the same medium. The SVF cells were then seeded at 7000 cells/cm^2 in an Upcell dish, which is coated with temperature-responsive polymers (CellSeed, Tokyo, Japan). The culture area was 1.9 cm^2 for mouse cells and 8.8 cm^2 for rat cells. Seven days after passage, the SVF cells were induced to differentiate into the adipogenic lineage by adding 0.87 μM insulin, 0.25 μM dexamethasone, 500 μM isobutylmethylxanthine (IBMX), and 5 μM Pioglitazone (Sigma-Aldrich) for 48 hours. Seven days after induction, the adipocytes were induced to spontaneously detach by placing the plates at 20 °C for 1 hour, yielding a scaffold-free sheet-shaped monolayer of induced adipocyte cell-sheet (iACS) that could be used as a graft. Finally, two iACS were layered to make a thicker sheet for grafting. Both WT mouse-derived iACS and APN-KO mouse-derived iACS were either assessed *in vitro* or labeled using a PKH26 red fluorescent linker kit (Sigma-Aldrich) prior to transplantation.

Enzyme-Linked Immunosorbent Assay

To determine the content of the secreted factors, enzyme-linked immunosorbent assays (ELISA) were performed. The collected culture supernatant of the WT-iACS, WT SVC cell-sheet or KO-iACS was centrifuged to remove debris and contaminating cells. For APN, samples were diluted 1:200 and analyzed (W, n=5; K, n=8). APN content of plasma samples from iACS-received APN-KO mice were analyzed with no dilutions (W 1 mo, n=4; W 3 mo, n=3; K

1 mo, n=4). An APN ELISA kit was purchased from Otsuka Pharmaceutical (Tokushima, Japan) according to the manufacturer's instructions. Content of HGF, VEGF, leptin, IL-6, IL-10, and TNFα in the culture supernatants were also analyzed by ELISA kit (R&D systems, MN, USA) with no dilutions (W, n=8-12; K, n=6-9).

Animal experiments

Myocardial infarction (MI) model of mice was created by left anterior descending artery (LAD) ligation as described previously.[3] Mice (10-15-weeks old, male C57BL/6J) were anesthetized by inhalation of isoflurane (1.5%, 1L/min, Mylan Inc., Pittsburgh, PA) provided by an anesthetic gas machine (DS Pharma, Osaka, Japan). The anesthetized mice were intubated in an endotracheal manner, and positive pressure ventilation was maintained with a ventilator (room air, 90 cycles/minutes, tidal volume 1 ml, Shinano, Tokyo, Japan). Then, the heart was exposed through a left lateral thoracotomy. With minimal manipulation of the fat pad surrounding the heart, the LAD component could easily be visualized. LAD was ligated with an 8-0 prolene suture (Johnson & Johnson, NJ, USA) at 1 mm distal to the left atrial appendage, immediately after bifurcation of the major left coronary artery. The myocardial ischemic area was visually assessed, to confirm that the LAD ligation had consistent ischemic effects. Procedure-related mortality, which occurred prior to chest closure, was consistently 6% in all the experimental

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groups, suggesting a consistent level of acute myocardial ischemia. Within 5 minutes after LAD ligation, the mice were randomly allocated into 3 groups; those that underwent transplantation of WT-iACS (W group; n=40), those that underwent transplantation of KO-iACS (K group; n=40) and those that underwent sham transplantation (C group; n=43). The pericardium was closed to prevent the dislocation of iACS. The mice were allowed to recover under care. In the experiment of iACS implantation to APN-KO mice, MI was not induced.

The mice were euthanized at 2 and 28 days after surgery by intravenous injection of pentobarbital (200 mg/kg body weight; DS Pharma) and 30 mM of potassium chloride (Wako Pure Chemical Industries, Osaka, Japan) to cause cardiac arrest in diastole under terminal anesthesia, and the heart was excised.

