digestion, and then it significantly decreased after 40 min (in comparison to the ratio at 20 min of digestion). This outcome indicates the toxic effect of the digestion process on exocrine and endocrine cells in the digested fluid. Many factors, including the activation of endogenous serine proteases, have recently been reported to influence the outcome of islet isolation (3,13,15,20, 22,23). In fact, we observed a greater number and variety of proteins at 20 min or later in the digestion process than at 0–20 min (Fig. 6).

We also examined the toxicity of the environment against islets in the digestion circuit during the digestion phase. We selected 40 islets from the digested fluid at 25 and 50 min and compared the ATP content from each time period. The ATP content of the islets selected at 50 min was significantly less than that obtained at the 25-min point. Therefore, prolonged digestion damages endocrine as well as exocrine cells in the digestion circuit. These results support the idea that collagenase digestion should be stopped earlier to obtain viable islets.

We measured adenosine contents in relation to islet count and yield using an overdigestion model of the porcine pancreas. Our findings clarified the chronological changes of these parameters and demonstrated that the conventional time for the termination of digestion correlated with the peak of the islet count changing ratio. Furthermore, the conventional time for the termination of digestion was preceded by a greater than threefold increase in the ATP changing ratio, which is therefore considered to be a useful objective indicator for the termination of the digestion phase. Further study is needed to compare the yield of islets and the in vivo and in vitro function of islets between the findings obtained with the conventional isolation method and islet isolation using the ATP measurements.

ACKNOWLEDGMENTS: This work was supported in part by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology and in part by a Grant-in-Aid for Research on Human Genome, Tissue Engineering Food Biotechnology, Health Sciences Research Grants, Ministry of Health, Labor and Welfare of Japan. The reagents of adenosine nucleotides and luciferase were provided by Kikkoman Corp. The authors declare no conflict of interest.

REFERENCES

1. Brandhorst, D.; Brandhorst, H.; Hering, B. J.; Federlin, K.; Bretzel, R. G. Islet isolation from the pancreas of large mammals and humans: 10 years of experience. Exp. Clin. Endocrinol. Diabetes 103(Suppl. 2):3–14; 1995.
2. Brandhorst, D.; Brandhorst, H.; Hering, B. J.; Federlin, K.; Bretzel, R. G. Large variability of the intracellular ATP content of human islets isolated from different donors. J. Mol. Med. 77:93–95; 1999.
3. Brandhorst, H.; Friberg, A.; Andersson, H. H.; Felldin, M.; Foss, A.; Salmela, K.; Lundgren, T.; Tibell, A.; Tufveson, G.; Korsgren, O.; Brandhorst, D. The importance of tryptic-like activity in purified enzyme blends for efficient islet isolation. Transplantation 87:370–375; 2009.
4. Eckhard, M.; Brandhorst, D.; Winter, D.; Jaeger, C.; Hahr, H.; Bretzel, R. G.; Brendel, M. D. The role of current product release criteria for identification of human islet preparations suitable for clinical transplantation. Transplant. Proc. 36:1528–1531; 2004.
5. Goto, M.; Holgersson, J.; Kumagai-Braesch, M.; Korsgren, O. The ATP/ADP ratio: A novel predictive assay for quality assessment of isolated pancreatic islets. Am. J. Transplant. 6:2483–2487; 2006.
6. Goto, T.; Tanioka, Y.; Sakai, T.; Terai, S.; Kamoda, Y.; Li, S.; Tanaka, T.; Tsujimura, T.; Matsumoto, I.; Fujino, Y.; Suzuki, Y.; Kuroda, Y. Application of the two-layer method on pancreas digestion results in improved islet yield and maintained viability of isolated islets. Transplantation 83:754–758; 2006.
7. Hering, B. J.; Kandaswamy, R.; Ansite, J. D.; Eckman, P. M.; Nakano, M.; Sawada, T.; Matsumoto, I.; Ihm, S. H.; Zhang, H. J.; Parkey, J.; Hunter, D. W.; Sutherland, D. E. R. Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. JAMA 293:830–835; 2005.
8. Ishii, S.; Saito, T.; Ise, K.; Sato, Y.; Tsuchiya, T.; Kenjo, A.; Kimura, T.; Anazawa, T.; Suzuki, S.; Terashima, M.; Gotoh, M. Evaluation of energy state of islet independent of size using newly developed ATP bioluminescence assay. Transplant. Proc. 37:3499–3450; 2005.
9. Ishii, S.; Sato, Y.; Terashima, M.; Saito, T.; Suzuki, S.; Murakami, S.; Gotoh, M. A novel method for determination of ATP, ADP, and AMP contents of a single pancreatic islet before transplantation. Transplant. Proc. 36: 1191–1193; 2004.
10. Kim, J. A new possible method to detect the rate of insulin release from mouse pancreatic islet cells using zinquin, a specific fluorescent probe for zinc. Jpn. J. Vet. Res. 49: 55; 2001.
11. Kim, J. H.; Park, S. G.; Lee, H. N.; Lee, Y. Y.; Park, H. S.; Kim, H. I.; Yu, J. E.; Kim, S. H.; Park, C. G.; Ha, J.; Kim, S. J.; Park, K. S. ATP measurement predicts porcine islet transplantation outcome in nude mice. Transplantation 87:166–169; 2009.
12. Kim, J. Y.; Lee, J. I.; Jeong, J. H.; Fang, Y.; Ju, M. K.; Kim, S. J.; Huh, K. H.; Kim, M. S.; Kim, Y. S. Improved yield and functional parameters of rat pancreas islets isolated under intramuscular anesthesia. Cell Transplant. 19: 743–750; 2010
13. Kin, T.; Johnson, P. R. V.; Shapiro, A. M. J.; Lakey, J. R. T. Factors influencing the collagenase digestion phase of human islet isolation. Transplantation 83:7–12; 2007.
14. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685; 1970.
15. Lakey, J. R. T.; Helms, L. M. H.; Kin, T.; Korbutt, G. S.; Rajotte, R. V.; Shapiro, A. M. J.; Warnock, G. L. Serine-protease inhibition during islet isolation increases islet yield from human pancreases with prolonged ischemia. Transplantation 72:565–570; 2001.
16. Mahler, R.; Franke, F. E.; Hering, B. J.; Brandhorst, D.; Brandhorst, H.; Brendel, M. D.; Federlin, K.; Schulz, A.; Bretzel, R. G. Evidence for a significant correlation of donor pancreas morphology and the yield of isolated purified human islets. J. Mol. Med. 77:87–89; 1999.
17. Matsumoto, S.; Noguchi, H.; Naziruddin, B.; Onaca, N.; Jackson, A.; Hatanaka, N.; Okitsu, T.; Kobayashi, N.; Klintmalm, G.; Levy, M. Improvement of pancreatic islet cell isolation for transplantation. Bayl. Univ. Med. Cent. Proc. 20:357–362; 2007.

18. Nano, R.; Clissi, B.; Melzi, R.; Calori, G.; Maffi, P.; Antonioli, B.; Marzorati, S.; Aldrighetti, L.; Freschi, M.; Grochowiecki, T.; Socci, C.; Secchi, A.; Di Carlo, V.; Bonifacio, E.; Bertuzzi, F. Islet isolation for allotransplantation: Variables associated with successful islet yield and graft function. Diabetologia 48:906–912; 2005.
19. 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.
20. Noguchi, H.; Ueda, M.; Hayashi, S.; Kobayashi, N.; Nagata, H.; Iwanaga, Y.; Okitsu, T.; Matsumoto, S. Comparison of M-Kyoto solution and histidine-tryptophan-ketoglutarate solution with a trypsin inhibitor for pancreas preservation in islet transplantation. Transplantation 84: 655–658; 2007.
21. Noguchi, H.; Ueda, M.; Hayashi, S.; Kobayashi, N.; Okitsu, T.; Iwanaga, Y.; Nagata, H.; Nakai, Y.; Matsumoto, S. Ductal injection of presetvation solution increases islet yields in islet isolation and improves islet graft function. Cell Transplant. 17:69–81; 2008.
22. Rose, N. L.; Palcic, M. M.; Lakey, R. T. Evaluating the effect of serine protease on collagenase activity during human islet isolation. Cell Transplant. 11:821–826; 2002.
23. Rose, N. L.; Palcic, M. M.; Shapiro, A. M. J.; Lakey, J. R. T. Endogenous pancreatic enzyme activity levels show no significant effect on human islet isolation yield. Cell Transplant. 13:153–160; 2004.
24. Sakakibara, T.; Murakami, S.; Imai, K. Enumeration of bacterial cell numbers by amplified firefly bioluminescence without cultivation. Anal. Biochem. 312:48–56; 2003.
25. Shapiro, A. M. J.; Lakey, J. R. T.; 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.
26. Zalewski, P. D.; Forbes, I. J.; Seamark, R. F.; Borlinghaus, R.; Betts, W. H.; Lincoln, S. F.; Ward, A. D. Flux of intracellular labile zinc during apoptosis (gene-directed cell death) revealed by a specific chemical probe, zinquin. Chem. Biol. 1:153–161; 1994.

Mitomycin C Treatment Significantly Reduces Central Damage of Islets in Culture

Takaharu Saito, MD, PhD, Takuro Saito, MD, PhD,* Yoshihiro Sato, MD, PhD,* Kazuya Ise, MD, PhD,* Takayuki Anazawa, MD, PhD,* Ikuro Oshibe, MD, PhD,* Junichiro Haga, MD,* Masaya Yamamoto, MD, PhD,† Satoshi Waguri, MD, PhD,† and Mitsukazu Gotoh, MD, PhD**

Objectives: We recently reported that mitomycin C (MMC) treatment and subsequent culture of islets significantly prolongs graft survival in allotransplantation and xenotransplantation models. The present study was performed to determine the changes in morphology and signal transduction in pancreatic islets after MMC treatment.

Methods: Freshly isolated rat islets were treated with 10 μg/mL MMC for 30 minutes and then cultured for up to 3 days. The samples were processed for immunohistologic studies and electron microscopic examination at various times after treatment. A DNA fragmentation assay was performed to detect apoptotic cell death. Western blotting was performed to determine the effects of MMC on signal transduction.

Results: As early as 4 hours after culture, the islets showed central damage; most cells were necrotic and stained with anti–high mobility group box 1 antibody, and a few were apoptotic. The ratio of the damaged area to the whole area was significantly decreased after MMC treatment. Western blotting showed that MMC treatment increased the levels of activated forms of p53 and $p21^{waf1}$, whereas levels of the activated forms of Akt and caspase-3 were unchanged.

Conclusions: Mitomycin C treatment protects islets from the progression of central damage during culture. The p53–$p21^{waf1}$ pathway might be involved in these effects.

Key Words: Mitomycin C, rat islet, necrosis, HMGB1, p53, $p21^{waf1}$

Abbreviations: MMC - mitomycin C, HMGB1 - high mobility group box 1

(*Pancreas* 2012;41: 245–252)

Multicenter trials using the Edmonton protocol have revealed that islet transplantation can successfully restore long-term endogenous insulin production and glycemic stability in subjects with type 1 diabetes mellitus. However, insulin independence is usually unsustainable because the rate of complete graft survival at 5 years after graft is less than 10%.[1] Therefore, the steps involved in the Edmonton protocol are currently being reevaluated for further improvement.

