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

digestion. Many neutrophils were activated (red-brown staining) by collagenase digestion in islet isolation using both UW and ET-Kyoto solutions (Fig. 2A), but their number (cells/field) was significantly reduced by the addition of 20 μM of sivelestat in ET-Kyoto solution (UW: 10.2±1.1, S-UW: 6.8±0.4, ET-Kyoto: 9.2±1.3, S-Kyoto: 3.6±0.5) (UW, ET-Kyoto vs. S-Kyoto: $p<0.001$, S-UW vs. S-Kyoto: $p<0.01$) (Fig. 2B). These results suggest a marked increase in enzyme activity by collagenase digestion in all types of isolation solutions (Fig. 2C). However, the enzyme activity was significantly suppressed in S-Kyoto islet isolation than those in other isolations (UW, S-UW and ET-Kyoto-isolation).

Sivelestat suppresses apoptosis in islets after warm digestion during islet isolation

Many TUNEL-positive cells were identified in the islets at the end of warm digestion (Fig. 3A), whereas no such cells were detected before digestion (data not shown). Quantitative analysis indicated a significantly lower number of TUNEL-positive cells within the islets of the S-Kyoto group (6.6±1.5) compared with those of UW, S-UW, and ET-Kyoto groups (23.1±3.9, 20.8±5.0, and 15.3±2.6, respectively) ($p<0.001$, each) (Fig. 3B).

Improvement of islet yield elicited by islet isolation with S-Kyoto solution

To investigate the beneficial effects of sivelestat in islet isolation, islet isolations were performed using either UW or ET-Kyoto solution, with or without 20 μM of sivelestat. Islet yield, isolation index and islet purity after purification were significantly improved by S-Kyoto islet isolation (Table 1). The recovery rate of

purification was also significantly improved by S-Kyoto islet isolation, compared to those by other isolation solutions (UW: $55.8\pm10.4\%$, S-UW: $56.6\pm12.7\%$, ET-Kyoto: $56.6\pm4.6\%$, S-Kyoto: $76.0\pm5.2\%$). Based on the results of UW isolation groups (UW, S-UW), no beneficial effects of sivelestat were elicited under these islet isolation parameters. With regard to islet size distribution, the percentage of large islets (>150 nm in diameter) in the S-Kyoto group was markedly higher than that in other isolation groups (Fig. 4A).

To further examine the morphological changes in isolated islets, SEM was employed. As shown in Figure 4B, islets obtained from the UW groups (UW, S-UW) were poor, based on the irregular islet surface and detection of islet damage during the isolation procedure. Similarly, islets obtained from ET-Kyoto isolation had irregular shape. On the other hand, islets isolated by S-Kyoto solution were well preserved, with round and smooth surface.

Improvement of islet viability elicited by islet isolation with S-Kyoto solution

Next, we evaluated islet viability using fluorescence labeling with TMRE and 7-AAD (Fig. 5). In the UW groups (UW, S-UW), no significant difference was noted in islet viability assay using TMRE and 7-AAD between with or without sivelestat (UW (n=5) vs. S-UW (n=5) ($60.0\pm5.6\%$ vs. $63.3\pm4.4\%$, not significant). However, the percentage of dead cells, represented by 7-AAD-positive cells, was significantly reduced in S-UW isolation group [UW (n=5) vs. S-UW (n=5), $25.8\pm5.3\%$ vs. $17.2\pm1.3\%$, $p<0.05$]. In contrast, the viability of islets isolated with S-Kyoto solution was significantly better relative to that of ET-Kyoto isolation [ET-Kyoto (n=5) vs. S-

Kyoto (n=5), 67.0±1.2% vs. 75.4±2.0%, p<0.001]. Moreover, the percentage of dead

cells in the S-Kyoto group was also lower compared with that in ET-Kyoto isolation

[ET-Kyoto (n-5) vs. S-Kyoto (n=5), 18.6±2.2% vs. 11.6±2.4%, p<0.01]. Similar

findings of islet viability were noted by MTS assay (Fig. 6A). The highest viability of

fresh islets was noted with S-Kyoto solution [ET-Kyoto (n=5) vs. S-Kyoto (n=5),

0.172±0.013 vs. 0.206±0.026, p<0.05, Fig. 6A]. With regard to the *in vitro* culture

after islet isolation, although islet viability in each group decreased gradually, that of

the S-Kyoto group was well preserved compared with other isolation groups (Fig. 6B).

