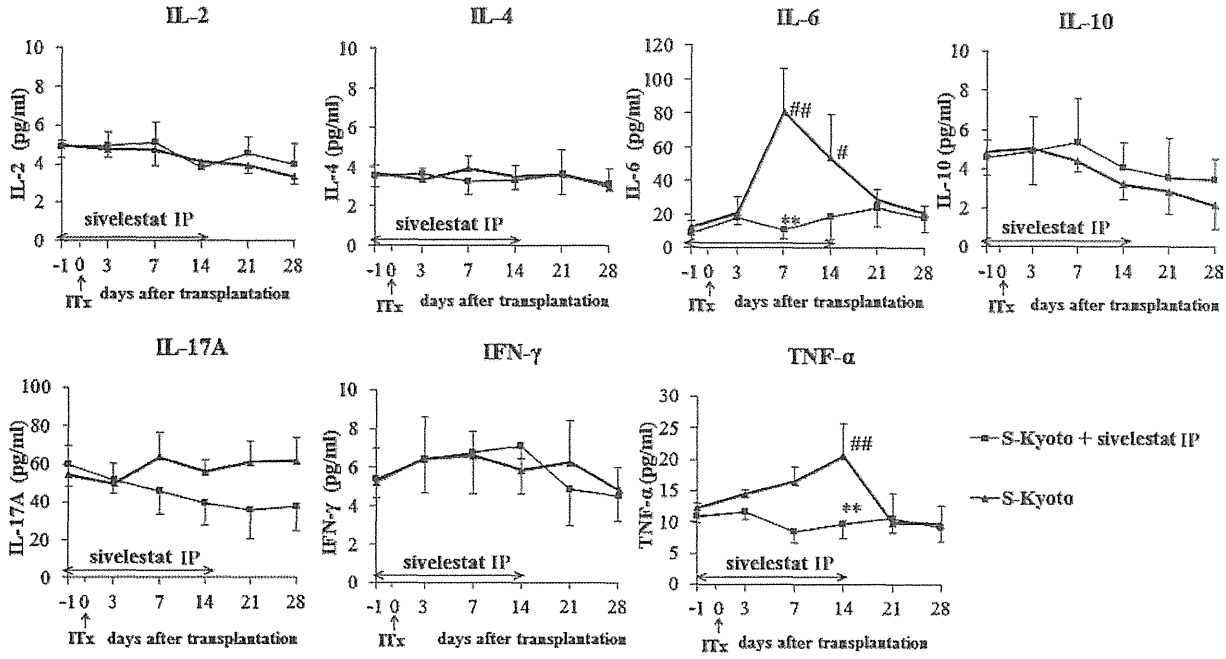


**Figure 9.** Assessment of proinflammatory cytokines in sera of islet mouse recipients after islet transplantation (ITx). The levels of proinflammatory cytokines (IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF-α) in the sera of islet recipients were measured at pretransplantation day 1 and posttransplantation days 4, 7, 14, 21, and 28. Data are mean ± SD of three independent samples; $^{**}p<0.01$, versus nonsivelestat IP group, $\#p<0.05$ and $\#\#p<0.01$, versus before transplantation (day −1).

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 upregulation 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, IL-4, IL-6, IL-10, IL-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 (Figs. 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 α1 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, C), 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 sivelestat 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.

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## REFERENCES

1. Abdelli, S.; Ansite, J.; Roduit, R.; Borsello, T.; Matsumoto, I.; Sawada, T.; Allaman-Pillet, N.; Henry, H.; Beckmann, J. S.; Hering B. J.; Bonny, C. Intracellular stress signaling pathways activated during human islet preparation and following acute cytokine exposure. Diabetes 53:2815–2823; 2004.
2. Andrikopoulos, S.; Blair, A. R.; Deluca, N.; Fam, B. C.; Proietto, J. Evaluating the glucose tolerance test in mice. Am. J. Physiol. Endocrinol. Metab. 295:E1323–E1332; 2008.
3. Barshes, N. R.; Wyllie, S.; Goss, J. A. Inflammation-mediated dysfunction and apoptosis in pancreatic islet transplantation: Implications for intrahepatic grafts. J. Leukoc. Biol. 77:587–597; 2005.
4. Bottino, R.; Balamurugan, A. N.; Tse, H.; Thirunavukkarasu, C.; Ge, X.; Profozich, J.; Milton, M.; Ziegenfuss, A.; Trucco, M.; Piganelli, J. D. Response of human islets to isolation stress and the effect of antioxidant treatment. Diabetes 53:2559–2568; 2004.
5. Devaney, J. M.; Greene, C. M.; Taggart, C. C.; Carroll, T. P.; O'Neill, S. J.; McElvaney, N. G.; Neutrophil elastase upregulates interleukin-8 via toll-like receptor 4. FEBS Lett. 544:129–132; 2003.
6. Farmer, D. G.; Amersi, F.; Kupiec-Weglinski, J.; Busuttil, R. W. Current status of ischemia and reperfusion injury in the liver. Transplant. Rev. 14:106–126; 2000.
7. Gao, Q.; Ma, L. L.; Gao, X.; Yan, W.; Williams, P.; Yin, D. P. TLR 4 mediates early graft failure after intraportal islet transplantation. Am. J. Transplant. 10:1588–1596; 2010.
8. Hering, B. J. Achieving and maintaining insulin independence in human islet transplant recipients. Transplantation 79:1296–1297; 2005.
9. Ichii, H.; Pileggi, A.; Molano, R. D.; Baidal, D. A.; Khan, A.; Kuroda, Y.; Inverardi, L.; Goss, J. A.; Alejandro, R.; Ricordi, C. Rescue purification maximizes the use of human islet preparations for transplantation. Am. J. Transplant. 5:21–30; 2005.