On day 2, the specimens for RNA analyses were cut transversely, and then the apex-side specimens were dissected to remove the right ventricular free wall, and part in the three pieces of infarction, peri-infarction, and remote, and soaked in RNA Later (Qiagen, Hilden, Germany, W, n=4; K, n=4; C, n=6). The specimens for CD11b staining were cut into 4 segments, embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan), and snap frozen in liquid nitrogen (W, n=4; K, n=4; C, n=6). The appropriate segments used for gene expression or histological analyses on day 2 were also used for TTC staining (W, n=8; K, n=7; C, n=9). The specimens used for the cytokine-array analysis were snap frozen in liquid nitrogen on day 2 (W,

n=4; K, n=5; C, n=6). The remaining mice were used for survival-rate analysis (W, n=24; K, n=26; C, n=25), but cases of accidental death were excluded. Twenty-eight days after treatment, 18 mice from each group were randomly chosen for cardiac performance analysis by echocardiography. Histological analyses were also performed at 28 days (W, n=6; K, n=8; C, n=5).

MI was also created in rats (8-week-old, female LEW/Sea) by the same method described above, except the tidal volume was 4 ml. Five minutes after LAD ligation, either two layers of iACS were transplanted onto the LV (n=9) or a sham transplantation was performed (n=6). Four weeks after the operation, a hemodynamic assessment was performed.

Quantitative real time PCR

Total RNA was isolated from the stored specimens using the RNeasy Mini Kit (Qiagen) and reverse transcribed with Ominiscript Reverse transcriptase (Qiagen). Quantitative PCR was performed with the ABI 7500 Fast Real-Time PCR System (Life Technologies) using Taqman Universal Master Mix (Life Technologies). Measurement copy number of mRNA was performed in triplicate. The primers and probes are shown in the Table. All probes were designed with a 5' fluorogenic probe 6FAM and a 3' quencher TAMURA. The results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Determination of infarct size

Freshly excised hearts from the W, K, and C groups 2 days after transplantation were washed in PBS and dissected into four transverse slices. The slices were then stained for 5 min at 37°C with 1% 2,3,5-triphenyltetrrazolium chloride (TTC; Sigma-Aldrich) to determine the infarct area. The stained slices were photographed, and then the infarct area was determined by computerized planimetry using MetaMorph Software (Molecular Device, CA, USA).

Histological analysis

Histological analyses of the hearts were performed 2 and 28 days after transplantation. The hearts and cell-sheets were cut into 8-μm sections. The sections were stained with antibodies for APN (1:1000 dilution; Otsuka Pharama), CD11b (1:100 dilution; Abcam, Cambridge, UK), or CD31 (1:200 dilution; Abcam). The secondary antibody was Alexa 488 goat anti-rabbit (1 μg/ml; Life Technologies). Counterstaining was with 6-diamidino-2-phenylindole (DAPI; 1 μg/ml; Life Technologies). Images were captured by fluorescence microscopy (Keyence, Osaka, Japan). Routine hematoxylin-eosin staining was performed. Masson's trichrome staining was performed to analyze the collagen accumulation. The collagen volume fraction in the peri-infarct area was calculated as the percentage of the myocardium. The data were collected

from 10 individual views per heart at a magnification of ×200. Furthermore, the heart sections were stained with Periodic acid-Schiff (PAS) to assess the cardiomyocyte size. Cardiomyocyte size at a magnification of ×400 was average from 50 myocytes per sample. MetaMorph Software was used for quantitative morphometric analysis.