Improvement of islet function elicited by islet isolation with S-Kyoto solution

Next, we evaluated insulin function of islets using static glucose change.

Although no significant difference was observed in insulin concentration under low

glucose (2.8 mM) using each solution, insulin concentration under high glucose (20

mM) and the SI improved significantly by S-Kyoto islet isolation in comparison with

those of other isolation solutions (Table 2). Based on the results of UW isolation

groups (UW, S-UW), no beneficial effects of sivelestat were elicited as determined by

the SI.

Prolongation of islet graft survival in S-Kyoto group and in in vivo experiments

Transplantation of 500 allogeneic islets of the ET-Kyoto group was associated

with graft survival of 7.4±1.1 days (Fig. 7A, Table 3). A significant prolongation of

islet graft survival was noted after transplantation of the S-Kyoto group, compared

with islet grafts of the ET-Kyoto group (11.2±1.3 days, p<0.05). Importantly, islet

transplantation coupled with 15-day intraperitoneal administration of 2 mg/day sivelestat elicited significant prolongation of graft survival, compared with both ET-Kyoto and S-Kyoto groups, respectively (Figure 7A, Table 3). Next, NE activity in sera of recipient mice was measured. NE activity increased gradually after islet transplantation in the S-Kyoto group. However, sivelestat significantly suppressed the enzyme activity measured at day 28 after transplantation (Fig. 7B). These results suggest that prolongation of islet graft survival in the sivelestat ip group is due to inhibition of NE enzyme activity and the anti-inflammatory properties of sivelestat.

To examine islet graft function *in vivo*, IPGTT was performed at day 7 after transplantation. In non-diabetic wild-type mice, injection of glucose induced hyperglycemia with the peak blood glucose level recorded at 15 min, but the level returned to normal at 60-120 min after the injection (Fig. 7C). A similar pattern was observed in mice transplanted with S-Kyoto and S-Kyoto and treated with sivelestat. On the other hand, blood glucose levels of mice transplanted with ET-Kyoto group and untreated diabetic mice were significantly higher than those of the control wild-type mice before injection and at 15, 60 and 120 min after injection of glucose (Fig. 7C). Immunohistochemical staining for insulin confirmed that islet transplantation with S-Kyoto and S-Kyoto with sivelestat prolonged graft survival. Small islet grafts with many infiltrating inflammatory cells were detected at day 7 after transplantation in the ET-Kyoto group, whereas insulin-positive islet grafts with well preserved islet structure were found in the S-Kyoto and S-Kyoto with sivelestat groups (Fig. 7D).

Sivelestat suppresses proinflammatory cytokines after warm digestion during islet isolation

We measured the levels of proinflammatory cytokines (IL-2, 4, 6, 10, 17A, IFN-γ, TNF-α) in the isolation solution at each step of islet isolation, including before warm digestion, at the end of warm digestion and after purification. The levels at the end of warm digestion were markedly higher than before warm digestion (Fig. 8). On the other hand, IL-6 and TNF-α level were significantly lower at the end of warm digestion in S-Kyoto solution compared with other isolation solutions such as UW and ET-Kyoto (Fig. 8).

Sivelestat suppresses proinflammatory cytokines in serum of islet recipients after transplantation

The serum levels of IL-6 and TNF-α in islet recipients were significantly higher at 7 and 14 post-transplantation days than before transplantation (day -1), whereas no significant increases were noted in the levels of other cytokines except IL-6 and TNF-α (Fig. 9). The serum levels of IL-6 and TNF-α were significantly lower in the sivelestat intraperitoneal (ip) group compared with non-sivelestat ip group at 7 and 14 post-transplantation days (Fig. 9).