10. Ito, T.; Omori, K.; Rawson, J.; Todorov, I.; Asari, S.; Kuroda, A.; Shintaku, J.; Itakura, S.; Ferreri, K.; Kandeel, F.; Mullen, Y. Improvement of canine islet yield by donor pancreas infusion with a p38 MAPK inhibitor. Transplantation. 86:321–329; 2008.
11. Kawabata, K.; Hagio, T.; Matsumoto, S.; Nakao, S.; Orita, S.; Aze, Y.; Ohno, H. Delayed neutrophil elastase inhibition prevents subsequent progression of acute lung injury induced by endotoxin inhalation in hamsters. Am. J. Respir. Crit. Care Med. 161:2013–2018; 2000.
12. Kawabata, K.; Suzuki, M.; Sugitani, M.; Imaki, K.; Toda, M.; Miyamoto, T. ONO-5046, a novel inhibitor of human neutrophil elastase. Biochem. Biophys. Res. Commun. 17: 814–820; 1991.
13. Kenmochi, T.; Miyamoto, M.; Sasaki, H.; Une, S.; Nakagawa, Y.; Moldovan, S.; Benhamou, P. Y.; Brunicardi, F. C.; Tanaka, H.; Mullen, Y. LAP-1 cold preservation solution for isolation of high-quality human pancreatic islets. Pancreas 17:367–377; 1998.
14. Khandoga, A.; Huettinger, S.; Khandoga, A. G.; Li, H.; Butz, S.; Jauch, K. W.; Vestweber, D.; Krombach, F. Leukocyte transmigration in inflamed liver: A role for endothelial cell-selective adhesion molecule. J. Hepatol. 50:755–765; 2009.
15. Kin, T.; Johnson, P. R.; Shapiro, A. M.; Lakey, J. R. Factors influencing the collagenase digestion phase of human islet isolation. Transplantation 83:7–12; 2007.
16. Kin, T.; Zhai, X.; Murdoch, T. B.; Salam, A.; Shapiro, A. M.; Lakey, J. R. Enhancing the success of human islet isolation through optimization and characterization of pancreas dissociation enzyme. Am. J. Transplant. 7:1233–1241; 2007.
17. Kono, T.; Okada, S.; Saito, M. Neutrophil elastase inhibitor, sivelestat sodium hydrate prevents ischemia-reperfusion injury in the rat bladder. Mol. Cell. Biochem. 311:87–92; 2008.
18. Korsgren, O.; Lundgren, T.; Felldin, M.; Foss, A.; Isaksson, B.; Permert, J.; Persson, N. H.; Rafael, E.; Rydén, M.; Salmela, K.; Tibell, A.; Tufveson, G.; Nilsson, B. Optimising islet engraftment is critical for successful clinical islet transplantation. Diabetologia 51:227–232; 2008.
19. Krüger, B.; Yin, N.; Zhang, N.; Yadav, A.; Coward, W.; Lal, G.; Zang, W.; Heeger, P. S.; Bromberg, J. S.; Murphy, B.; Schröppel, B. Islet-expressed TLR 2 and TLR 4 sense injury and mediate early graft failure after transplantation. Eur. J. Immunol. 40:2914–2924; 2010.
20. Kusaka, M.; Pratschke, J.; Wilhelm, M. J.; Ziai, F.; Zandi-Nejad, K.; Mackenzie, H. S.; Hancock, W. W.; Tilney, N. L. Activation of inflammatory mediators in rat renal isografts by donor brain death. Transplantation 69:405–410; 2000.
21. Kwon, A. H.; Qiu, Z. Neutrophil elastase inhibitor prevents endotoxin-induced liver injury following experimental partial hepatectomy. Br. J. Surg. 94:609–619; 2007.
22. Lakey, J. R.; Helm, L. M.; Kin, T.; Korbutt, G. S.; Rajotte, R. V.; Shapiro, A. M.; Warnock, G. L. Serine-protease inhibition during islet isolation increases islet yield from human pancreases with prolonged ischemia. Transplantation 72: 565–570; 2001.
23. Lakey, J. R.; Rajotte, R. V.; Warnock, G. L.; Kneteman, N. M. Human pancreas preservation prior to islet isolation. Cold ischemic tolerance. Transplantation 59:689–694; 1995.
24. Land, W. G. The role of postischemic reperfusion injury and other nonantigen-dependent inflammatory pathways in transplantation. Transplantation 79:505–514; 2005.
25. Lawrence, M. C.; Naziruddin, B.; Levy, M. F.; Jackson, A.; McGlynn, K. Calcineurin/nuclear factor of activated T cells and MAPK signaling induce TNF-{alpha} gene expression in pancreatic islet endocrine cells. J. Biol. Chem. 286:1025–1036; 2011.
26. Li, D. S.; Yuan, Y. H.; Tu, H. J.; Liang, Q. L.; Dai, L. J. A protocol for islet isolation from mouse pancreas. Nat. Protoc. 4:1649–1652; 2009.
27. Linn, T.; Schmitz, J.; Hauck-Schmalenberger, I.; Lai, Y.; Bretzel, R. G.; Brandhorst, H.; Brandhorst, D. Ischemia is linked to inflammation and induction of angiogenesis in pancreatic islets. Clin. Exp. Immunol. 144:179–187; 2006.
28. Matsumoto, S.; Noguchi, H.; Shimoda, M.; Ikemoto, T.; Naziruddin, B.; Jackson, A.; Tamura, Y.; Olson, G.; Fujita, Y.; Chujo, D.; Takita, M.; Kobayashi, N.; Onaca, N.; Levy, M. Seven consecutive successful clinical islet isolations with pancreatic ductal injection. Cell Transplant. 19:291–297; 2010.
29. Moberg, L.; Korsgren, O.; Nilsson, B. Neutrophil granulocytes are the predominant cell type infiltrating pancreatic islets in contact with ABO-compatible blood. Clin. Exp. Immunol. 142:125–131; 2005.
30. Mori, H.; Nagahiro, I.; Osaragi, T.; Kotani, K.; Nakanishi, H.; Sano, Y.; Date, H.; Shimizu, N. Addition of a neutrophil elastase inhibitor to the organ flushing solution decreases lung reperfusion injury in rat lung transplantation. Eur. J. Cardiothorac. Surg. 32:791–795; 2007.
31. Morini, S.; Braun, M.; Onori, P.; Cicalese, L.; Elias, G.; Gaudio, E.; Rastellini, C. Morphological changes of isolated rat pancreatic islets: A structural, ultrastructural and morphometric study. J. Anat. 209:381–392; 2006.
32. Morohoshi, Y.; Matsuoka, K.; Chinen, H.; Kamada, N.; Sato, T.; Hisamatsu, T.; Okamoto, S.; Inoue, N.; Takaishi, H.; Ogata, H.; Iwao, Y.; Hibi, T. Inhibition of neutrophil elastase prevents the development of murine dextran sulfate sodium-induced colitis. J. Gastroenterol. 41:318–324; 2006.
33. Noguchi, H.; Nakai, Y.; Matsumoto, S.; Kawaguchi, M.; Ueda, M.; Okitsu, T.; Iwanaga, Y.; Yonekawa, Y.; Nagata, H.; Minami, K.; Masui, Y.; Futaki, S.; Tanaka, K. Cell Permeable peptide of JNK inhibitor prevents islet apoptosis immediately after isolation and improves islet graft function. Am. J. Transplant. 5:1848–1855; 2005.
34. Noguchi, H.; Naziruddin, B.; Onaca, N.; Jackson, A.; Shimoda, M.; Ikemoto, T.; Fujita, Y.; Kobayashi, N.; Levy, M. F.; Matsumoto, S. Comparison of modified Celsior solution and M-Kyoto solution for pancreas preservation in human islet isolation. Cell Transplant. 19:751–758; 2010.
35. Noguchi, H.; Ueda, M.; Nakai, Y.; Iwanaga, Y.; Okitsu, T.; Nagata, H.; Yonekawa, Y.; Kobayashi, N.; Nakamura, T.; Wada, H.; Matsumoto, S. Modified two–zlayer preservation method (M-Kyoto/PFC) improves islet yields in islet isolation. Am. J. Transplant. 6:496–504; 2006.
36. Ohmura, Y.; Tanemura, M.; Kawaguchi, N.; Machida, T.; Tanida, T.; Deguchi, T.; Wada, H.; Kobayashi, S.; Marubashi, S.; Eguchi, H.; Takeda, Y.; Matsuura, N.; Ito, T.; Nagano, H.; Doki, Y.; Mori, M. Combined transplantation of pancreatic islets and adipose tissue-derived stem cells enhances the survival and insulin function of islet grafts in diabetic mice. Transplantation 90:1366–1373; 2010.
37. Paraskevas, S.; Maysinger, D.; Wang, R.; Duguid, T. P.; Rosenberg, L. Cell loss in isolated human islets occurs by apoptosis. Pancreas 20:270–276; 2000.