It is unclear whether freshly isolated islets or cultured islets are superior for clinical islet transplantation. Cultured islets were found to be less immunogenic in most experimental studies[2,3]; however, islet loss in culture was marked in many experimental studies and in clinical settings.[3,4] Recently, Kin et al[4] reported that the islet equivalent (IE) was decreased to 86% after culture for 20 hours (median), and the risk factors for islet loss were related to donor isolation factors. Bottino et al[5] reported that the DNA content in islet preparations after culture for 24 hours was reduced by up to 80%. Their assay might reflect dispersed islets and central damage of islets, of which the latter is usually found during islet culture.[6–10] Most of the previous studies suggested that this process involves apoptotic events.[11–13]

Meanwhile, we have reported that mitomycin C (MMC) treatment before islet culture significantly prolongs graft survival in allotransplantation and xenotransplantation models[14–17] and that 10 μg/mL of MMC was optimal to maintain in vitro insulin secretory capacity and to prolong graft survival. This prolonged survival was obtained when islets were cultured for 20 hours to 3 days, but not when they were transplanted immediately.[16]

In this study, we treated islets with MMC and cultured them for 3 days. Here, we showed that the central damage was mostly due to necrotic events being associated with the intracellular expression of high mobility group box 1 (HMGB1) rather than apoptotic events and that MMC treatment significantly reduced these events, probably through a mechanism involving the p53–$p21^{waf1}$ pathway.

MATERIALS AND METHODS

Animals

Male Wistar (Jcl) rats (CLEA Japan, Inc, Tokyo, Japan), 8 to 9 weeks old, were used for islet isolation. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Fukushima Medical University, and all procedures in the experiment were performed according to the guidelines of the National Council's Guide for the Care and Use of Laboratory Animals.

Islet Isolation, MMC Treatment, and Culture

The islets were isolated using a stationary collagenase digestion protocol previously reported.[18] The islets obtained after the second Ficoll separation, devoid of major immunogenic contaminants, were incubated for 30 minutes without or with 10 μg/mL of MMC (Kyowa Hakko Kogyo, Tokyo, Japan). The islets were then washed 3 times with RPMI-1640 containing 10% fetal bovine serum and cultured in complete medium (RPMI-1640 with HEPES [10 mM], L-glutamine [2 mM], penicillin [100 U/mL], streptomycin sulfate [100 μg/mL], amphotericin B [0.5 μg/mL], and 10% fetal bovine serum] for 0, 4, 8, or 20 hours or 3 days at 37°C under 5% CO_2/95% air in a humidified atmosphere. The culture medium was changed once every other day. For the evaluation of islet survival in culture, approximately 200 islets per culture condition were seeded in a 6-well culture plate (Corning Coster Co, Cambridge, Mass). On 0, 4, 8, or 20 hours or 3 days in culture, islet counting was performed using an optical graticule (n = 6 in each group). The

From the Departments of *Surgery 1 and †Anatomy and Histology, Fukushima Medical University, Fukushima, Japan.
Received for publication September 17, 2010; accepted May 11, 2011.
Reprints: Mitsukazu Gotoh, MD, PhD, Department of Surgery 1, Fukushima Medical University, 1 Hikarigaoka, Fukushima 960-1295, Japan (e-mail: mgotoh@fmu.ac.jp).
This work was supported by a research grant from Fukushima Medical University and in part by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology and in part by a Grant-in-Aid for Research on Human Genome, Tissue Engineering Food Biotechnology, Health Sciences Research Grants, Ministry of Health, Labour and Welfare of Japan.
The authors declare no conflict of interest.

crude number of islets was converted into a standard number of islet equivalents (IEQ; diameter standardized to 150 μm).

Histology and Immunohistochemistry of Cultured Islets

Paraffin Embedding of Islets and Immunohistologic Staining of Islets

Cultured islets were transferred to microtubes and collected by centrifugation. They were then fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sections (4 μm thick) were cut from each block. Sections (≥50 μm apart) were stained with hematoxylin and eosin (HE). For immunohistologic staining of insulin, glucagon, and HMGB1, we used purified rabbit anti-insulin (H-86; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), antiglucagon (catalog number 11184; Progen, Biotechnik, Heidelberg, Germany), and anti-HMGB1 (product number 326052219; Shino-test Corp, Kanagawa, Japan) polyclonal antibodies. To detect each molecule, the sections were stained using a peroxidase-labeled biotin-avidin detection system Histofine Simple Stain Rat MAX PO® in accordance with the manufacturer's instructions (Nichirei Biosciences, Inc, Tokyo, Japan).

TUNEL Assay

In situ detection of DNA fragmentation was performed on 4-μm-thick paraffin sections of islets using the In Situ Apoptosis Detection Kit (Takara Bio, Inc, Shiga, Japan) according to the method described in the manual. To identify regions of central damage in the islets, the sections were counterstained with eosin. Cells positive for the TUNEL reaction with nuclear condensation were counted as apoptotic cells per section.

Evaluation of Diameter and Area of Islets

To assess the area of central damage in islets, we randomly selected paraffin sections (≥100 μm apart) with HE staining. For approximately 200 islets per culture condition, we measured the diameter, damaged areas, and the entire area of the individual islets by Microanalyzer software (Nihon Poladigital Co, Tokyo, Japan). We also calculated the ratio of the damaged area to the whole islet area.

Transmission Electron Microscopic Observation

Morphologic examination of islets was done by transmission electron microscopy. Approximately 600 islets per culture condition were fixed in a solution containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4); osmicated, en bloc–stained with uracyl acetate; dehydrated in graded alcohols; and embedded in Epon 812 (TAAB, Berkshire, UK). The approximately 100-nm-thick sections were picked up on a copper grid, stained with lead citrate and uranyl acetate to enhance contrast, and were viewed and photographed with an electron microscope (JEM-1200EX; JEOL Ltd, Tokyo, Japan).

DNA Fragmentation Assay

Nucleosomal ladder detection of apoptotic cells was performed using an Apoptotic DNA Ladder Extraction Kit (BioVision Research Products, Mountain View, Calif) and the supplied reagents according to the method described in the manual.

Western Blotting

After each culture period, islet samples (about 600 islets per culture condition) were centrifuged, and the supernatant was discarded to leave the pellet containing the islets. The pellet was then frozen in liquid nitrogen and stored at −80°C until use. The frozen pellet was dissolved in a lysis buffer mixture (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 15% glycerol, 5 mM EDTA, 1% Triton X-100 [Sigma-Aldrich Co, St Louis, Mo]), containing protease inhibitor cocktail (Roche Diagnostics Co, Mannheim, Germany), phosphatase inhibitor cocktail (Sigma-Aldrich Co), and loading buffer (66 mM Tris-HCl, 10% glycerol, 2% SDS, 100 mM DL-dithiothreitol, 0.001% bromophenol blue) and boiled for 5 minutes. The sample was centrifuged for 1 minute at 15,000 rpm at 4°C, and the supernatant was applied to gel electrophoresis. Equal volumes of the supernatant were loaded onto 5% to 20% polyacrylamide gels, run at 90 V for 5 minutes and then at 150 V for1 hour. The proteins were transferred to a nitrocellulose membrane (Amersham Biosciences Co, Chalfont St Giles, Buckinghamshire, United Kingdom) at 25 V for 16 hours. The membranes were blocked with 7.0% nonfat dried milk in PBS containing 0.1% Tween-20 for 30 minutes at room temperature

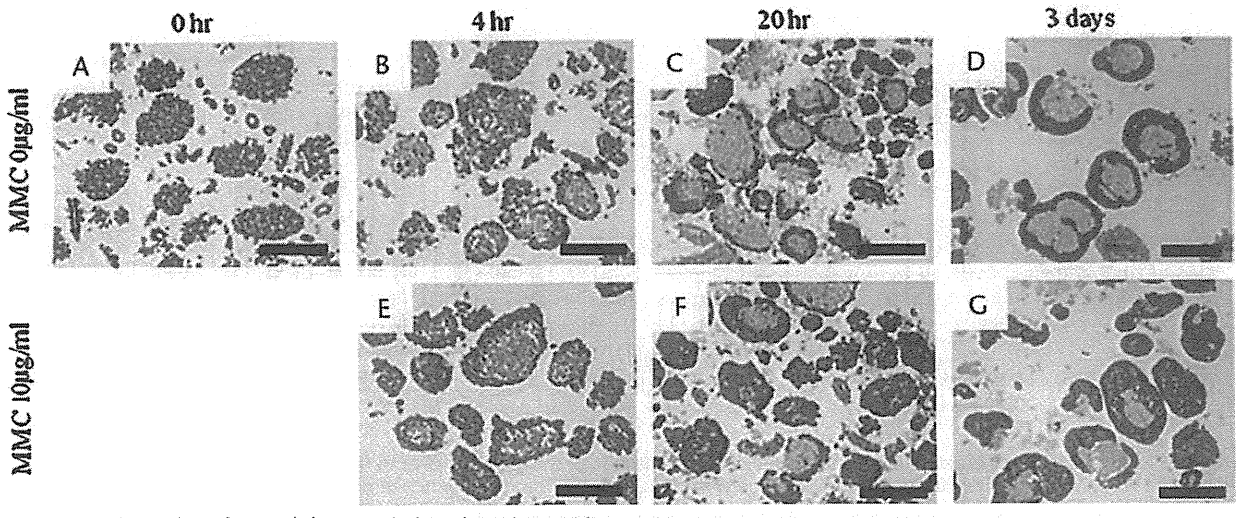


FIGURE 1. Formation of central damage during islet culture with or without MMC pretreatment. Isolated islets were untreated (A–D) or treated (E–G) with 10 μg/mL MMC for 30 minutes. They were immediately fixed (A) or cultured for 4 hours (B and E), 20 hours (C and F), and 3 days (D and G) before fixation as indicated. They were processed for HE staining. Note that the islets show central damage as early as 4 hours after culture and that the fraction of central damaged area is apparently reduced by MMC treatment at 20 hours and 3 days after isolation. Bars, 200 μm.

and then incubated with anti–phospho-p53 (Ser15; Cell Signaling Technology, Inc, Danvers, Mass; dilution 1:100), anti-$p21^{waf1}$ (Thermo Fisher Scientific, Inc, Waltham, Mass; dilution 1:100), anti–caspase-3 (Cell Signaling Technology, Inc; dilution 1:500), anti–phospho-Akt (Ser473) (Cell Signaling Technology, Inc; dilution 1:500), and anti–β-actin (Millipore Co, Billerica, Mass; dilution 1:750) in blocking buffer for 24 hours at 4°C. The membranes were washed with PBS containing 0.1% Tween-20 twice for 10 minutes each and then incubated for 1 hour at room temperature with horseradish peroxidase–conjugated antirabbit antibody (GE Healthcare Co, Chalfont St Giles, Buckinghamshire, UK; dilution 1:1000) or antimouse antibody conjugated with horseradish peroxidase (GE Healthcare Co.; dilution 1:1,000). After washing for another 30 minutes, the blot signals were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc) and LAS-1000 (Fujifilm Co, Tokyo, Japan). Quantitation was performed with ImageJ software (National Institutes of Health, Bethesda, Md). For statistical analysis, the signal intensities were normalized to β-actin that was used as internal control.

Statistical Analysis

Data are expressed as means ± SE. The number of TUNEL-positive cells and the ratio of the damaged area to the whole islet area were compared between the different experimental groups using unpaired Student *t* test. Signal intensities in the Western blot analysis were compared between experimental groups with or without MMC using paired Student *t* test. Values of $P < 0.05$ were considered statistically significant. All statistical analyses were performed using SPSS 16.0 for Windows software (SPSS, Inc, Chicago, Ill).