DISCUSSION

Islet transplantation is currently one of the most attractive strategies for the treatment of type 1 diabetes. One important key to achieving successful insulin-independence after islet transplantation is acquiring a sufficient donor islet mass (1,10). The number and quality of islets recovered from isolation are influenced by several factors (10). Tissue damage of donor pancreas starts to occur as early as the onset of brain death associated with hypotension, peripheral vasoconstriction, tissue ischemia, and release of stress hormones and inflammatory mediators (10,20,24,38). Moreover, at the time of procurement, donor pancreas is exposed to warm ischemia after donor cross-clamping and cold ischemia storage in preservation solution, such as UW, ET-Kyoto solution (23,40). Furthermore, during islet isolation, warm digestion, trauma and hypoxia, may cause cell damage in isolated islets similar to ischemia/reperfusion injury (IRI) of other transplant organs (10). Therefore, we need to design an efficient isolation method to reduce cell damage toward donor pancreata.

The major finding of the present study was identification of the crucial role of NE in islet isolation and transplantation. First, we showed a marked increase in NE activity during islet isolation, especially at the end of warm digestion by collagenase (Fig. 2C), and that NE was cytotoxic against isolated islets (Fig. 1A). Second, the addition of sivelestat to the isolation solution during islet isolation inhibited NE activity (Fig. 2C) and significantly improved islet yields, islet viability and insulin function of isolated islets (Tables 1 and 2). Furthermore, we also showed that

treatment of recipient animals with sivelestat significantly prolonged the survival of

insulin secreting islet allografts (Fig. 7A, Table 3)

In clinical liver transplantation, IRI, an exogenous antigen-independent

inflammatory event, remains a major problem, because IRI causes early transplant

graft failure and can lead to a higher incidence of both acute and chronic rejections

(6,54,55). The mechanisms underlying liver IRI involve leukocyte accumulation and

activation of neutrophils, Kupffer cells (macrophages), and T cells, secretion of

proinflammatory cytokines and chemokines, complement activation and activation of

vascular cell adhesion molecules (49,51,53-55). Recent reports also indicated that the

cross-talk interaction between NE and toll like receptor (TLR) 4 promotes liver

damage, and that NE inhibitors, such as sivelestat, ameliorated the hepatocellular

damage by reducing the expression of proinflammatory cytokines, chemokines, and

TLR4 (24,54,55).

In islet transplantation, proinflammatory cytokines such as TNF-α and IL-1β

induce β-cell apoptosis (1,3,27). These proinflammatory cytokines are produced by

acinar and ductal cells (10). Moreover, in isolated islets, resident macrophages and

monocytes produce cytokines (3,10,25). During islet isolation, the pancreatic tissues

are warmed up to 37°C for collagenase digestion (10). As shown in Figure 2, many

neutrophils were activated and released NE at the end of warm digestion. The released

NE caused injury to membrane components of macrophages, acinar cells and islets.

Activated macrophages and acinar cells produce proinflammatory cytokines, including

TNF-α and IL-6 (3). In fact, significantly high levels of TNF-α and IL-6 were found

in isolation solution at the end of warm digestion compared with those before warm

digestion (Fig. 8). Moreover, NE may also serve as a putative endogenous TLR4 ligand, causing TLR4 up-regulation on macrophages and islets during the isolation process (5,39,54,55). Such excessive expression of TNF-α and TLR 4 affects the surrounding islets causing their apoptosis (7,19). Indeed, to detect apoptotic cells during islet isolation, TUNEL staining was performed before and at the end of warm digestion. Many TUNEL-positive cells were detected in UW solution at the end of warm digestion, whereas no such cells were detected in pancreatic tissue before warm digestion (Fig. 3A). What is the mechanism of NE-induced islet injury ? Sivelestat effectively prevented the cross-talk between NE and inflammation responses, including the expression of proinflammatory cytokines (TNF-α, IL-6) and TLR4, resulting in a significant improvement in islet yields, islet viability and insulin function in islet isolation with S-Kyoto solution. Furthermore, analysis of serum cytokine production profile (IL-2, 4, 6, 10, 17A, IFN-γ, TNF-α) in islet recipient mice showed significant increase in IL-6 and TNF-α production in serum at 1 and 2 weeks after islet transplantation, compared with those before islet transplantation (day -1), whereas no such increase was observed in the production of other cytokines before/after islet transplantation (Fig. 9). As shown in Figure 7B, NE activity increased gradually after islet transplantation. However, sivelestat significantly suppressed NE activity and the increase in IL-6 and TNF-α production in mice (Fig. 7B and 9). Taken together, local inflammatory reaction, resulting in functional loss of islet grafts occurred after transplantation and treatment with sivelestat inhibited such inflammatory reactions, as evidenced by reduced IL-6 and TNF-α production, and resulted in prolongation of islet allograft survival.