38. Pratschke, J.; Wilhelm, M. J.; Kusaka, M.; Basker, M.; Cooper, D. K.; Hancock, W. W.; Tilney, N. L. Brain death and its influence on donor organ quality and outcome after transplantation. Transplantation 67:343–348; 1999.
39. Ribeiro-Gomes, F. L.; Moniz-de-Souza, M. C.; Alexandre-Moreira, M. S.; Dias, W. B.; Lopes, M. F.; Nunes, M. P.; Lungarella, G.; Dos Reis, G. A. Neutrophils activate macrophages for intracellular killing of *Leishmania major* through recruitment of TLR 4 by neutrophil elastase. J. Immunol. 179:3988–3994; 2007.
40. Ricordi, C. Quantitative and qualitative standards for islet isolation assessment in humans and large mammals. Pancreas 6:242–244; 1991.
41. Ricordi, C.; Strom, T. B. Clinical islet transplantation: Advances and immunological challenges. Nat. Rev. Immunol. 4:259–268; 2004.
42. Rosenberg, L.; Wang, R.; Paraskevas, S.; Maysinger, D. Structural and functional changes resulting from islet isolation lead to islet cell death. Surgery 126:393–398; 1999.
43. Ryan, E. A.; Lakey, J. R.; Paty, B. W.; Imes, S.; Korbutt, G. S.; Kneteman, N. M.; Bigam, D.; Rajotte, R. V.; Shapiro, A. M. Successful islet transplantation: Continued insulin reserve provides long-term glycemic control. Diabetes 51:2148–2157; 2002.
44. Ryan, E. A.; Lakey, J. R.; Rajotte, R. V.; Korbutt, G. S.; Kin, T.; Imes, S.; Rabinovitch, A.; Elliott, J. F.; Bigam, D.; Kneteman, N. M.; Warnock, G. L.; Larsen, I.; Shapiro, A. M. Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. Diabetes 50:710–719; 2001.
45. Ryan, E. A.; Paty, B. W.; Senior, P. A.; Bigam, D.; Alfadhli, E.; Kneteman, N. M.; Lakey, J. R.; Shapiro, A. M. Five-year follow-up after clinical islet transplantation. Diabetes 54:2060–2069; 2005.
46. Shapiro, A. M.; Lakey, J. R.; Paty, B. W.; Senior, P. A.; Bigam, D. L.; Ryan, E. A. Strategic opportunities in clinical islet transplantation. Transplantation 79:1304–1307; 2005.
47. Shapiro, A. M.; Lakey, J. R.; Ryan, E. A.; Korbutt, G. S.; Toth, E.; Warnock, G. L.; Kneteman, N. M.; Rajotte, R. V. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N. Engl. J. Med. 343:230–238; 2000.
48. Shapiro, A. M.; Ricordi, C.; Hering, B. J.; Auchincloss, H.; Lindblad, R.; Robertson, R. P.; Secchi, A.; Brendel, M. D.; Berney, T.; Brennan, D. C.; Cagliero, E.; Alejandro, R.; Ryan, E. A.; DiMercurio, B.; Morel, P.; Polonsky, K. S.; Reems, J. A.; Bretzel, R. G.; Bertuzzi, F.; Froud, T.; Kandaswamy, R.; Sutherland, D. E.; Eisenbarth, G.; Segal, M.; Preiksaitis, J.; Korbutt, G. S.; Barton, F. B.; Viviano, L.; Seyfert-Margolis, V.; Bluestone, J.; Lakey, J. R. International trial of the Edomonton protocol for islet transplantation. N. Engl. J. Med. 355:1318–1330; 2006.
49. Shimoda, M.; Iwasaki, Y.; Okada, T.; Sawada, T.; Kubota, K. Protective effect of sivelestat in a porcine hepatectomy model prepared using an intermittent Pringle method. Eur. J. Pharmacol. 587:248–252; 2008.
50. Tamakuma, S.; Ogawa, M.; Aikawa, N.; Kubota, T.; Hirasawa, H.; Ishizaka, A.; Tanaka, N.; Hamada, C.; Matsuoka, S.; Abiru, T. Relationship between neutrophil elastase and acute lung injury in humans. Pulm. Pharmacol. Ther. 17:271–279; 2004.
51. Teoh, N. C.; Farrell, G. C. Hepatic ischemia reperfusion injury: Pathogenic mechanisms and basis for hepatoprotection. J. Gastroenterol. Hepatol. 18:891–902; 2003.
52. Thomas, F.; Wu, J.; Contreras, J. L.; Smyth, C.; Bilbao, G.; He, J.; Thomas, J. A tripartite anoikis-like mechanism causes early isolated islet apoptosis. Surgery 130:333–338; 2001.
53. Tsuchihashi, S.; Ke, B.; Kaldas, F.; Flynn, E.; Busuttil, R. W.; Briscoe, D. M.; Kupiec-Weglinski, J. W. Vascular endothelial growth factor antagonist modulates leukocyte trafficking and protects mouse livers against ischemia/reperfusion injury. Am. J. Pathol. 168:695–705; 2006.
54. Uchida, Y.; Freitas, M. C.; Zhao, D.; Busuttil, R. W.; Kupiec-Weglinski, J. W. The inhibition of neutrophil elastase ameliorates mouse liver damage due to ischemia and reperfusion. Liver Transpl. 15:939–947; 2009.
55. Uchida, Y.; Freitas, M. C.; Zhao, D.; Busuttil, R. W.; Kupiec-Weglinski, J. W. The protective function of neutrophil elastase inhibitor in liver Ischemia/reperfusion injury. Transplantation 89:1050–1056; 2010.
56. Wang, P.; Henning, S. M.; Heber, D. Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. PLoS One 5:e10202; 2010.
57. Wu, Y.; Han, B.; Luo, H.; Roduit, R.; Salcedo, T. W.; Moore, P. A.; Zhang, J.; Wu, J. DcR3/TR6 effectively prevents islet primary nonfunction after transplantation. Diabetes 52:2279–2286; 2003.
58. Yin, D.; Ding, J. W.; Shen, J.; Ma, L.; Hara, M.; Chong, A. S. Liver ischemia contributes to early islet failure following intraportal transplantation: Benefits of liver ischemic-preconditioning. Am. J. Transplant. 6:60–68; 2006.
59. Yoshimura, K.; Nakagawa, S.; Koyama, S.; Kobayashi, T.; Homma, T. Roles of neutrophil elastase and superoxide anion in leukotriene B4-induced lung injury in rabbit. J. Appl. Physiol. 76:91–96; 1994.
60. Zeiher, B. G.; Artigas, A.; Vincent, J. L.; Dmitrienko, A.; Jackson, K.; Thompson, B. T.; Bernard, G. Neutrophil elastase inhibition in acute lung injury: Results of the STRIVE study. Crit. Care Med. 32:1695–1702; 2004.