RESULTS

Histologic Changes in Cultured Islets and the Effect of MMC Treatment

Immediately after isolation, HE staining revealed the islets were normal in appearance (Fig. 1). Regions of central damage in islets that had less prominent staining and were more easily distinguishable from the surrounding normal area were observed at 4 hours and were still visible after culture for 3 days. Pyknotic

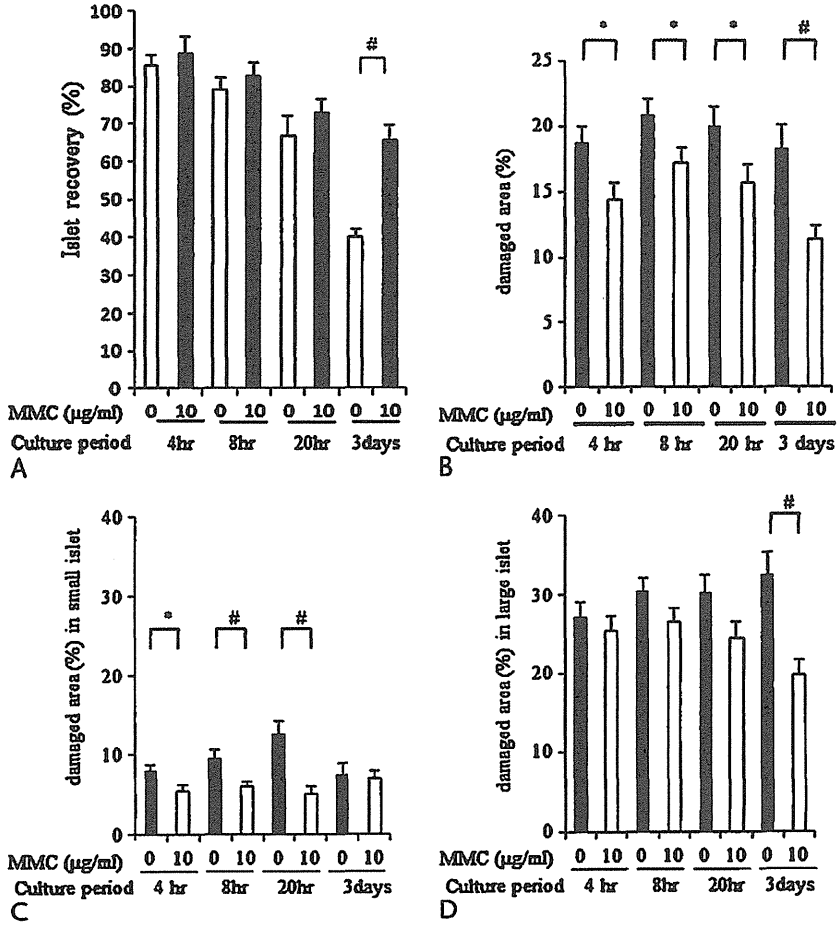
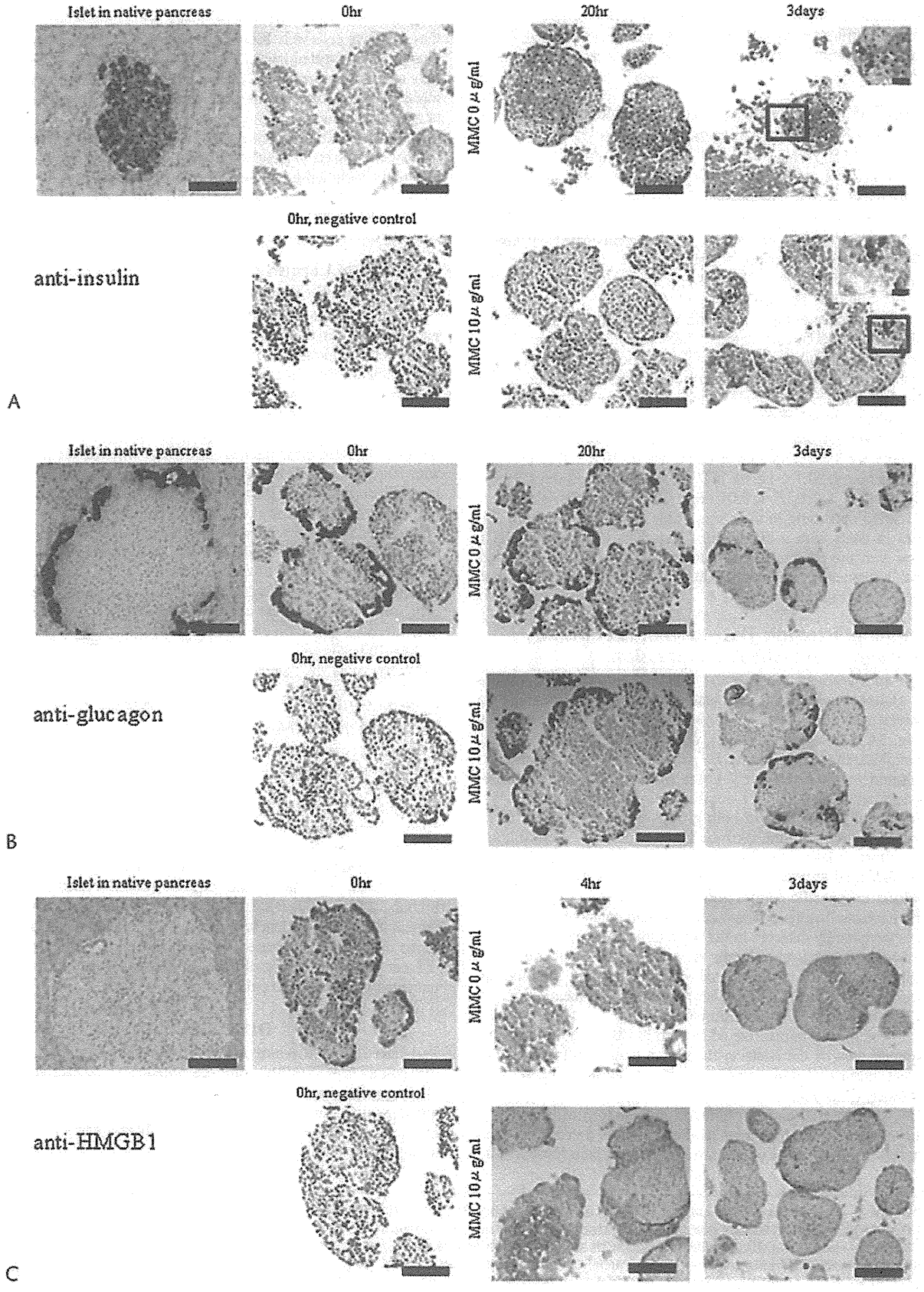


FIGURE 2. A, Islet recovery during culture and quantification of the ratio of the damaged area to the whole islet. The percentages of IEQ recoveries for up to 3 days of culture were compared between the 2 groups. The recovery rate of islet treated with MMC before the culture significantly improved on 3 days compared with untreated islets ($P < 0.01$). Images of the HE-stained islets shown in Figure 1 were captured, and the fraction of the damaged central area was quantified using Microanalyzer software and expressed as a mean ± SE. B, A significant decrease in the ratio of the damaged area is noted for the MMC-treated islets compared with the untreated islets (n = 170–260 islets per group). $^{*}P < 0.05$. $^{\#}P < 0.01$. C and D, When the islets shown in Figure 1 were classified as either small (C; diameter <100 μm; n = 90–130 islets per group) or large (D; diameter >100 μm; n = 70–150 islets per group), a significant decrease in the ratio of damaged area to the whole area is observed in the small islets cultured for 4, 8, or 20 hours and in large islets cultured for 3 days. $^{*}P < 0.05$. $^{\#}P < 0.01$.



cell, an indicator of apoptosis, was only rarely observed in the central damaged area. Mitomycin C treatment seemed to reduce the fraction of damaged areas of islets at each culture time (Fig. 1).

Effect of MMC Treatment on Islet Recovery and the Ratio of the Damaged Area to the Whole Islet Area

Figure 2A shows the percentages of IEQ recoveries for up to 3 days of culture. The recovery rate of islet treated with MMC before the culture significantly improved on 3 days compared with untreated islets (untreated vs 10 μg/mL MMC: 4 hours, 85.5 ± 2.7 vs 88.7 ± 4.3; 8 hours, 79.2 ± 2.9 vs 82.9 ± 3.4; 20 hours, 66.9 ± 5.2 vs 72.8 ± 4.0; 3 days, 40.3 ± 1.9 vs 65.8 ± 4.0, $P < 0.0001$; Fig. 2A).

We next quantified the ratio of the damaged area to the whole islet area in each group. There was no significant difference between culture times (4, 8, or 20 hours or 3 days), suggesting that the damage had occurred and was completed within the first 4 hours of culture. Treating islets with MMC before the culture significantly reduced the ratios in all culture times compared with untreated islets (untreated vs 10 μg/mL MMC: 4 hours, 18.8 ± 1.2 vs 14.4 ± 1.2; 8 hours, 20.8 ± 1.2 vs 17.2 ± 1.2; 20 hours, 20.1 ± 1.5 vs 15.7 ± 1.4, $P < 0.05$; 3 days, 18.3 ± 1.8 vs 11.3 ± 1.1, $P < 0.01$; Fig. 2B). When the islets were classified according to their size, these changes were more evident in islets with a diameter of less than 100 μm (Fig. 2C). However, the changes were not significantly different in islets with a diameter greater than 100 μm, except for those cultured for 3 days (Fig. 2D).

Immunohistologic Detection of Islet Insulin, Glucagon, and HMGB1 Expression

To investigate whether the damaged area contains insulin- or glucagon-expressing cells and whether the cell damage involves necrotic events, we examined the expression of insulin (Fig. 3A), glucagon (Fig. 3B), and HMGB1 (Fig. 3C) by immunohistochemistry in native pancreatic islets and in islets cultured for 20 hours (or 4 hours for HMGB1 expression) or 3 days after isolation. The number of insulin-positive cells was significantly decreased in both groups immediately after isolation compared with those of the intact pancreas. After 20 hours of culture, relatively diffuse staining was observed in the central damaged area of the islets in both groups, which probably reflects nonspecific signals and/or insulin retained by unknown mechanisms. Insulin-positive cells containing more intense signals were hardly detected in the surrounding survived cells. After 3 days of culture, several cells survived in the peripheral region and contained an insulin signal with an apparent granular appearance (insets in Fig. 3A). These were more frequently found in MMC-treated islets than in untreated islets.

The glucagon-positive cells were mostly located in the peripheral area of the islets in the native pancreas and remained intact, compared with insulin-positive cells, immediately after isolation (Fig. 3B).

The HMGB1 signal was found in the nuclei of islet cells in the intact pancreas. Immediately after isolation, a strong HMGB1 signal was found in the cytoplasm of some islet cells located in the peripheral and central areas of the islets. After culture for 4 hours, these positive cells were more frequently found in untreated islets than in MMC-treated islets. After culture for 20 hours or 3 days, only some of the peripheral islet cells showed a strong signal for HMGB1, while the cells in the central damaged area often contained nuclear signals.

These findings indicate that the central damaged area was mainly composed of insulin-positive cells, which were vulnerable to the isolation procedure. Furthermore, it is suggested from the HE-observation that at least some of the cells in the central part of islet undergo necrotic cell death soon after the isolation procedure.

TUNEL, DNA Fragmentation, and Electron Microscopic Examination of the Islets

The TUNEL assay revealed that the apoptotic cells were mainly located in the periphery of the islets, but there were only 0 to 4 cells apoptotic per islet section (Figs. 4A, B). There were no significant differences in the number of apoptotic cells between the untreated and the MMC-treated groups at any culture time for up to 3 days (Fig. 4C). In the DNA fragmentation assay, the islets treated with 100 μg/mL MMC and cultured for 20 hours showed clear nuclear laddering, whereas those treated with 10 μg/mL MMC or left untreated showed only slight laddering, with no significant difference between the 2 groups (Fig. 4D).

Electron microscopy of islets cultured for 4 hours revealed that the cells located within the central damaged area exhibited necrotic features such as cell swelling, low cytoplasmic electron density, and ballooning of the mitochondria (Fig. 4G).[19] Similar changes were observed at day 3 of culture. In contrast, apoptotic cells identified by nuclear condensation and cell shrinkage were only rarely observed on electron microscopic analysis (5–6 cells in 500–700 cells within each islet) during culture for up to 3 days, regardless of MMC treatment.