Myocardial echocardiography

Echocardiography examinations were performed 4 weeks after cell transplantation by an investigator blinded to the group identities (n=18 each). Two-dimensional, targeted M-mode tracings were obtained at the level of the papillary muscles with an echocardiography system equipped with a 12-MHz transducer (GE Healthcare, WI, USA). The left ventricular (LV) dimensions were measured following the method of the American Society of Echocardiology from at least 3 consecutive cardiac cycles. Three readings were obtained from each mouse and averaged. The LV ejection fraction (EF) was calculated as $(LVDd^3\text{-}LVDs^3)/LVDd^3\times100$, where LVDd is the LV end-diastolic dimension and LVDs is the LV end-systolic dimension.[4]

Hemodynamic analysis

Four weeks after LAD ligation and cell-sheet transplantation, rats (iACS-treated group, n=9; sham-treated group, n=6) were anesthetized and ventilated. A silk thread was placed under the

inferior vena cava to change the LV preload. The conductance catheter (Unique Medical, Tokyo, Japan) was inserted through the LV apex toward the aortic valve along the longitudinal axis of the LV cavity, then fixed. A Millar 1.4 F pressure-tip catheter (Millar Instruments, TX, USA) was also inserted from the LV anterior and fixed. The conductance system and the pressure transducer controller (Integral 3; Unique Medical) were set as previously reported.[5] The conductance, pressure, and intracardiac electrocardiographic signals were analyzed with the Integral 3 software. The baseline indices were initially measured under stable hemodynamic conditions, then the pressure-volume loop was drawn during inferior vena caval occlusion, and analyzed. The following indices were calculated: dP/dtmax, dP/dtmin, the time constant of isovolumic relaxation (τ), and the end-systolic pressure-volume relationship (ESPVR).

Evaluation of survival rate after the operation

To evaluate the life-saving effect of the implantation, survival rates were determined. The mice were housed for 50 weeks after the operation. The survival rates of the mice in the W, K, and C groups were evaluated by the Kaplan–Meier method. The comparison among the three groups was analyzed by the overall log-rank test, and the pairwise comparisons were performed by log-rank test with the Benjamin-Hochberg multiplicity correction.

Cytokine antibody array

Protein was isolated from the stored specimens of mouse heart at -80°C. The whole heart tissues were homogenized in Tissue Extraction Reaction Reagent I (Life Technologies), centrifuged, and finally passed 0.22-μm filter (Millipore, MA, USA). Protein contents were measured by Milliplex Mouse Cytokine/Chemokine Panel Pre-mixed 32Plex, according the manufacturer's instructions (Millipore).

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Table. Primers and probes used in this study.

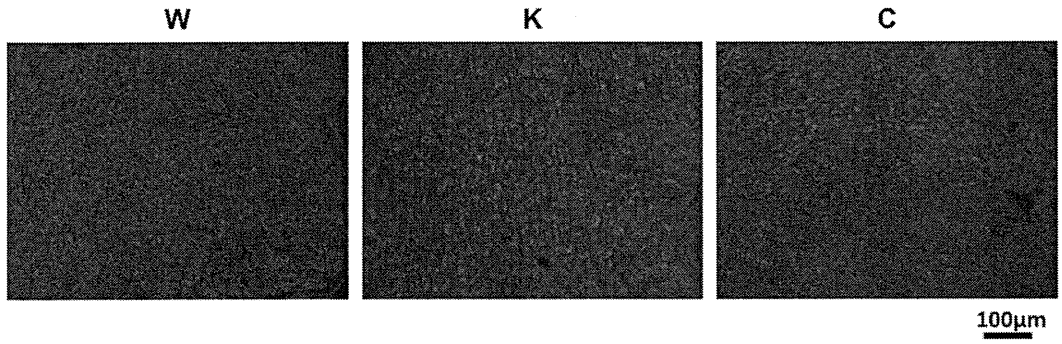
	Forward primer	Reverse Primer	Probe
GAPDH	GCATGGCCTTCCGTGTTC	GATGCCTGCTTCACCACCTT	CCGCCTGGAGAAACCTGCCAAGTATG
TNF-α	CCACCACGCTCTTCTGTCTACT	TTGGTGGTTTGCTACGACGT	CCCAGACCCTCACACTCAGATCATCTTC