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Brief Communication

# Pretreatment of Donor Islets With the $Na^+/Ca^{2+}$ Exchanger Inhibitor Improves the Efficiency of Islet Transplantation

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**Pancreatic islet transplantation is an attractive therapy for the treatment of insulin-dependent diabetes mellitus. However, the low efficiency of this procedure necessitating sequential transplantations of islets with the use of 2–3 donors for a single recipient, mainly due to the early loss of transplanted islets, hampers its clinical application. Previously, we have shown in mice that a large amount of HMGB1 is released from islets soon after their transplantation and that this triggers innate immune rejection with activation of DC, NKT cells and neutrophils to produce IFN-γ, ultimately leading to the early loss of transplanted islets. Thus, HMGB1 release plays an initial pivotal role in this process; however, its mechanism remains unclear. Here we demonstrate that release of HMGB1 from transplanted islets is due to hypoxic damage resulting from $Ca^{2+}$ influx into β cells through the $Na^+/Ca^{2+}$ exchanger (NCX). Moreover, the hypoxia-induced β cell damage was prevented by pretreatment with an NCX-specific inhibitor prior to transplantation, resulting in protection and long-term survival of transplanted mouse and human islets when grafted into mice. These findings suggest a novel strategy with potentially great impact to improve the efficiency of islet transplantation in clinical settings by targeting donor islets rather than recipients.**

**Key words: Early loss of transplanted islets, hypoxia, islet transplantation, $Na^+/Ca^{2+}$ exchanger**

**Abbreviations: IDDM, insulin-dependent diabetes mellitus; HMGB1, high-mobility group box 1 protein; NCX, $Na^+/Ca^{2+}$ exchanger; STZ, streptozotocin.**

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## Introduction

Pancreatic islet transplantation has now become a procedure of choice for the treatment of insulin-dependent diabetes mellitus (IDDM) since Shapiro et al. (1) reported that insulin independence could be achieved after sequential transplantations of islets combined with a novel immunosuppressive regime. Since then, the rate of insulin independence among IDDM recipients has improved to reach 50–60% at 5 years after islet transplantation (2–4). Currently, however, insulin independence has been achieved in limited patients with IDDM after islet transplantation from a single donor, and therefore sequential transplantation of islets with the use of 2–3 donors is still required for the treatment of a single recipient (1–4). Thus, this low efficiency has been a major obstacle facing clinical islet transplantation.

Previously, we have shown in mice that the low efficiency of islet transplantation is mainly due to early graft loss in the liver, the site of islet transplantation. This is caused by innate immune rejection of transplanted islets in concert with activation of DC, NKT cells and neutrophils to produce IFN-γ (5), and is triggered by high-mobility group box 1 protein (HMGB1) released from islets soon after their transplantation (6). Importantly, by targeting this HMGB1-DC-NKT cell-neutrophil-IFN-γ-mediated pathway, the efficiency of islet transplantation was sufficiently enhanced to the point that islet transplantation from one donor to one recipient became feasible in mice (5,6). However, the upstream events that cause HMGB1 release from transplanted islets soon after transplantation remain unknown.

Since the site of clinical islet transplantation is the portal vein in the liver (1–4), we speculated that transplanted islets are immediately exposed to low oxygen tension and therefore the transplanted β cells are easily damaged by hypoxia, which may induce HMGB1 release to activate innate immune cells thus causing the early loss of transplanted islets. Furthermore, accumulating evidence has shown that hypoxic cell death is caused by an increase in intracellular calcium ($[Ca^{2+}]_i$) (7), suggesting that pathways involved in the influx of calcium ions may be a therapeutic target of intervention. Among calcium channels and transporters, we focused on the sodium ($Na^+$)-calcium ($Ca^{2+}$) exchanger (NCX), since inhibition of this membrane protein has been reported to help prevent cell death resulting from ischemia-reperfusion injury (8–10).

NCX is a major $Ca^{2+}$-regulatory protein expressed by all excitable and many nonexcitable cells and transports $Ca^{2+}$ across the plasma membrane (11). Pancreatic islet β cells express NCX (12) and it participates not only in the maintenance of glucose homeostasis by regulated $Ca^{2+}$-dependent secretion of insulin (13), but also in cell survival or death through its anti- or pro-apoptotic effects, respectively (14,15).

In the present study, we hypothesized that NCX on transplanted islets mediates ischemic islet cell death soon after transplantation, and that this outcome might be prevented by *in vitro* pretreatment of donor islets prior to transplantation with a specific NCX inhibitor. This is indeed the case; thus, the present study identifies a novel strategy to improve the efficiency of islet transplantation by targeting donor islets rather than recipients.

## Materials and Methods

### *Mice*

Male C57BL/6 and NOD/*scid* mice were purchased from Charles River Japan (Kanagawa, Japan). Mouse insulin promoter (MIP)-GFP mice (C57BL/6 background) (16) were purchased from Jackson Laboratory. Mice were kept under specific pathogen-free conditions and used at 8–16 weeks of age. All experiments were in accordance with protocols approved by the Animal Care and Use Committee at Fukuoka University.

### *Mouse islet isolation and in vitro culture*

Islets were isolated from the mouse pancreas (17,18) and those with a diameter of 150–250 μm were hand-picked and used for the experiments. Isolated islets were cultured at 24°C in a $CO_2$-incubator (5% $CO_2$ + 95% air) in medium (D-MEM, Nissui) supplemented with 10% FBS. After overnight culture, the medium was changed to that containing 0.2% bovine serum albumin (BSA, Sigma, St. Louis, MO) and islets were further incubated for 3 h in medium containing a specific NCX inhibitor, SEA0400 (8) or vehicle (DMSO). Islets were then cultured for 6–12 h at 37°C in the normoxic $CO_2$-incubator (95% air, 5% $CO_2$) or in a hypoxic chamber (Belco, Vineland, NJ) gassed with 1% $O_2$ + 5% $CO_2$ + 94% $N_2$.

### *Fluorescent micrographs*

Islets were stained with HO342 (Sigma) and PI (Sigma) and observed under fluorescent microscopy.

### *HMGB1 measurement*

The amount of HMGB1 in the culture medium and the serum of recipient mice was measured with an ELISA kit (Shino-test Co., Sagamihara, Japan) (19).

### *Ca imaging*

Isolated islets from the MIP-GFP mouse pancreas were further dispersed into single cells (20). The cells were incubated in Krebs Ringer bicarbonate buffer (HKRB) with 1 μM Fura-2AM (Invitrogen, Carlsbad, CA) for 30 min at 37°C. To measure the $[Na^+]_i$-dependent $Ca^{2+}$ influx, cells were perfused with $Ca^{2+}$-free HKRB for 20 min and then switched to a $Na^+$-free buffer HKRB in the presence of 1 μM ouabain. Fura-2AM was excited at 340 and 380 nm, and the emissions at >510 nm were captured every 500 ms using an Aquacosmos system (Hamamatsu Photonics, Hamamatsu, Japan).

### *Mouse islet transplantation*

The 200 C57BL/6 islets pretreated with SEA0400 or DMSO alone were transplanted into the liver via the portal vein (21) of syngenic recipient mice made diabetic with STZ (Sigma) (180 mg/kg). The nonfasting plasma glucose levels were measured three times a week after the injection of STZ and once a week after islet transplantation using a GlucoCard DIA meter (Arkray, Kyoto, Japan).

### *Liver MNC preparation and flow cytometry*

Preparation of liver MNCs and antibodies used are described in the Supplemental Materials and Methods.

### *Human islet experiments*

Human islets were provided by Prodo Lab (Irvine, CA) and were cultured at 24°C in CMRL1066 medium (Mediatech, Manassas, VA) containing 0.2% human albumin for 2–3 days prior to the following experiments.

For islet transplantation, three batches of human islets in total were used (Table S1). For each batch, one or two transplants using SEA0400- or vehicle-treated islets (1000 IEQ) were performed. Human islets were cultured in the presence of 10 μM SEA0400 or vehicle (DMSO) for 24 h and grafted beneath the capsule of one kidney of STZ (170 mg/kg, iv injection)-induced diabetic male NOD/*scid* mice (22). Nonfasting plasma glucose and body weight were measured once a week after transplantation. The kidneys of recipient mice bearing human islets were removed and fixed in 10% formalin, embedded in paraffin and sectioned for histological analysis.