Assessment of p53 to $p21^{waf1}$ Pathway in Islet Treated With MMC

Mitomycin C treatment increased the levels of phosphorylated p53 protein by 3- to 5-fold and that of $p21^{waf1}$ protein by 2-fold at 8 and 20 hours of culture (Fig. 5). The ratio of signal intensities to β-actin in MMC-treated to untreated islets at 8 and 20 hours of culture was calculated in 3 separate experiments and showed significant differences (paired Student t test) for p53 (MMC-treated/untreated: 8 hours, 3.28 ± 0.04, $P < 0.05$; 20 hours, 4.00 ± 0.88, $P < 0.05$) and $p21^{waf1}$ (MMC-treated/untreated: 8 hours, 2.19 ± 0.09, $P < 0.05$; 20 hours, 1.94 ± 0.10, $P < 0.05$) protein levels. The protein levels of phosphorylated Akt and active forms of caspase-3 increased with culture period, but these levels did not show significant differences between MMC-treated and untreated groups at each culture time.

DISCUSSION

Islet loss is a major limitation for successful islet transplantation. Islet viability could be affected at various steps including pancreas retrieval, preservation, islet isolation, culture, and transplantation. In this study, we showed that central damage of islets was morphologically recognizable as early as 4 hours after starting the islet culture. The damaged area was mainly

FIGURE 3. Immunohistochemical study of islet expression of insulin, glucagon, and HMGB1. Pancreatic tissue (native islet) and isolated islets cultured for the periods as indicated with or without 10 μg/mL MMC pretreatment were fixed and embedded in paraffin, followed by immunohistochemical staining with antibodies against insulin (A), glucagon (B), and HMGB1 (C). Tissue samples were counterstained with hematoxylin to detect nuclei. Some sections obtained from samples at 0 hour after isolation were stained without the primary antibodies (negative control). In "3 days" of (A), the boxed areas are enlarged in insets. Bars, 100 μm; 20 μm in the insets.

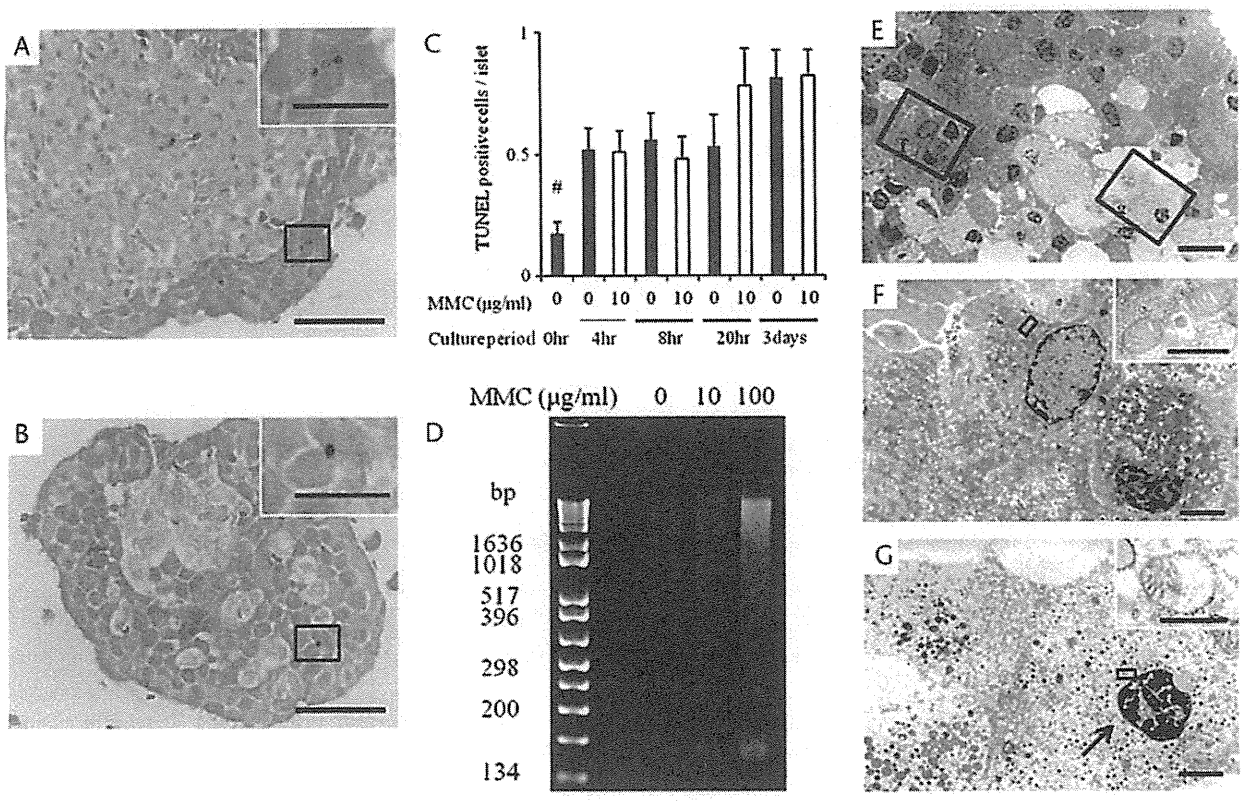


FIGURE 4. TUNEL, DNA fragmentation, and electron microscopic examination of the islets. Untreated (A) and MMC-treated (B) islets were cultured for 20 hours and fixed for TUNEL staining. They were counterstained with eosin to define the areas of central damage. The region marked by the black square is enlarged in the inset. Note that only a few TUNEL-positive cells are observed outside the region of central damage in the untreated (A) and MMC-treated (B) islets. Bars, 50 μm; 20 μm in the inset. C, The number of TUNEL-positive cells per islet section was counted in the untreated (black column) and MMC-treated (white column) islets (n = 60–90 islets sections per group). The number of TUNEL-positive cells of cultured islets was significantly greater than that of fresh islets ($^{\#}P < 0.01$). However, no significant difference was noted between the untreated and MMC-treated islets. D, The DNA fragmentation assay was performed to detect apoptotic cell death. Islets treated with 0, 10, or 100 μg/mL MMC were cultured for 20 hours and lysed for DNA extraction. Islets treated with 100 μg/mL MMC showed detectable DNA laddering. Representative data of 3 separate experiments are shown. Isolated islets were cultured for 4 hours without MMC pretreatment and were processed for electron microscopy. A peripheral region of an islet containing both damaged and undamaged regions is shown in E. Undamaged (boxed region f) and damaged (boxed region g) regions were magnified and are shown in F and G, respectively. Cells in the peripheral region (F) appear to be normal with intact nuclei and mitochondria (inset), whereas those in the damaged region (G) are necrotic showing cell enlargement, low cytoplasmic electron density, mitochondrial swelling (inset), and slight nuclear condensation (arrow). Bars, 10 μm (E); 2 μm (F and G); 0.5 μm (inset).

composed of necrotic cells rather than apoptotic cells, which were only rarely observed in the region peripheral to area of central damage. Furthermore, MMC treatment significantly reduced islet loss during culture and the area of central damage and increased the level of activated p53 and $p21^{waf1}$, suggesting that the central damage occurs through pathway related to the cell cycle. This is the first report to show that MMC treatment protects islets during culture.

When we analyzed the extent of islet damage according to islet size, we found that the smaller islets were less vulnerable to damage. The ratio of central damage of small islets (diameter <100 μm) was approximately 10%, whereas that of large islets (diameter >100 μm) was up to 30% after culture for 3 days. This is supported by the earlier findings reported by MacGregor et al[6] who found that the extent of central damage after culture for 24 hours was lower in rat islets with a diameter less than 125 μm compared with islets with a diameter greater than 150 μm. This is also consistent with the findings reported by Lehmann et al[7] who used human islets. Taken together, it can be speculated that the smaller diffusion barrier associated with smaller islets may help to prevent central damage.[6]

Numerous studies have been conducted in an attempt to prevent or reduce the extent of central damage of islets in culture. These studies have focused on improving oxygen supply by using less medium, hyperoxygen culture, and oxygen delivery proteins[7,9,20] and by lowering the culture temperature to reduce oxygen demand.[10] The use of extracellular matrix from ductal components has also been reported to provide better extracellular conditions.[8] Accordingly, MMC treatment could offer an additional means of preventing central damage during islet culture.

We found that the area of central damage was mainly composed of necrotic cells but contained a few apoptotic cells. To date, the contribution of necrotic or apoptotic cells to this event was inconsistent between studies. A high apoptotic index (15%–45%) was observed in islets obtained from canine or human pancreata and cultured for 3 days.[11–13] In contrast, only a few apoptotic cells per islet were noted in human islets cultured for 24 hours.[21] On the other hand, in a model using canine islets,

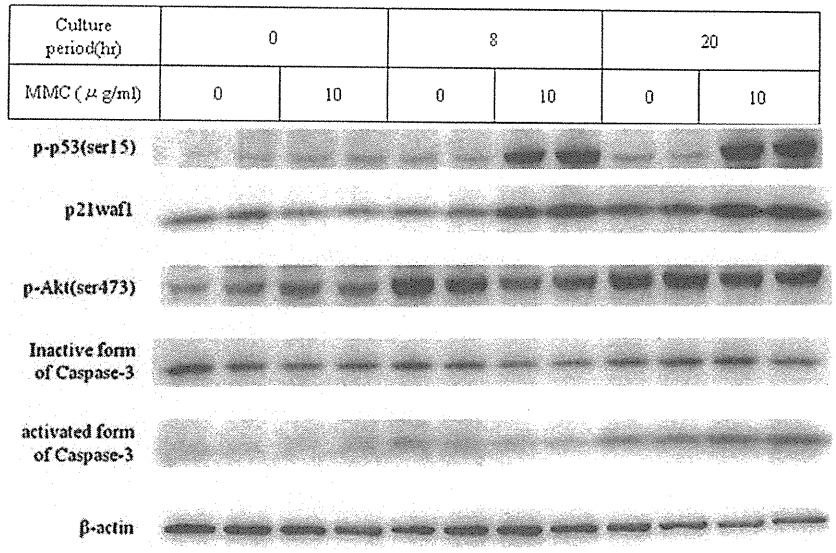


FIGURE 5. Western blotting for p53, $p21^{waf1}$, Akt, caspase-3, and β-actin protein expression in cultured islets. Untreated and MMC-treated islets were cultured for 0, 8, or 20 hours. Islets were lysed and subjected to Western blotting in duplicate using the antibodies indicated. Significant increases in the expression of phospho-p53 (Ser15) and $p21^{waf1}$ were observed in islets treated with MMC and cultured for 8 or 20 hours as compared with untreated islets (see text). The blots shown are representative of 3 independent experiments.

annexin V/propidium iodide staining revealed that the number of necrotic cells exceeded that of apoptotic cells when islets were cultured for 12 to 72 hours.[22] In a human islet culture model that was used to test the effects of antioxidant treatment, necrotic and apoptotic events continued after isolation and culture, as revealed by the poly (ADP-ribose) polymerase cleavage assay.[23]

Apoptotic cells are characterized by chromatin condensation, cell volume reduction, and cellular fragmentation, leading to cell shrinkage.[19,24] Meanwhile, necrotic cells are characterized by increased cell volume, decreased cytoplasmic density, and mitochondrial swelling with minor nuclear condensation.[19] In this study, most of the cells in the central damaged area showed features characteristic of necrotic cells, and only a few cells showed characteristics of apoptotic cells, indicating that the area was mostly composed of necrotic cells. In the DNA fragmentation assay, the islets treated with MMC at a toxic concentration of 100 μg/mL and cultured for 20 hours showed a DNA laddering, whereas those treated with or without 10 μg/mL MMC showed only faint laddering. These results were consistently observed, even when we cultured islets in smaller amounts of medium (data not shown), which has been reported to improve oxygen supply to the cells.[20]

High mobility group box 1 was originally identified as a chromosomal protein facilitating the binding of transcription factors to their cognate DNA sequences.[25] Recently, Scaffidi et al[26] showed that HMGB1 is released from necrotic but not apoptotic cells and that it induces inflammatory responses in vivo. Similarly, Steer et al[27] reported that interleukin-1 stimulated β-cell necrosis and the release of the immunologic adjuvant HMGB1. Together with these results, we can reasonably assume that the regions of islets showing central damage during culture are mainly composed of necrotic cells that release HMGB1 and that MMC treatment reduces the extent of damage to prolong graft survival in vivo. This was confirmed in the recent work by Matsuoka et al[28] who showed that HMGB1 is involved in the initial events of early loss of transplanted islets in mice.

