warm digestion, at the end of warm digestion and after purification. Moreover, serum samples were collected from islet recipients at day 1 before transplantation and days 4, 7, 14, 21, 28 after transplantation. These samples were frozen immediately at -80°C until analysis. Proinflammatory cytokine (IL-2, 4, 6, 10, 17A, IFN-γ, TNF-α) levels in these samples were measured using the BD™ Cytometric Bead Array Mouse Th1/Th2/Th17 CBA kit (BD Biosciences, San Jose, CA) and analyzed on a FACS Calibur Flow cytometer (BD Immunocytometry).

Statistical analysis

All experiments were done using each independent mouse. One mouse was used in each experiment (n=1). Values were expressed as mean ± SD. Differences between groups were examined for statistical significance using the two-tailed unpaired Student's t-test or one-way analysis of the variance (ANOVA) followed by Bonferroni's post hoc test when multiple comparisons were made. A P value less than 0.05 denoted the presence of significant difference.

RESULTS

Direct cytotoxicity of neutrophil elastase and optimum effective concentration of sivelestat

To assess the direct cytotoxicity of NE against isolated islets, LDH assay was performed at various concentrations of this enzyme. NE caused 43-48% killing at both 5 and 10 μg/ml. To prevent this killing, sivelestat was added to the culture medium at 2, 20 or 200 μM. The cytotoxicity induced by 10 μg/ml of NE could not be inhibited by sivelestat (Fig. 1A). However, the cytotoxicity induced by less than 5 μg/ml of NE was significantly abrogated by either 20 or 200 μM of sivelestat, but not by 2 μM of sivelestat (Fig. 1A).

To elect the optimum concentration of sivelestat for islet isolation, islet isolation was performed by the addition of various concentrations of sivelestat to the isolation solution (Fig. 1B). Islet yields using a high concentration of sivelestat (200 μM or 2 mM) were significantly lower than that with 20 μM of sivelestat. In contrast, no significant improvement in islet yield was observed by 2 μM of sivelestat isolation compared with that of the control group (ET-Kyoto isolation). These findings correlated with those from LDH assay and thus the following experiments were performed using 20 μM of sivelestat.

Activation of neutrophils in pancreas during collagenase digestion

Naphthol AS-D chloroacetate esterase staining was performed to demonstrate whether resident neutrophils in the pancreas tissue are activated by collagenase