### *Statistical analysis*

The statistical significance of differences was determined by a one- or two-tailed Student's t-test. The statistical significance with respect to the rate of normoglycemia in streptozotocin-induced diabetic NOD/*scid* mice after transplantation of human islets was determined by Fisher's exact test.

## Results

### *HMGB1 release from hypoxia-damaged islets is prevented by SEA0400, a specific NCX inhibitor*

In order to investigate whether the early loss of islets after transplantation is caused by hypoxia-mediated damage to the islet cells during transplantation, we first focused on the $Na^+/Ca^{2+}$ exchanger (NCX), which plays a central role in elevating intracellular calcium during ischemia and reperfusion in various tissues (8–10).

Three isoforms of NCX have been described (11); therefore, our initial experiment was to determine which NCX type is expressed on and functions in mouse pancreatic islet cells. A quantitative real-time PCR analysis revealed that islet cells predominantly express *Ncx1* transcripts (Figure S1a), which is reflected in the predominance of NCX1 protein expression in β cells (Figure S1b). To address the question of whether a specific NCX inhibitor, SEA0400 (8), can suppress NCX function in β cells, single cells from mouse islets genetically engineered to express GFP under the MIP (16) were prepared and assayed (Figure 1a). The intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) of $GFP^+$ β cells was stable when the cells were cultured in $Na^+$-containing medium, but increased in $Na^+$-free medium (Figure 1b). This increase in $[Ca^{2+}]_i$ was almost completely blocked by the addition of SEA0400 (Figure 1b). The $Ca^{2+}$ influx via NCX on islet cells was also confirmed under the conditions of hypoxia/reoxygenation by measuring $Ca^{2+}$ influx in the MIN6 islet cell line (23) using the calcium indicator GCaMP2 (24) (Figure S2a).

Since NCX on β cells is functional and its function can be blocked by SEA0400, we investigated whether pretreatment of islets with SEA0400 prior to hypoxic culture has any beneficial effect on their survival. For these studies, isolated islets were cultured at 37°C inside a chamber filled with hypoxic gas (1%$O_2$, 5%$CO_2$ and 94%$N_2$). Under these conditions, the $O_2$ concentration in the culture medium inside the chamber dropped to 20 mmHg within 30 min and was maintained at that level for more than 12 hrs (Figure S2b). As a control for normoxia conditions, islets were cultured at 37°C in a $CO_2$-incubator gassed with 5% $CO_2$ and 95% air. Islet cell death was assessed by fluorescent microscopy using the DNA binding dyes propidium iodide (PI) and Hoechst 33342 (HO 342) (25); the nuclei of all cells are stained blue by HO342, whereas only dead cell nuclei are stained red by PI. When isolated islets were cultured under normoxia conditions, only a few PI positive cells were observed (Figure 2a, left column). In marked contrast, under hypoxic conditions the number of PI positive islet cells was significantly increased at 6 h (Figure 2a, right upper panel). When isolated islets were incubated under normoxia conditions for 3 h in the presence of SEA0400 (10 μM), then transferred into the hypoxic chamber and cultured thereafter in the absence of SEA0400, the number of PI positive cells was markedly decreased at 6 h compared with islets treated with vehicle,

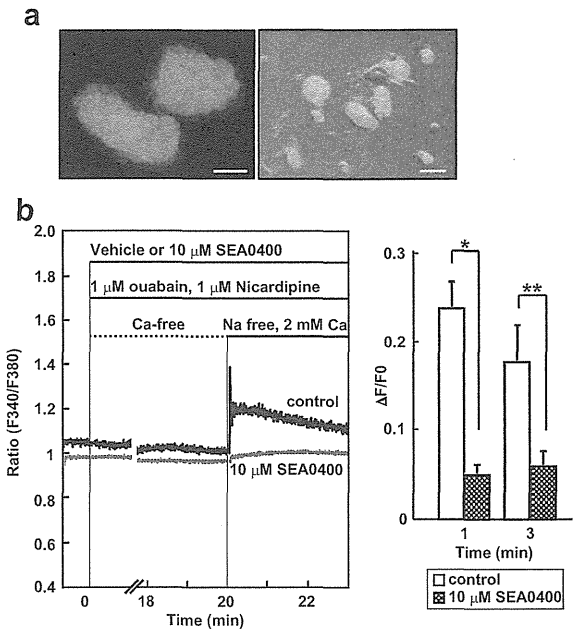


**Figure 1: The function of NCX is blocked by the specific NCX inhibitor, SEA0400** . (A) Fluorescent-micrographs of an isolated islet (left) and dispersed islet single cells (right) from MIP-GFP mice. Original magnification; x200. Bars indicate 50 (left) and 10 μm (right). (B) $[Na^+]_i$-dependent $Ca^{2+}$ influx via NCX in $GFP^+$-β cells. Fura-2AM-loaded cells were exposed to $Ca^{2+}$-free HKRB for 20 min and then to $Na^+$-free HKRB, with or without 10 μM SEA0400. Typical traces (left panel) and average data (right panel, n = 3) of $Ca^{2+}$ signals were shown in the left and right column, respectively. Data are expressed as the mean ± SE of $Ca^{2+}$ influx at 1 and 3 min. *$p < 0.01$ (n = 3) and **$p < 0.05$ (n = 3).

dimethyl sulfoxide (DMSO) (Figure 2a, right, middle, and lower panels).

In order to validate the beneficial effect of SEA0400 on islet cell survival, we measured the amount of HMGB1 in the culture medium, since our previous study demonstrated that HMGB1 is abundant within the islets and is released into the medium in parallel with the degree of β cell damage (6). The amount of HMGB1 in the medium of islets pretreated with SEA0400 followed by culture under hypoxia conditions was significantly lower than that seen when islets were similarly cultured but pretreated with DMSO (Figure 2b), clearly demonstrating that the pretreatment of islet cells with the NCX inhibitor maintained viability and inhibited HMGB1 release.

### *Pretreatment of donor islets with SEA0400 in vitro prevents their early loss after transplantation*

The above results strongly suggested that pretreatment of islets with SEA0400 *in vitro* might prevent their early loss after transplantation *in vivo*. To assess possible beneficial effects of SEA0400-pretreatment, streptozotocin (STZ)-

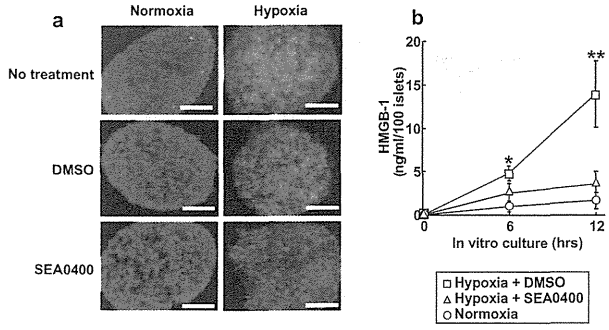


**Figure 2: Treatment with the NCX-specific inhibitor, SEA0400, helps prevent *in vitro* hypoxic islet cell death** . (A) Fluorescent micrographs of islets without treatment (upper panel) and pretreated with DMSO (middle panel) or SEA0400 10 μM (lower panel), cultured in normoxia (left columns) or in hypoxia (right columns) for 6 h and stained with HO342 (blue) and PI (red). A representative micrograph of an islet in each group is shown. The experiment was repeated three times. Original magnification; 200×. Bars indicate 50 μm. (B) The amount of HMGB1 in the culture medium of islets in normoxia versus hypoxia measured by ELISA. Islets pretreated with 10 μM SEA0400 or vehicle (DMSO) were cultured in hypoxia (open triangle and square) or normoxia (open circle) and the medium was collected at the times indicated. The values are expressed as the mean ± SD (n = 5). *p < 0.01. **p < 0.001. Representative data of the two experiments are shown.