Mitomycin C has been shown to induce cell cycle arrest, DNA repair, and cell apoptosis by the phosphorylation of p53 at serine 15.[29–32] Mitomycin C treatment and subsequent culture for 8 or 20 hours upregulated the active form of p53 and that of the downstream gene $p21^{waf1}$. Meanwhile, the active form of caspase-3 was not significantly affected by MMC. This indicates that the activation of p53 was directed at cell cycle arrest rather than apoptotic processes through the activation of caspase-3.[33] This is supported by the finding that the active form of Akt, which inhibits apoptosis and enhances cell cycle progression, was not enhanced.[34]

In response to genotoxic stress, the activation of p53 and subsequent activation of $p21^{waf1}$ induced cell cycle arrest by inhibiting kinase activity because $p21^{waf1}$ forms a complex with cyclin-Cdk or with PCNA.[35,36] Cell cycle arrest induces hypometabolism, which protects the cells from various stressors.[37] For example, hypothermic culture of Chinese hamster ovary cells at 30°C induces G_1 arrest, which prevents cell death.[38,39] Ischemic preconditioning of the PC12 cell line also induces cell cycle arrest and protects against cell death after reperfusion.[40] On the basis of these results, we speculate that some pathway leading to cell cycle arrest might be induced by MMC, which would protect the islets in culture and reduce release of HMGB1 leading to prolonged graft survival.

ACKNOWLEDGMENTS

The authors thank Yoko Ishii, Yukiko Kikuta, Yoshie Saito, Hidemi I, Atsuko Yabashi, and Katsuyuki Kanno for their excellent technical assistance and Prof Yoichi Yasunami for his precious comments on histologic evaluation of HMGB1.

REFERENCES

1. Ryan EA, Paty BW, Senior PA, et al. Five-year follow-up after clinical islet transplantation. *Diabetes*. 2005;54:2060–2069.
2. Marchetti P, Scharp DW, Pfiffner K, et al. Cryogenic storage of isolated, purified porcine pancreatic islets. *Transplantation*. 1994;57:340–346.
3. Andersson A. Reversal of hyperglycemia by intrasplenic transplantation of 4-week-cultured allogeneic mouse islets. *Diabetes*. 1982;31(suppl 4):55–59.

4. Kin T, Senior P, O'Gorman D, et al. Risk factors for islet loss during culture prior to transplantation. *Transpl Int.* 2008;21:1029–1035.
5. Bottino R, Balamurugan AN, Bertera S, et al. Preservation of human islet cell functional mass by anti-oxidative action of a novel SOD mimic compound. *Diabetes.* 2002;51:2561–2567.
6. MacGregor RR, Williams SJ, Tong PY, et al. Small rat islets are superior to large islets in in vitro function and in transplantation outcomes. *Am J Physiol Endocrinol Metab.* 2006;290:E771–E779.
7. Lehmann R, Zuellig RA, Kugelmeier P, et al. Superiority of small islets in human islet transplantation. *Diabetes.* 2007;56:594–603.
8. Ilieva A, Yuan S, Wang RN, et al. Pancreatic islet cell survival following islet isolation: the role of cellular interactions in the pancreas. *J Endocrinol.* 1999;161:357–364.
9. Stagner JI, Parthasarathy SN, Wyler K, et al. Protection from ischemic cell death by the induction of cytoglobin. *Transplant Proc.* 2005;37:3452–3453.
10. Ono J, Lacy PE, Michael HEB, et al. Studies of the functional and morphologic status of islets maintained at 24°C for four weeks in vitro. *Am J Pathol.* 1979;97:489–504.
11. Wang RN, Rosenberg L. Maintenance of beta-cell function and survival following islet isolation requires re-establishment of the islet-matrix relationship. *J Endocrinol.* 1999;163:181–190.
12. Paraskevas S, Maysinger D, Wang R, et al. Cell loss in isolated human islets occurs by apoptosis. *Pancreas.* 2000;20:270–276.
13. Farilla L, Bulotta A, Hirshberg B, et al. Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets. *Endocrinology.* 2003;144:5149–5158.
14. Matsuyama S, Gunji T, Ise K, et al. Permanent acceptance of mitomycin C–treated islet allograft. *Transplantation.* 2003;76:65–71.
15. Grtochowiecki T, Gotoh M, Dono K, et al. Pretreatment of crude pancreatic islets with mitomycin C prolongs graft survival time in xenogeneic rat-to-mouse model. *Transplantation.* 1999;67:1474–1477.
16. Gunji T, Saito T, Sato Y, et al. Mitomycin-C treatment followed by culture produces long-term survival of islet xenografts in a rat-to mouse model. *Cell Transplant.* 2008;17:619–629.
17. Ise K, Kanazawa Y, Sato Y, et al. Survival of mitomycin C–treated pancreatic islet xenografts is mediated by increased expression of transforming growth factor-β. *Transplantation.* 2004;77:907–914.
18. Gotoh M, Maki T, Kiyoizumi T. An improved method for isolation of mouse pancreatic islets. *Transplantation.* 1985;40:437–438.
19. Okada Y, Maeno E, Shimizu T, et al. Receptor-mediated control of regulatory volume decrease (RVD) and apoptotic volume decrease (AVD). *J Physiol.* 2001;532:3–16.
20. Yasunami Y, Lacy PE, Davie JM, et al. Prolongation of islet xenograft survival (rat to mouse) by in vitro culture at 37°C. *Transplantation.* 1983;35:281–284.
21. Meier JJ, Ritzel RA, Maedler K, et al. Increased vulnerability of newly forming beta cells to cytokine-induced cell death. *Diabetologia.* 2006;49:83–89.
22. Aikin R, Rosenberg L, Paraskevas S, et al. Inhibition of caspase-mediated PARP-1 cleavage results in increased necrosis in isolated islets of Langerhans. *J Mol Med.* 2004;82:389–397.
23. Bottino R, Balamurugan AN, Tse H, et al. Response of human islets to isolation stress and the effect of antioxidant treatment. *Diabetes.* 2004;53:2559–2568.
24. Benson RSP, Heer S, Dive C, et al. Characterization of cell volume loss in CEM-C7A cells during dexamethasone-induced apoptosis. *Am J Physiol Cell Physiol.* 1996;270:C1190–C1203.
25. Bianchi ME, Beltrame M, Paonessa G. Specific recognition of cruciform DNA by nuclear protein HMG1. *Science.* 1989; 243:1056–1059.
26. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature.* 2002;418:191–195.
27. Steer SA, Scarim AL, Chambers KT, et al. Interleukin-1 stimulates beta-cell necrosis and release of the immunological adjuvant HMGB1. *PLoS Med.* 2006;3(2):e17.
28. 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(3):735–743.
29. Sun X, Shimizu H, Yamamoto KI. Identification of a novel p53 promoter element involved in genotoxic stress-inducible p53 gene expression. *Mol Cell Biol.* 1995;15:4489–4496.
30. Shieh SY, Ikeda M, Taya Y, et al. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell.* 1997;91:325–334.
31. Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell.* 1997;88:323–331.
32. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. *Nature.* 2000;408:307–310.
33. Emamaullee JA, Shapiro AM. Interventional strategies to prevent β-cell apoptosis in islet transplantation. *Diabetes.* 2006;55:1907–1914.
34. Franke TF, Hornik CP, Segev L, et al. PI3K/Akt and apoptosis: Size matters. *Oncogene.* 2003;22:8983–8998.
35. El-Deiry WS, Tokino T, Velculescu VE, et al. WAF1, a potential mediator of p53 tumor suppression. *Cell.* 1993;75:817–825.
36. Harper JW, Adami GR, Wei N, et al. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G_1 cyclin-dependent kinases. *Cell.* 1993;75:805–816.
37. Storey KB, Storey JM. Tribute to P. L. Lutz: Putting life on 'pause'—molecular regulation of hypometabolism. *J Exp Biol.* 2007;210:1700–1714.
38. Kaufmann H, Mazur X, Fussenegger M, et al. Influence of low temperature on productivity, proteome and protein phosphorylation of CHO cells. *Biotechnol Bioeng.* 1999;63:573–582.
39. Moore A, Mercer J, Dutina G, et al. Effects of temperature shift on cell cycle, apoptosis and nucleotide pools in CHO cell batch cultures. *Cytotechnology.* 1997;23:47–54.
40. Hillion JA, Li Y, Maric D, et al. Involvement of Akt in preconditioning-induced tolerance to ischemia in PC12 cells. *J Cereb Blood Flow Metab.* 2006;26:1323–1331.

Cell Transplantation, Vol. 21, pp. 473–482, 2012
Printed in the USA.

0963-6897/12 $90.00 + .00
DOI: http://dx.doi.org/10.3727/096368911X605385
E-ISSN 1555-3892
www.cognizantcommunication.com

A Model to Evaluate Toxic Factors Influencing Islets During Collagenase Digestion: The Role of Serine Protease Inhibitor in the Protection of Islets

Manabu Tsukada,* Takuro Saito,* Kazuya Ise,* Akira Kenjo,*
Takashi Kimura,* Yoshihiro Satoh,* Takaharu Saito,* Takayuki Anazawa,*
Ikuro Oshibe,* Shigeya Suzuki,† Yasuhiro Hashimoto,‡ and Mitsukazu Gotoh*

*Department of Surgery, Fukushima Medical University, Fukushima City, Fukushima, Japan
†Research and Development Division, Kikkoman Co. Ltd, Noda City, Chiba, Japan
‡Department of Biochemistry, Fukushima Medical University, Fukushima City, Fukushima, Japan

The recovery of all of the islets contained in a pancreas is the goal of islet isolation for transplantation. This study reveals an environment that injures the isolated islets during digestion and proposes a new model for optimal islet isolation. Islets were isolated from Wistar rat pancreases by stationary collagenase digestion while the digestion time was varied at 15, 30, 60, and 120 min. The digested pancreas and islets were analyzed histologically and adenosine nucleotides were measured. Overnight cultured islets (40 islets) were cocultured for 30 min with the supernatants obtained from pancreatic collagenase digestion at different digestion periods in order to assess the toxic environment. The peak yields of islets were obtained at 30 min of digestion. The histological study of digested pancreas showed that the exocrine cells lost their cellular integrity at 120 min of digestion, but the islet cells were left intact. Accordingly, the ATP levels of the pancreatic tissue decreased during the digestion period. The coculture experiment demonstrated that the islets cultured with the supernatants from the collagenase digestion showed digestion time-dependent disruption of the cellular integrity of islets in accordance with a rapid decrease of ATP levels in the islets. The addition of serine protease inhibitors into this coculture clearly showed protection of islets, which maintained high ATP levels in association with intact membrane integrity as assessed by AO/PI staining. Morphological deterioration of islets as well as a marked ATP decrease was evident in the entire digested pancreas as well as in islets cocultured in the supernatants from the collagenase digestion. Various factors toxic to the islets can therefore be analyzed in future experiments using this coculture model for obtaining a good yield of viable islets.

Key words: Islet isolation; Collagenase digestion; Adenosine triphosphate (ATP); Trypsin; Pefabloc; Ulinastatin

INTRODUCTION

Islet isolation is a crucial step for islet transplantation (1,15). Although there have been substantial advances in islet isolation technology during the past decades (11, 20), it is still difficult to recover all of the islets contained in a pancreas (13). In fact, even in the leading centers, a transplantable yield of isolated islets is obtained from less than 50% of the processed pancreases (18,19). A major obstacle to successful human islet isolation has thus been the variability of the collagenase digestion phase of islet isolation (10).