Supplementary figure legends

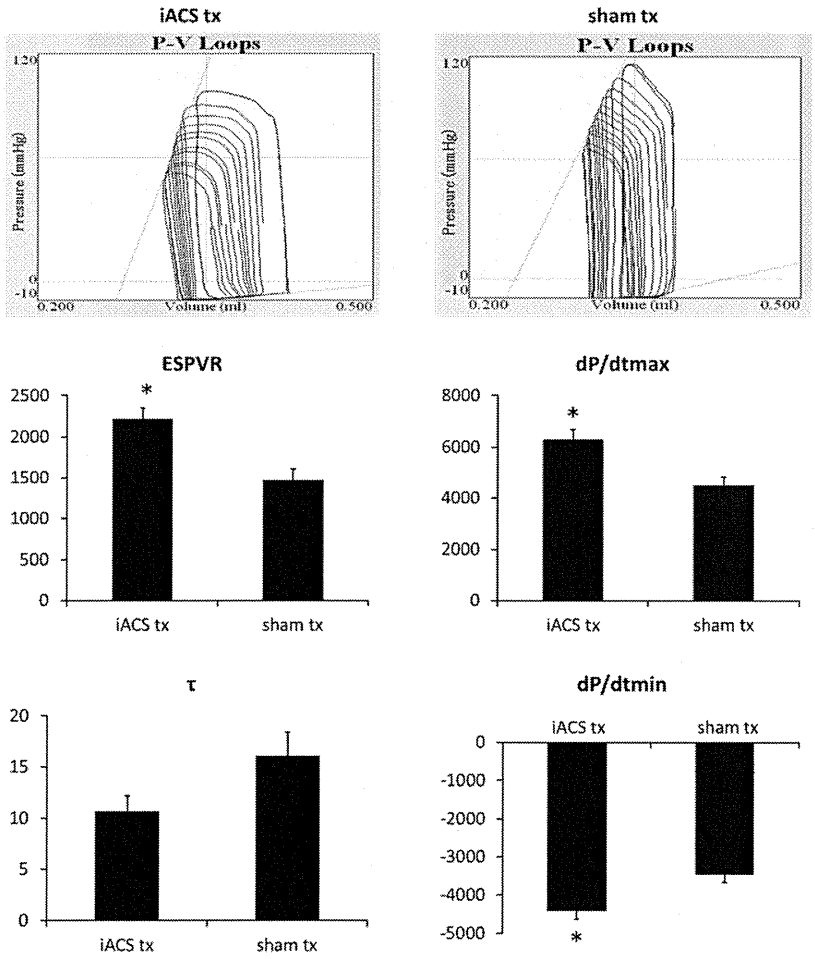
Supplementary figure 1. Capillary formation in the iACS-treated heart. Four weeks after infarction and iACS transplantation, CD31 staining was performed to assess angiogenesis. Representative images from each group are shown. The density of CD31-positive capillaries was the same among the groups. Green,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



Supplementary figure 2



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

Tomohiko Machida[1], Masahiro Tanemura[1,4,5], Yoshiaki Ohmura[1], Tsukasa Tanida[1], Hiroshi Wada[1], Shogo Kobayashi[1], Shigeru Marubashi[1], Hidetoshi Eguchi[1], Toshinori Ito[2], Hiroaki Nagano[1], Masaki Mori[1], Yuichiro Doki[1], Yoshiki Sawa[3]

Departments of [1]Gastroenterological Surgery, [2]Complementary and Alternative Medicine, and [3]Cardiovascular Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka, 565-0871, JAPAN

Department of [4]Surgery and Institute for Clinical Research, National Hospital Organization Kure Medical Center, 3-1, Aoyamacho, Kure, Hiroshima, 737-0023, JAPAN

Running head: Beneficial effect of sivelestatin islet transplantation

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

[5]**Corresponding author:**

Masahiro Tanemura, MD, PhD

Department of [1]Gastroenterological Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, JAPAN

Tel: +81-6-6879-3251, Fax: +81-6-6879-3259

E-mail: mtanemura64@yahoo.co.jp .

T. Machida and M. Tanemura contributed equally to this work.

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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).