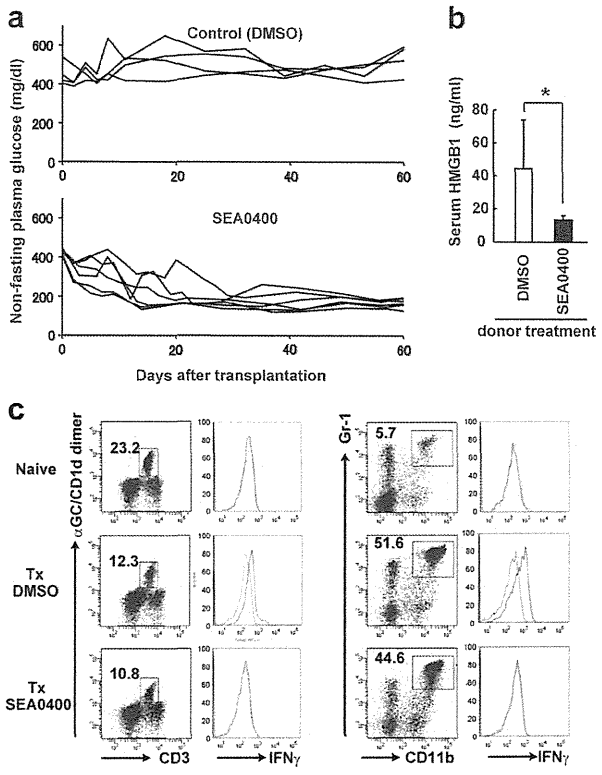


**Figure 3: Pretreatment of donor islets with SEA0400 *in vitro* prevents their early loss after transplantation** . (A) Nonfasting plasma glucose levels of STZ-induced diabetic mice (C57BL/6) transplanted with 200 syngeneic islets pretreated with DMSO (upper panel) or 10 μM SEA0400 (lower panel). Individual lines represent glucose levels of each animal. (B) Serum concentration of HMGB1 in diabetic mice receiving 400 islets pretreated with SEA0400 or vehicle (DMSO) at 6 h after transplantation. In this particular experiment, 400 islets were used so that the islet-derived HMGB1 could be readily detected. The values are expressed as the mean ± SD in each group (n = 4). *p < 0.005. (C) FACS analysis of liver MNCs from naïve mice (first row) and from diabetic mice at 6 h after transplantation of 200 syngeneic islets pretreated with vehicle (DMSO) (second row) or with 10 μM SEA0400 (third row). NKT ($CD1d\text{-}dimer^+$ $CD3^+$) cells and $Gr\text{-}1^+$ $CD11b^+$ cells were analyzed for IFN-γ production (second and fourth columns). Representative data from two to three experiments are shown.

induced diabetic mice were transplanted with 200 islets from a single donor, a dose that is normally not sufficient to normalize hyperglycemia (5,6). As expected, the diabetic mice receiving 200 islets pretreated with DMSO remained hyperglycemic (Figure 3a, upper panel). In striking contrast, diabetic recipients of 200 islets pretreated with 10 μM SEA0400 for 3 h prior to transplantation became normoglycemic (Figure 3a, lower panel). These findings suggest that islets pretreated with SEA0400 *in vitro* prior to transplantation are less damaged *in vivo*, resulting in the inhibition of HMGB1 release and protection from attack by the innate immune system.

In fact, when the SEA0400-pretreated islet cells were transplanted, the cellular cascade of HMGB1-mediated NKT cell-dependent IFN-γ production by $Gr\text{-}1^+$ $CD11b^+$cells (neutrophils), which is essential for the early loss of transplanted islets (5), was not activated. The serum level of HMGB1 at 6 h after transplantation of SEA0400-pretreated islets was significantly lower than those of control mice receiving DMSO-treated islets (Figure 3b). Moreover, there was no detectable IFN-γ production by NKT cells or neutrophils in mice receiving SEA0400-pretreated islets, whereas it was up-regulated in both cell types in mice receiving control islets (Figure 3c).

In order to confirm that NCX actually plays an essential role in the early loss of transplanted islets, we used genetically engineered $Ncx1^{+/-}$ $GFP^+$ β cells obtained by crossing $Ncx1^{+/-}$ (26) and MIP-GFP mice (16) and found that the function of NCX is suppressed and that $Ncx1^{+/-}$ islets, similar to wild-type islets treated with SEA0400, are resistant to *in vitro* and *in vivo* hypoxic cell death (Figure S3).

One possibility for the amelioration of hyperglycemia in diabetic mice receiving donor islets pretreated with SEA0400 was augmented insulin release from transplanted islets. Therefore, we examined whether the SEA0400 treatment alters the amount of insulin released from islets *in vitro* in response to glucose and found that there was no effect (Figure S4), indicating that the beneficial effect of SEA0400 is due to prevention of early graft loss.