Islet isolation involves an enzymatic and mechanical digestion to release islets from the surrounding exocrine tissue, followed by a separation procedure to purify the islets. The collagenase digestion is critical to islet isolation, although it is potentially harmful to the islets (10). However, no monitoring system has yet been established.

The content of adenosines reflects the viability in various organ transplants (6–8). Assessment of the levels of adenosine triphosphate (ATP) or the adenosine diphosphate (ADP)/ATP ratio is useful for prediction of transplantation outcome in islet transplantation (3). A new method was recently established for determination of adenosine levels in isolated islets (6,7). This method is a bioluminescent enzymatic cycling assay, which allows quick measurement in samples as small as a single islet (6,7). This method allows the measurement of

Received March 31, 2010; final acceptance July 18, 2011.
Address correspondence to Mitsukazu Gotoh, M.D., Ph.D., Department of Surgery 1, Fukushima Medical University, 1 Hikarigaoka, Fukushima 960-1295, Japan. Tel: 81-24-547-1254; Fax: 81-24-548-2735; E-mail: mgotoh@fmu.ac.jp

the levels of adenosines during the digestion phase not only in pancreatic tissue but also in isolated islets in experimental models using rats.

The collagenase digestion phase results in the release of endogenous pancreatic enzymes from the exocrine tissues (2,12,16,17,21–23). A strong increase of such enzyme activity, in particular serine proteases involved in the digestion process, is detrimental to the islet isolation outcome (2). However, the effect of the addition of serine protease inhibitors prior to digestion still remains controversial (12,16,17,21–23).

The aim of this study was to examine the changes in the energy status of islets during the digestion procedure, to elucidate the extracellular components of the digestive procedure as a supernatant to determine whether this supernatant is toxic to the islets and, if it is, whether a serine protease inhibitor could reduce the toxicity to the islets. This model provides a simple system to elucidate the mechanism of overdigestion and to select compounds for protecting islets during collagenase digestion.

MATERIALS AND METHODS

Animals

Wistar rats (8 weeks, male, provided by CLEA Japan, Inc., Tokyo, Japan) were used for islet isolation under anesthesia with ether inhalation. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Fukushima Medical University, and all procedures in this experiment were performed according to the National Research Council's guidelines.

Histological Study and Energy Levels of the Pancreatic Tissue During Collagenase Digestion

Pancreatic grafts were taken from Wistar rats and were digested by the stationary method using 2 mg/ml collagenase (collagenase S-1, Nitta Gelatin Inc., Osaka, Japan). Pancreatic tissue samples for histological study and measurement of adenosine levels were taken at 0, 15, 30, 60, and 120 min after starting the digestion. Samples were fixed in paraformaldehyde for a histological study. Samples were taken and stored in deep freezer until the adenosine levels were measured.

Assessment of the Toxicity of Digested Supernatant During Collagenase Digestion on Overnight Cultured Islets

Islets were isolated and purified from Wistar rats by the stationary digestion method as previously reported (4), and cultured in RPMI in a 37°C incubator overnight. The 40 islets were cultured for 30 min at 37°C in Hanks balanced salt solution (HBSS), HBSS with 2 mg/ml collagenase, and in supernatants obtained from the collagenase digestion at 15, 30, 60, and 120 min after starting digestion. After 30 min of incubation, these islets were collected by gentle centrifugation (500 rpm for 1 min) and used for the histological analysis and measurement of adenosines.

Histological Analysis of Islets

The cultured islets and the treated islets were transferred into 1.5-ml Falcon microtubes, and collected by centrifugation at 500 rpm for 1 min. These islets were then fixed in 4% paraformaldehyde for 1 day, dehydrated, and embedded in paraffin. The sections were stained with hematoxylin and eosin (H&E) staining. For the immunohisotological staining of insulin, purified rabbit anti-insulin (H-86) (Santa Cruz Biotechnology, Inc., CA, USA) was used. To detect the insulin molecules the sections were stained using a peroxidase-labeled biotin-avidin detection system Histofine® Simple Stain Rat MAX PO(R) according to the instructions provided by the manufacturer (Nichirei Biosciences Inc. Tokyo, Japan).

Measurement of Adenosine Nucleotides (ANs)

Adenosine nucleotides were measured by the bioluminescence method using luciferase, as provided by Kikkoman Corp., Chiba, Japan (24) and the luminescence Mithras LB940 photon counter (Berthold Technologies GmbH & Co KG, Bad Wildbad, Germany). This method provides rapid results and has sufficient sensitivity to detect the ATP content even in one islet cell (6,7). ATP, ATP + adenosine monophosphate (AMP), and total adenosine nucleotide (TAN) content were measured by each reagent individually. Energy charge (EC) was calculated by obtaining the contents of ADP and AMP by subtraction of each of the measurable components. Twenty microliters of each sample was diluted with 80 µl of Tris buffer and 100 µl reagent was added immediately before the assay. It took 10 s to react with the ANs and the luminescence remained stable for 2 min. In the case of pancreatic tissue, samples were added with 0.5 N $HClO_4$ and thoroughly homogenized for 30 s. Next, it was centrifuged for 5 min at 15,000 rpm and then the supernatant was taken and stored in liquid nitrogen. Samples were transferred and stored in a deep freezer until processed. It was diluted 1000 times and measured. Islets were placed in 500 µl of culture medium plus 55 µl of 0.5 N $HClO_4$ for freezing at –80°C until processed. After centrifugation, the samples were assayed without dilution.

Measurement of Trypsin Activity

Aliquots of supernatant from the warm digested pancreatic tissue at 15, 30, 60, and 120 min after stationary

digestion were collected and frozen until the measurement of trypsin activity. The trypsin activity was measured by absorption spectrophotometry (λ405 nm; Model 680 Series Microplate Readers Bio-Rad Laboratories, CA, USA) using the trypsin substrate kit containing carbobenzoxy-glycyl-D-alanine-*p*-nitroanilide-acetate, SPECTROZYME®TRY (American Diagnostica Inc., Stamford, CT, USA). The assay was calibrated using dilutions of a stock solution of trypsin from the bovine pancreas (Sigma-Aldrich Co., St. Louis, MO, USA) in 200 µl of 50 mM Tris buffer at 37°C to generate a standard curve. Absorbance was measured at 1, 5, and 10 min and the trypsin activity was calculated to compare it with the standard curve.

Evaluation of the Effect of Serine Protease Inhibitors on Isolated Islets in a Toxic Environment During Collagenase Digestion

Overnight cultured islets were cultured for 30 min at 37°C in HBSS, HBSS with 2 mg/ml collagenase, and supernatants obtained from collagenase digestion at 15, 30, 60, and 120 min after starting of digestion. To assess the protective effect of serine protease inhibitor on islets in the presence of digested supernatant, Pefabloc (Pefabloc SC PLUS, Roche Diagnostics Co., Mannheim, Germany) or ulinastatin (MIRACLID Inc, Mochida Pharma., Tokyo, Japan) were added to the culture media. Pefabloc was used at 0.4 mM, which is the recommended concentration (21), and ulinastatin was added at a dose of 100 U/ml according to a previous report (17). After 30 min of incubation, these islets were collected by centrifugation and used for histological analysis and measurement of adenosines. The viability of islet cells was assessed by simultaneous use of the inclusion and exclusion dyes acridine orange (AO) and propidium iodide (PI), as described previously (5). Briefly, 40 islets were cultured with 0.67 µM AO and 75 µM PI for 30 min in HBSS or supernatants obtained after 15, 30, 60, and 120 of digestion. Next, the islets were examined by confocal microscopy (model FV300, Olympus, Japan). Fluorescent images were obtained under a fluorescent microscope (Eclipse E800, Nikon, Japan) and a confocal laser microscope (FV300, Olympus, Japan). A fluorescent illuminator consisting of a 100-W mercurial light source with a 490-nm excitation filter and a 510-nm barrier filter was used. This filter combination permits the simultaneous visualization of the green emission of AO and the red emission of PI.

Statistical Analysis

Data are expressed as the means ± SD. The ATP contents of pancreatic tissue and islets were compared using analysis of variance (ANOVA) and unpaired Student's *t*-test with Bonferroni correction. Values of $p < 0.05$ were considered to be statistically significant. All statistical analyses were performed using the Stat-view J 4.58 for Windows software package (Abacus Concepts Inc., Berkeley, CA, USA).

RESULTS

Islet Yield and Adenosine Content in Relation to the Digestion Period

The islet yields per rodent after 30, 60, and 120 min of stationary digestion method using collagenase were 1304 ± 409, 534 ± 156, and3.3 ± 0.9 islet equivalents (IEQ), respectively ($n = 3$), thus showing a significant difference between 30, 60, and 120 min of digestion period ($p < 0.05$). The ATP content of these islets, after 30, 60, and 120 min of stationary digestion, were 18.3 ± 0.2, 7.0 ± 0.5, and 3.5 ± 0.8 µM, respectively, also showing a significant difference between each digestion period ($p < 0.05$). Therefore, the islet yield and ATP content in the overdigestion period, 60 and 120 min of digestion, revealed a significantly low level in comparison to 30 min of digestion.

Histological Changes in Pancreas During Collagenase Digestion

The interstitial space of the pancreatic tissue after collagenase digestion was wider than that of the pancreatic tissue before distension, and the islets were separated from exocrine tissue after collagenase digestion. There were clearly many lobular structures in the pancreatic tissue after digestion. Half of the lobular structures were broken at 15 min of digestion and almost all disappeared after 30 min of digestion with the presence of islets free from the exocrine tissue. No lobular structures with destruction of exocrine tissue were obvious after 60 min of digestion. The densely stained nuclei and cytoplasmic swelling of exocrine cells were evident with significant damage to the surface of islets. Finally, the acinar cells were complete free from the lobar structures and islets were also disrupted after 120 min of digestion (Fig. 1).

Changes of ATP, TAN Content, and EC in the Pancreas During Collagenase Digestion

The ATP contents of the pancreatic tissue decreased during collagenase digestion, showing significant differences before and after digestion [ATP content before digestion: 1599 ± 431, 15 min of warm digested tissue (WDT): 238 ± 114, 30 min of WDT: 109 ± 27, 60 min of WDT: 72 ± 22, 120 min of WDT: 38 ± 23 µM, $p < 0.01$]. During digestion, ATP content after 120 min of WDT was significantly lower than that of 15 min and 30 min of WDT ($p < 0.05$) (Fig. 2a).