Trypsin released from pancreatic acinar cells directly destroys islets (35).

Previous studies demonstrated that inhibition of trypsin by pefabloc or ulinastatin

during pancreas digestion improved islet yield and reduced the fraction of embedded

islets (22,35). These findings suggest that trypsin may degrade pancreatic ductules,

resulting in reduced delivery of collagenase solution (35). However, the molecular

weight of the new recombinant NE inhibitor sivelestat is much lower than alpha-1

protease inhibitors, and appears to exert its cytoprotective effect in the

microenvironment between neutrophil and pancreatic tissues (12,30). In this study,

we examined the effects of the addition of sivelestat to ET-Kyoto solution. ET-Kyoto

solution has a high sodium/low potassium composition with comparatively low

viscosity (35). Therefore, it allows sufficient organ flushing after harvesting the

pancreatic tissues (28,35). Furthermore, we also examined the effects of the addition

of sivelestat to the UW solution. The results suggested that sivelestat also provided

cytoprotection when added to the UW solution. This conclusion was based on the

finding that the decrease in the number of activated neutrophils and neutrophil

elastase activity in the S-UW group tended to be larger than in the UW group (Fig. 2B

and 2C), albeit statistically insignificant. The islets isolated from the UW solution are

easily damaged by mechanical stress due to the high viscosity of the UW solution

(13). Indeed, in our experiment, membrane shear damage had a negative impact on

the isolated islets of the UW group during the isolation process, as shown in Figure

4B. This was probably due to one or more of the following reasons: 1) higher

viscosity of the UW solution relative to that of the ET-Kyoto solution, 2) the high

percentage of activated neutrophils present in the pancreatic tissue, and 3) the higher

level of neutrophil elastase released in the UW than in ET-Kyoto. We speculate that the balance between the amount of released neutrophil elastase and its inhibition by collagenase digestion tilts towards excess elastase activity when sivelestat is added to the UW solution. We also assessed the synergistic effects of ulinastatin and sivelestatin on improvement of islet yield and islet viability. However, no additive effects were observed in comparison with S-Kyoto solution alone and S-Kyoto solution with ulinastatin (data not shown).

As shown in Figure 4B, SEM showed well preserved islets that were isolated by S-Kyoto solution, as evident by their round and smooth surface. NE is reported to increase the permeability of vascular endothelial cells, a process known to be involved in tissue edema (30). Indeed, during islet isolation, inhibition of NE activity by sivelestat may maintain cell membrane stability and permeability of endothelial cells in isolated islets, preventing tissue edema, and leading to improvement of islet yield and insulin function of isolated islets.

In conclusion, we succeeded in isolating large numbers of islets using a new preservation solution, S-Kyoto solution, and in significant prolongation of islet graft survival in recipient mice treated with sivelestat. Our results emphasize the role of NE in the pathophysiology of islet damage during islet isolation and after transplantation. NE contributes to the accumulation of neutrophils and secretion of proinflammatory mediators during the isolation procedure and after islet transplantation. Therefore, treatment with NE inhibitors is potentially suitable for better harvest of transplantable islets and long-term islet allograft survival, allowing successful management of diabetes with islets from a single donor. We plan to assess in the near future the

beneficial effects of S-Kyoto solution and monotherapy with sivelestat in human islet transplantation.

DISCLOSURES: The authors declare no conflict of interest.

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