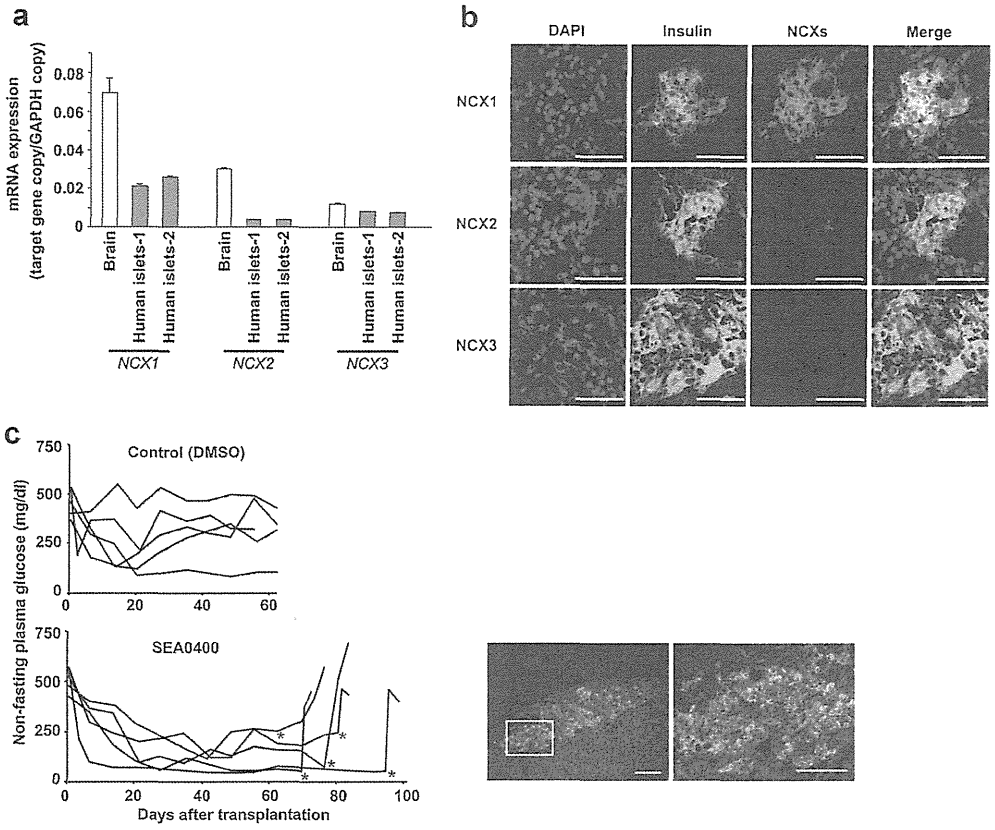


**Figure 4: Beneficial effect of SEA0400 on engraftment of human islets in NOD/*scid* mice** . (A) A quantitative real-time PCR analysis revealed that human islet cells predominantly express *NCX1* transcripts. (B) Immunohistochemical study of human pancreata revealed that human islet β cells express NCX1 protein. (C) Isolated human islets and streptozotocin-induced diabetic NOD/*scid* mice were used as donors and recipients, respectively. Individual lines represent the nonfasting plasma glucose levels of diabetic NOD/*scid* mice transplanted with 1000 IEQ (islet equivalent) human islets pretreated with SEA0400 or vehicle (DMSO) prior to transplantation (left column). *The kidneys bearing human islet grafts were removed on the day indicated. Human islet grafts pretreated with SEA0400 were retrieved at 72 days after transplantation, fixed and immunolabeled with anti-insulin (green) and anti-glucagon (red) antibodies, counterstained with DAPI (blue) (right column). *Scale bars represent 50 μm. The boxed area in the left image is shown at a higher magnification on the right.*

### *Beneficial effect of SEA0400 pretreatment on human islets grafted into NOD/scid mice*

A key question that we next investigated was whether the remarkable effect of SEA0400 pretreatment on survival of mouse islets would translate to human islets. Prior to transplantation experiments, we confirmed the expression of NCX1 by human islets and found that, as in mice, human islet cells predominantly express *NCX1* transcripts (Figure 4a) and protein (Figure 4b). In the islet transplantation experiments, human (Figure 4c) or mouse (Figure S5) islets were grafted beneath the kidney capsule of streptozotocin-induced diabetic NOD/*scid* mice. When the mice received 1000 IEQ (islet equivalent) human islets pretreated with SEA0400 prior to transplantation, their plasma glucose levels (5/5) became less than 250 mg/dL by 30 days after transplantation (Figure 4c), while the diabetic mice receiving vehicle (DMSO) treated islets (4/5) remained hyperglycemia. The difference in the rate of normoglycemia between the two groups of recipient mice was statistically significant ($p < 0.05$, by Fisher's exact test). In order to confirm that the normoglycemia of recipient mice is dependent on transplanted human islets, the kidneys bearing human islet grafts were removed at 70–100 days after transplantation, which promptly made the mice hyperglycemic again (Figure 4c, left panel). Morphologically, insulin-containing cells were abundant beneath the kidney capsule of normoglycemic (Figure 4c, right panel) compared with hyperglycemic recipient mice.

## Discussion

The findings presented here demonstrate that the release of HMGB1, an initial trigger of early loss of transplanted islets, is due to hypoxic death of the islet cells. This is mediated by the increase in intracellular $Ca^{2+}$ concentration of β cells through NCX1 and can be prevented by a specific NCX inhibitor, SEA0400. Importantly, the hyperglycemia of STZ-induced diabetes was ameliorated by transplantation of 200

mouse islets pretreated with SEA0400 *in vitro*, whereas the mice remained hyperglycemic if transplanted with 200 vehicle-treated islets. Furthermore, we found that human islet cells also predominantly express NCX1 and that the loss of human islet grafts is also prevented by pretreatment of the donor islets with SEA0400 *in vitro* prior to transplantation into immunodeficient mice. These preclinical results are quite promising and strongly suggest that the use of a NCX inhibitor could be applied to human donor islets to improve efficiency of islet transplantation in a clinical setting.

The early loss of transplanted islets has been a major concern to improve the efficiency of islet transplantation. Although our current study unveils the upstream event as to etiology in association with HMGB1 release from transplanted islets triggering innate immune responses in the liver, the site of islet transplantation, it is a matter of interest for the future study to dissect the roles of other inflammatory cytokines and chemokines in the early loss of transplanted islets in relation to HMGB1-mediated pathway. Furthermore, it is interesting to investigate whether the treatment with the NCX inhibitor improves the viability of islets and/or rescues their damage under stress conditions with a more comprehensive method as described (27).

Thus, the present study indicates that if the beneficial effect of the specific NCX inhibitor SEA0400 on the outcome of islet transplantation is also indeed the case clinically, it will have a tremendous impact on the treatment of patients with IDDM.

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## Disclosures

The authors of this manuscript have no conflict of interest to disclose as described by the *American Journal of Transplantation*.

## References

1. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000; 343: 230–238.
2. McCall M, Shapiro AM. Update on islet transplantation. Cold Spring Harb Perspect Med 2012; 2: a007823.
3. Barton FB, Rickels MR, Alejandro R, et al. Improvement in outcomes of clinical islet transplantation: 1999–2010. Diabetes Care 2012; 35: 1436–1445.
4. Bellin MD, Barton FB, Heitman A, et al. Potent induction immunotherapy promotes long-term insulin independence after islet transplantation in type 1 diabetes. Am J Transplant 2012; 12: 1576–1583.
5. Yasunami Y, Kojo S, Kitamura H, et al. Valpha14 NK T cell-triggered IFN-gamma production by Gr-$1^+$CD$11b^+$ cells mediates early graft loss of syngeneic transplanted islets. J Exp Med 2005; 202: 913–918.
6. Matsuoka N, Itoh T, Watarai H, et al. High-mobility group box 1 is involved in the initial events of early loss of transplanted islets in mice. J Clin Invest 2010; 120: 735–743.
7. Szydlowska K, Tymianski M. Calcium, ischemia and excitotoxicity. Cell Calcium 2010; 47: 122–129.
8. Matsuda T, Arakawa N, Takuma K, et al. SEA0400, a novel and selective inhibitor of the Na+-$Ca^{2+}$ exchanger, attenuates reperfusion injury in the in vitro and in vivo cerebral ischemic models. J Pharmacol Exp Ther 2001; 298: 249–256.
9. Yoshiyama M, Nakamura Y, Omura T, et al. Cardioprotective effect of SEA0400, a selective inhibitor of the Na(+)/Ca(2+) exchanger, on myocardial ischemia-reperfusion injury in rats. J Pharmacol Sci 2004; 95: 196–202.
10. Ogata M, Iwamoto T, Tazawa N, et al. A novel and selective $Na^+/Ca^{2+}$ exchange inhibitor, SEA0400, improves ischemia/reperfusion-induced renal injury. Eur J Pharmacol 2003; 478: 187–198.
11. Quednau BD, Nicoll DA, Philipson KD. The sodium/calcium exchanger family-SLC8. Pflugers Arch 2004; 447: 543–548.
12. Van Eylen F, Svoboda M, Herchuelz A. Identification, expression pattern and potential activity of Na/Ca exchanger isoforms in rat pancreatic B-cells. Cell Calcium 1997; 21: 185–193.
13. Hamming KS, Soliman D, Webster NJ, et al. Inhibition of beta-cell sodium-calcium exchange enhances glucose-dependent elevations in cytoplasmic calcium and insulin secretion. Diabetes 2010; 59: 1686–1693.
14. Diaz-Horta O, Kamagate A, Herchuelz A, Van Eylen F. Na/Ca exchanger overexpression induces endoplasmic reticulum-related apoptosis and caspase-12 activation in insulin-releasing BRIN-BD11 cells. Diabetes 2002; 51: 1815–1824.
15. Nguidjoe E, Sokolow S, Bigabwa S, et al. Heterozygous inactivation of the Na/Ca exchanger increases glucose-induced insulin release, beta-cell proliferation, and mass. Diabetes 2011; 60: 2076–2085.
16. Hara M, Wang X, Kawamura T, et al. Transgenic mice with green fluorescent protein-labeled pancreatic beta-cells. Am J Physiol Endocrinol Metab 2003; 284: E177–E183.
17. Sutton R, Peters M, McShane P, Gray DW, Morris PJ. Isolation of rat pancreatic islets by ductal injection of collagenase. Transplantation 1986; 42: 689–691.
18. Okeda T, Ono J, Takaki R, Todo S. Simple method for the collection of pancreatic islets by the use of Ficoll-Conray gradient. Endocrinol Jpn 1979; 26: 495–499.
19. Yamada S, Yakabe K, Ishii J, Imaizumi H, Maruyama I. New high mobility group box 1 assay system. Clin Chim Acta 2006; 372: 173–178.
20. Ono J, Takaki R, Fukuma M. Preparation of single cells from pancreatic islets of adult rat by the use of dispase. Endocrinol Jpn 1977; 24: 265–270.
21. Kemp CB, Knight MJ, Scharp DW, Lacy PE, Ballinger WF. Transplantation of isolated pancreatic islets into the portal vein of diabetic rats. Nature 1973; 244: 447.