The TAN content failed to show a significant difference between pancreatic tissue before digestion and 15

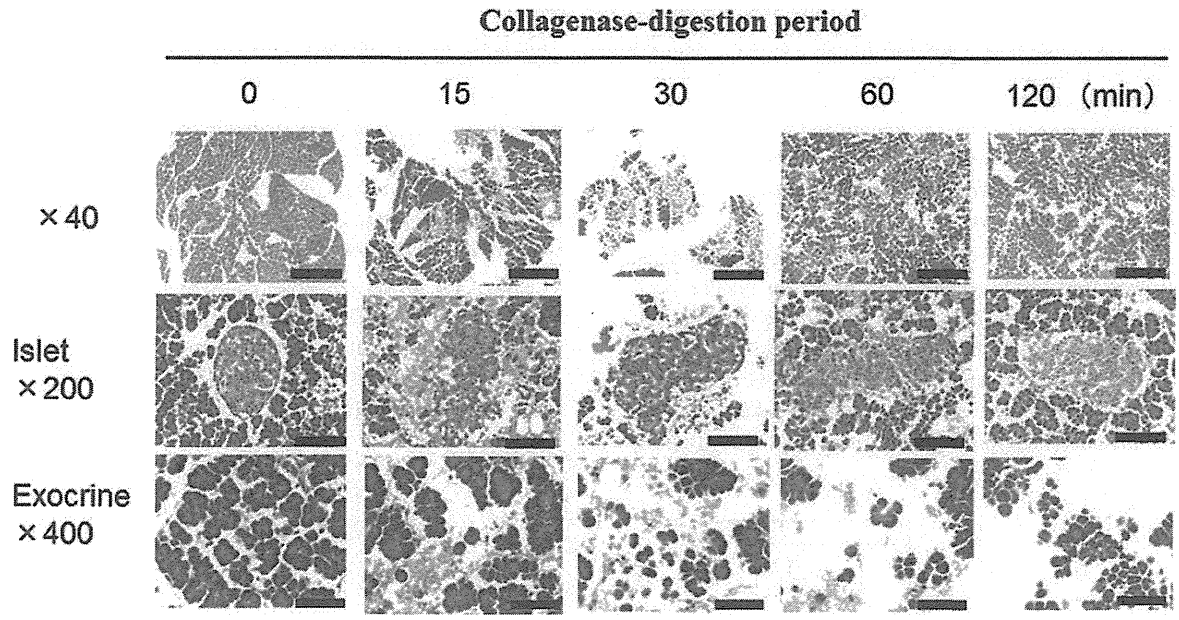


Figure 1. Histological changes in the pancreas during collagenase digestion. Hematoxylin and eosin staining of paraffin-embedded pancreatic tissue of Wister rat. Pancreata were distended through pancreatic duct by collagenase solution and digested for 15, 30, 60, and 120 min (40×, 200×, and 400× magnification, respectively). The lobular structures disappeared after 30 min of digestion with the presence of islets free from the exocrine tissue. The acinar cells were complete free from the lobar structures and the islets remained relatively intact after 120 min of digestion. Scale bars: 500 μm (top row), 100 μm (middle row), 50μm (bottom row).

min of WDT, but TAN content after 30, 60, and 120 min of WDT was significantly lower than that before digestion (TAN content before digestion: 3106 ± 194, 15 min of WDT: 2411 ± 419, 30 min of WDT: 1978 ± 199, 60 min of WDT: 990 ± 67, 120 min of WDT: 253 ± 42 μM, $p < 0.01$). The TAN contents revealed significant differences during digestion between 30, 60, and 120 min of WDT ($p < 0.01$), respectively (Fig. 2b).

The EC of the pancreatic tissue decreased during collagenase digestion showing a significant difference between before and after digestion (EC before digestion: 0.58 ± 0.06, 15 min of WDT: 0.16 ± 0.04, 30 min of WDT: 0.11 ± 0.10, 60 min of WDT: 0.14 ± 0.09, 120 min of WDT: 0.32 ± 0.04, $p < 0.01$) (Fig. 2c).

Changes in the Trypsin Activity in Supernatants of Digested Fluid During Collagenase Digestion

Endogenous enzymes were thought to induce a toxic environment during the digestion phase; thus, trypsin was selected as one of the factors that damaged the pancreatic tissue. Trypsin activity of digestion supernatant increased time dependently with the progression of digestion. The trypsin activities at 15, 30, 60, and 120 min were 339 ± 66.8, 795 ± 371.8, 1504 ± 257.4, and 1545 ± 800.2 U/ml, respectively, thereby showing significant differences between 15 and 60 min, and between 15 min and 120 min (Fig. 3).

Evaluation of Effect of the Serine Protease Inhibitors on the Morophological Changes of Isolated Islets in a Toxic Environment During Collagenase Digestion

The islets were used after an overnight culture in RPMI. Forty islets were cultured in HBSS or the digestion supernatants after 15, 30, 60, and 120 min of digestion for 30 min, with or without serine protease inhibitors to assess the effect of serine protease inhibitors. The islets cultured without serine protease inhibitors served as the control group.

Histologically, the islets in the control group showed limited changes in cultured with 15 and 30 min of digestion supernatant, but evident in cultured with 60 and 120 min of digestion supernatant, as described above (Fig. 4a). In contrast, the islets cultured with serine proteases (both Pefabloc and ulinastatin) showed very limited damage to their surface when cultured with 15, 30, and 60 min of digestion supernatant. Moreover, only limited damage to the islets with serine proteases was observed and they maintained their integrity even when cultured with a digestion supernatant for 120 min. An immunohistological study using anti-insulin antibody revealed diffusely positive cells for anti-insulin antibody within the islets, and almost the same staining level of insulin was observed regardless of whether or not serine proteases were added when cultured for 15, 30, and 60 min in the digestion supernatant. Only limited damage to the

islets with serine proteases was also observed when cultured with a digestion supernatant for 120 min, and the insulin positive cells in the islets with serine proteases also maintained their integrity (Fig. 4b).

Changes of ATP Content in Isolated Islets Cultured With Digestion Supernatant During Collagenase Digestion

The ATP levels of 40 islets cultured with collagenase decreased, but failed to show a significant difference in comparison to HBSS alone. In contrast, the ATP content of the islets decreased in accordance with the period of digestion showing a significant decrease when cultured with the pancreas digestion supernatant taken from 15, 30, 60, and 120 min of digestion in comparison to either HBSS or collagenase (ATP content in HBSS: 0.32 ± 0.05 μM, in collagenase: 0.24 ± 0.04 μM, in 15-min digested fluid: 0.17 ± 0.03 μM, 30-min digested fluid: 0.13 ± 0.02 μM, 60-min digested fluid: 0.07 ± 0.02 μM, and 120-min digested fluid: 0.03 ± 0.02, $p < 0.01$) (Fig. 5).

Evaluation of Effect of the Serine Protease Inhibitors on the ATP Content, AO/PI Staining of Isolated Sslets in a Toxic Environment During Collagenase Digestion

The ATP levels of these islets were measured, and expressed as the "ATP content ratio," which was calculated by the formula: ATP contents ratio = measured ATP content of islets/ATP content of islets cultured in HBSS for 30 min (Fig. 6). The ATP content ratio of islets in the control group decreased significantly after

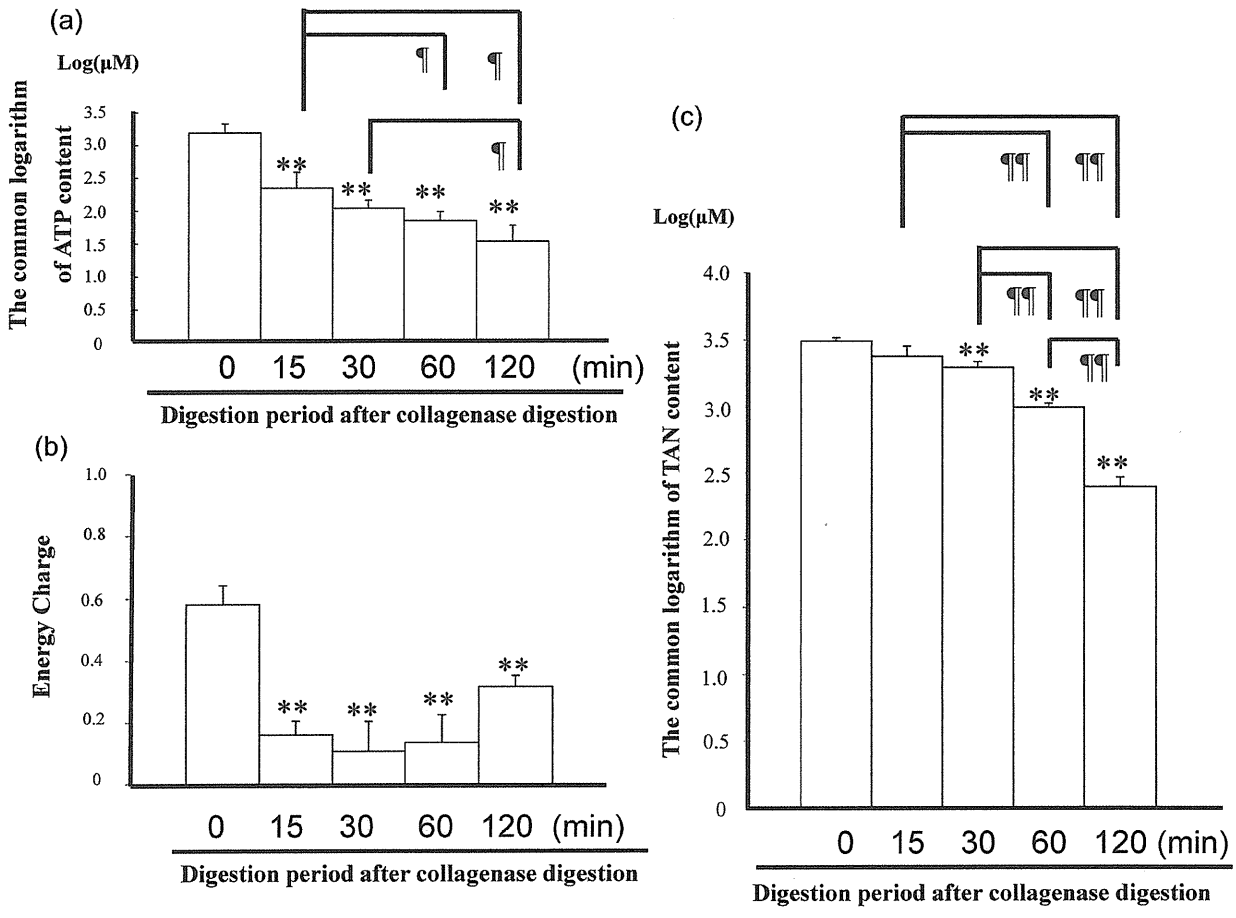


Figure 2. (a) Changes of adenosine triphosphate (ATP) content in pancreas during collagenase digestion ($n = 3$). ATP contents were adjusted by a common logarithm. The ATP contents of the pancreatic tissue decreased during collagenase digestion showing significant differences before and after digestion. **Significant difference in comparison to 0 min, $p < 0.01$. ¶Significant difference between digestion periods, $p < 0.05$. (b) Changes of total adenine nucleotide (TAN) content in the pancreas during collagenase digestion ($n = 3$). TAN contents were adjusted by the common logarithm. The TAN contents of pancreatic tissue revealed significant decrease during digestion periods. **Significant difference in comparison to 0 min, $p < 0.01$. ¶¶Significant difference between digestion periods, $p < 0.01$. (c) Changes of energy charge (EC) in the pancreas during collagenase digestion ($n = 3$). Time means digestion period after distension. The EC of the pancreatic tissue decreased during collagenase digestion. **Significant difference in comparison to 0 min, $p < 0.01$.

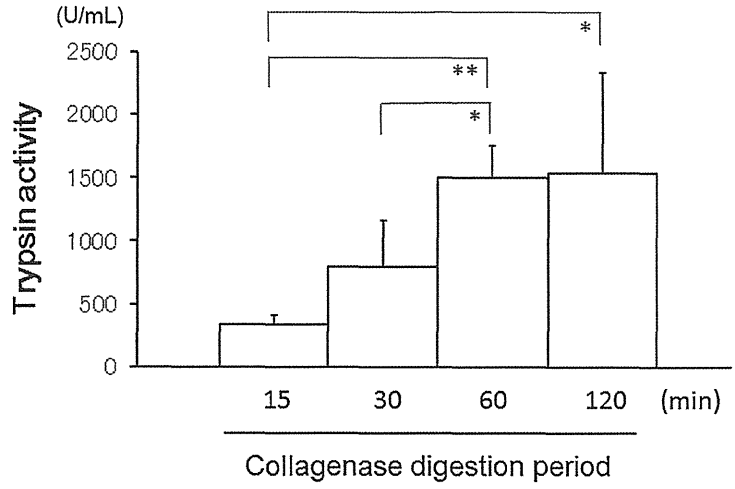


Figure 3. The trypsin activity in the supernatant of warm digested pancreatic tissue ($n = 4$). The trypsin activity of the digestion supernatant increased time dependently with the progression of digestion. Significant difference between the digestion periods: $*p < 0.05$, $**p < 0.01$.

culture with 15-, 30-, 60-, and 120-min digestion supernatant. In contrast, the ATP content ratio in the presence of Pefabloc or ulinastatin maintained the same level as that before digestion in the islets cultured for 15 min. Moreover, the ratio of islets with Pefabloc maintained the level even in islets cultured with 30 min digestion supernatant, thus showing a significant difference in comparison to the control islets cultured for the same period.