22. Gu W, Winters KA, Motani AS, et al. Glucagon receptor antagonist-mediated improvements in glycemic control are dependent on functional pancreatic GLP-1 receptor. Am J Physiol Endocrinol Metab 2010; 299: E624–E632.
23. Miyazaki J, Araki K, Yamato E, et al. Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. Endocrinology 1990; 127: 126–132.
24. Tallini YN, Ohkura M, Choi BR, et al. Imaging cellular signals in the heart in vivo: Cardiac expression of the high-signal $Ca^{2+}$ indicator GCaMP2. Proc Natl Acad Sci USA 2006; 103: 4753–4758.
25. Saldeen J. Cytokines induce both necrosis and apoptosis via a common Bcl-2-inhibitable pathway in rat insulin-producing cells. Endocrinology 2000; 141: 2003–2010.
26. Wakimoto K, Kobayashi K, Kuro OM, et al. Targeted disruption of $Na+/Ca^{2+}$ exchanger gene leads to cardiomyocyte apoptosis and defects in heartbeat. J Biol Chem 2000; 275: 36991–36998.
27. Ichii H, Inverardia L, Pileggia A, et al. A novel method for the assessment of cellular composition and beta-cell viability in human islet preparations. Am J Transplant 2005; 5: 1635–1645.

## Supporting Information

Additional Supporting Information may be found in the online version of this article.

**Figure S1: Ncx1 is the predominant Ncx isoform expressed by mouse islet cells.** (A) RT-PCR analysis of *Ncx1*, *Ncx2* and *Ncx3* expression. Isolated mouse islets (1000) were analyzed for mRNA levels by quantitative real-time PCR. Brain tissues and *Gapdh* were used as controls. (B) Fluorescent-micrographs of islets. Islets from wild-type C57BL/6 mouse pancreas were examined. The frozen sections were stained with DAPI (left column), anti-insulin (second column) and anti-NCXs (third column) (*Suppl ref 1*). The merged images are shown in the fourth column. Original magnification: 200×. Bars represent 50 μm.

**Figure S2: NCX on MIN6 insulinoma cells functions during hypoxia/reoxygenation and is suppressed by a specific inhibitor, SEA0400.** (A) $[Ca^{2+}]_i$ elevation induced by hypoxia/reoxygenation in MIN6 cells is suppressed by a specific NCX inhibitor SEA0400. Typical traces (left panel) and average data (right panel) of $Ca^{2+}$ signals when MIN6 cells expressing the Ca-indicator GCaMP2 were exposed to 5% $CO_2$/95% air after culturing under hypoxic conditions for 6 h. The cells were treated with SEA0400 (10 μM) or vehicle. Each column and bar represent the mean ± SD of individual cells (n = 6–7). The difference between the two groups was statistically significant (*$p < 0.001$). (B) Oxygen tension in the medium during culture in hypoxia. Culture dishes were placed inside the chamber maintained at 37°C and gassed with 1% $O_2$, 5% $CO_2$ and 94% $N_2$ and oxygen tension of the medium was measured continuously for more than 12 h using polarography (Unique Medical).

**Figure S3: Use of *Ncx1*$^{+/-}$ islets to examine the role of NCX1 in *in vitro* hypoxic death and *in vivo* islet cell death after transplantation.** (A) Dispersed cultured single cells prepared from *Ncx1*$^{+/+}$ or *Ncx1*$^{+/-}$ MIP-GFP$^+$ islets were used for the experiments. Typical traces (left panel) and average data (right panel) of $Ca^{2+}$ signals when *Ncx1*$^{+/-}$ MIP-GFP$^+$ islets were exposed to 5% $CO_2$/95% air after hypoxic conditions for 6 h are shown. Each column and bar represents the mean ± SE at the time indicated. The differences were statistically significant compared with the corresponding controls (*$p < 0.01$, n = 3 and **$p < 0.05$, n = 3). (B) Fluorescent-micrographs of WT and *Ncx1*$^{+/-}$ islets cultured for 6 h in normoxia or in hypoxia as indicated. Cultured islets were stained with HO342 and PI. Representative micrographs of WT and *Ncx1*$^{+/-}$ islets are shown. Original magnification, 200×. Bars indicate 100 μm. (C) Nonfasting plasma glucose levels of STZ-induced diabetic mice transplanted with 200 syngeneic WT or *Ncx1*$^{+/-}$ islets. Lines represent individual animals. (D) Serum HMGB1 concentrations of mice receiving WT or *Ncx1*$^{+/-}$ islets at 6 h after transplantation. The difference was statistically significant (*$p = <0.01$, n = 3 in each group). (E) Flow cytometry profiles of MNC from the livers of mice receiving 200 WT or *Ncx1*$^{+/-}$ islets at 6 h after transplantation into the liver. Representative data of two experiments are shown.

**Figure S4: The amount of insulin released from wild-type islets *in vitro* in response to glucose was not affected by treatment with SEA0400.** Isolated islets were cultured overnight and used for the *in vitro* experiments. Islets were maintained at 37°C in a $CO_2$-incubator (95% air + 5% $CO_2$) throughout the experiment. Islets were first preincubated with KRH buffer containing 50 mg/dL glucose supplemental with 0.2% BSA for 2 h. Then, islets were further incubated in the KRH buffer containing 50 (n = 4) or 300 mg/dL (n = 4) glucose for 1 hour. The amount of insulin released in the medium after the last incubation was measured by ELISA (Morinaga, Tokyo, Japan). The values are expressed as mean ± SD (n = 4).

**Figure S5: *In vitro* pretreatment of donor islets with SEA0400 helps prevent early loss of islet grafts transplanted beneath the kidney capsule of diabetic recipient mice.** Nonfasting plasma glucose levels of STZ-induced diabetic mice transplanted with 50 syngeneic WT islets treated with 10 μM SEA0400 or vehicle (DMSO) prior to transplantation are shown. Lines represent plasma glucose levels of individual mice. *The kidneys bearing islet grafts were removed at the day indicated.

**Table S1:** Donors and islet characteristics