The observation of the islets using confocal microscopy with AO/PI revealed that almost all of the islets in the control group stained with PI at the end of 30-min culture (Fig. 7). In contrast, the majority of islets cultured in the presence of Pefabloc or ulinastatin were stained with AO and showed a "green color," but they were not stained with PI, thus suggesting the protective effects of Pefabloc and ulinastatin against lethal damage.

The islet yields after 30 min of stationary digestion without serine protease, with Pefabloc or ulinastatin were 1304 ± 409, 1019 ± 259, and 851 ± 686 IEQ, respectively, thus showing no significant difference. However, the ATP content of these islets digested without serine protease, with Pefabloc or Ulinastatin was 1.8 ± 0.1, 2.3 ± 0.2, and 2.4 ± 0.4 μM, respectively, thus showing a significant difference between those islets without serine protease and those with Pefabloc ($p < 0.05$).

DISCUSSION

The procedure of human islet isolation is complex and it requires a great deal of experience to yield sufficient islets for transplantation (1,15). The technique of islet isolation has advanced (10,11,13,18–20). A tissue dissociation chamber and recirculation of enzyme solution established by Ricordi et al., and purified enzyme, Liberase HI, has been used worldwide (11,13,18–20). Although substantial advances in islet isolation technology have been achieved, it is still difficult to recover all of the islets contained in a pancreas. Many factors influencing the collagenase digestion phase have been reported (10), but little is known about the morphological changes and energy levels of pancreatic grafts or islet cells during digestion. This study focused on the digestion phase of islet isolation and demonstrated a reduction of ATP and EC levels of pancreatic graft during the digestion phase, and established an evaluation system of the toxicity of digestion supernatant involving endogenous pancreatic enzymes against isolated islets. Moreover, the effect of serine protease inhibitors has also been clarified using this newly established system.

The current study confirmed the decrease of islet yield after over digestion of the pancreatic graft, which was consistent with previously reports (9,25). Morphological changes in the islets were commonly observed in the peripheral cells of the isolated islets during isolation, including breakage of the plasma membrane and loss of cells (14). Secondly, islet fragmentation and disintegration was observed after 60 and 120 min of collagenase digestion. These changes are caused by the toxic environment during digestion phase, including elevation of endogenous pancreatic enzymes (2,10,12,17,21–23). In fact, the trypsin activity was observed to increase with the progression of digestion.

The ATP contents of pancreatic grafts or islets are used to evaluate the viability of the graft or isolated

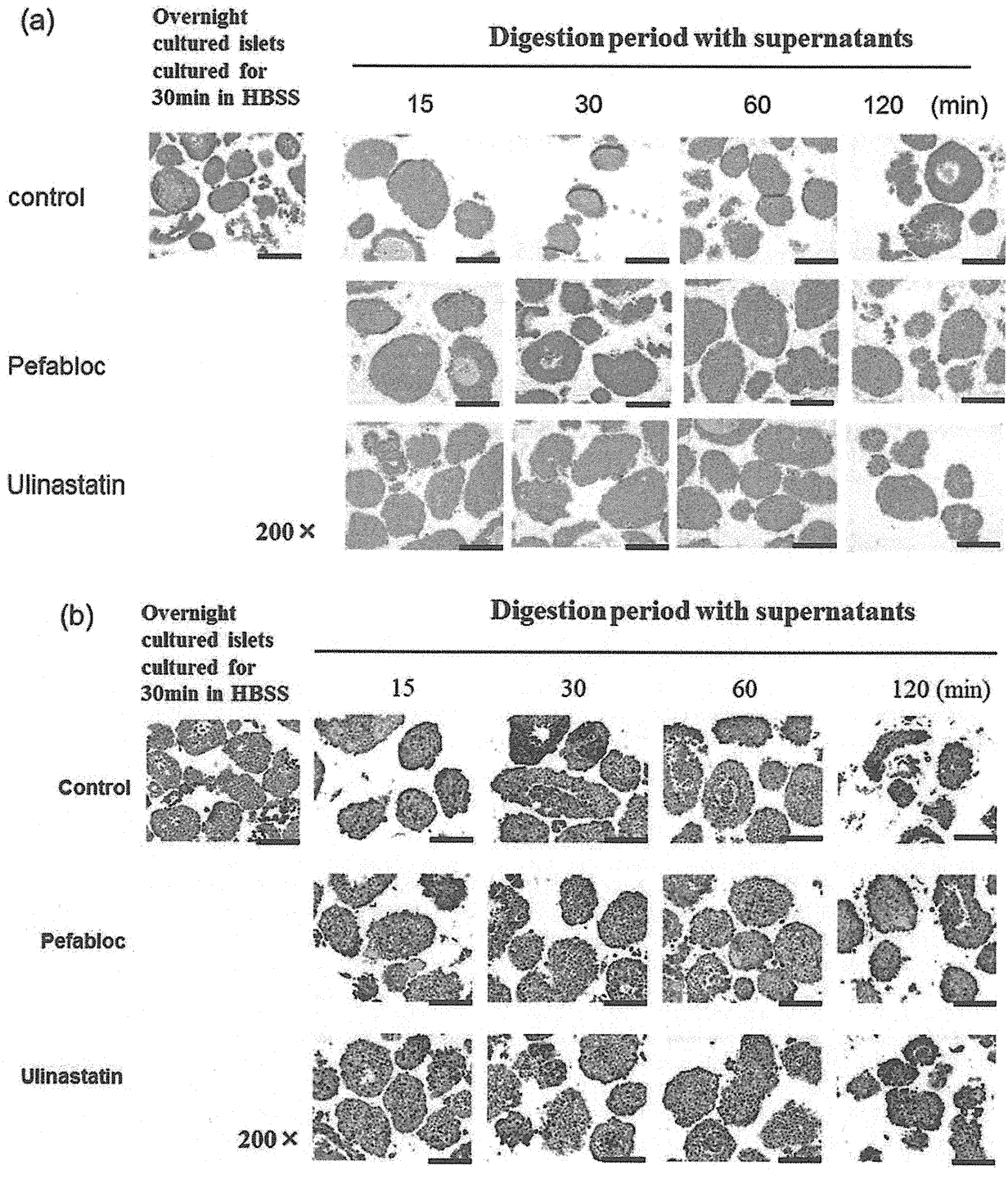


Figure 4. (a, b) Histological finding of isolated islets cultured with supernatants of digestion supernatant during collagenase digestion for 30 min, with or without serine protease inhibitors. H/E staining of paraffin-embedded pancreatic islets of Wister rat (a). Islets were cultured overnight and cultured in digestion supernatant for 30 min at 37°C, with or without serine proteases, Pefabloc or ulinastatin. The islets cultured with pancreatic digestion supernatant showed prominent damage leading to disruption of the cellular integrity of the islets in the control group. The islets cultured with serine proteases (both Pefabloc and ulinastatin) showed very limited damage to their surface compared to the control group. The immunohistological findings of the pancreatic islets of Wister rat using anti-insulin antibody (b) were similar to those of H/E staining. Scale bars: 100 μm.

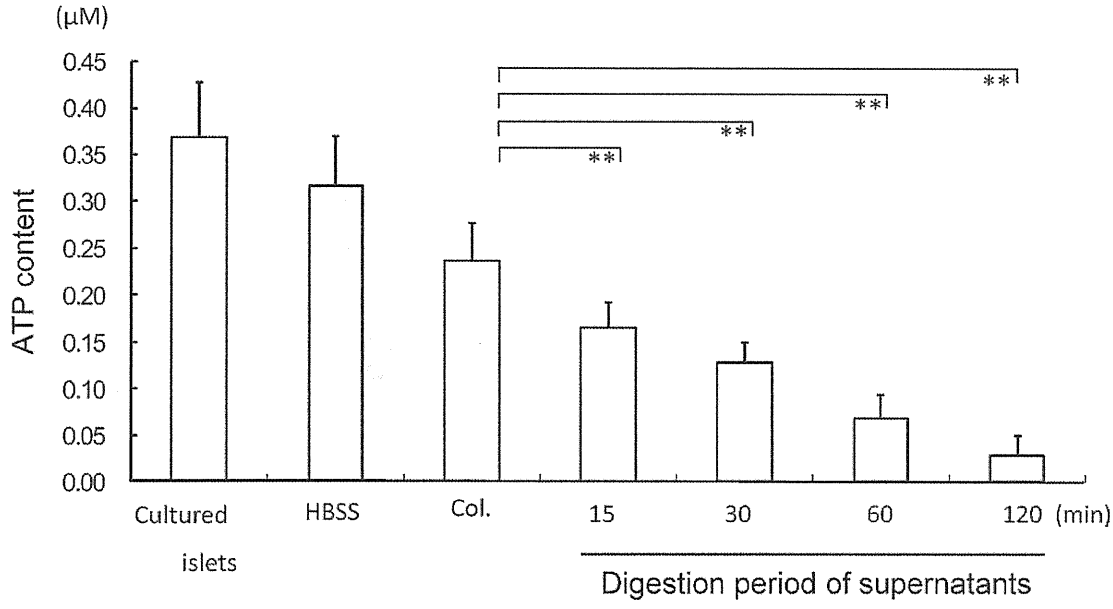


Figure 5. ATP content of 40 islets cultured with digestion supernatants during collagenase digestion for 30 min ($n = 3$). The ATP levels of 40 islets cultured with pancreas digestion supernatant decreased in a time-dependent fashion. Significant difference between digestion periods: $*p < 0.05$, $**p < 0.01$.

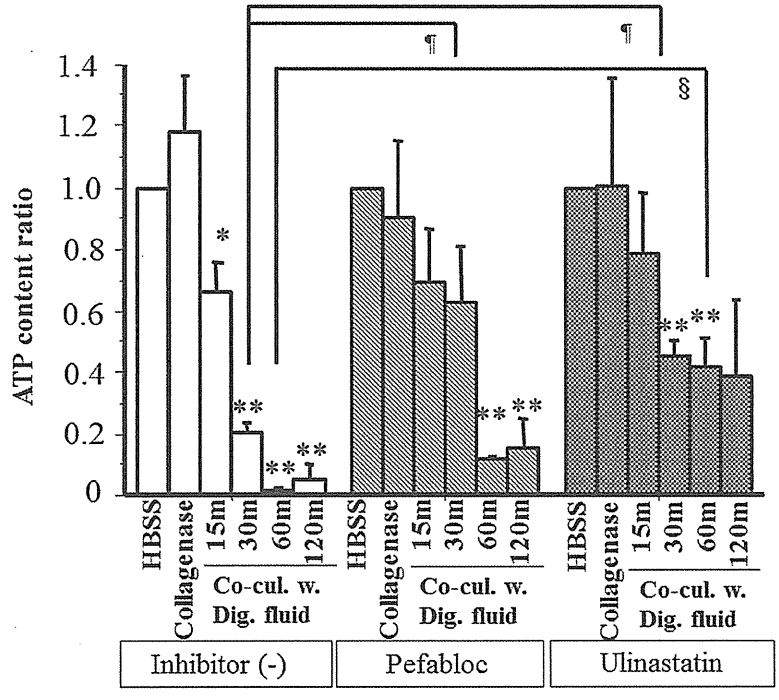


Figure 6. The ATP content of 40 islets cultured in digestion supernatants during collagenase digestion for 30 min, with or without serine protease inhibitors. Significant difference between digestion periods: $*p < 0.05$, $**p < 0.01$. ¶Significant difference in comparison to islets cultured with 30-min digestion supernatants in the control group, $p < 0.05$. §Significant difference in comparison to the islets cultured with 60-min digestion supernatants in the control group, $p < 0.05$. The ATP content ratio in the presence of Pefabloc or ulinastatin was maintained at relatively high levels in comparison to the control group.

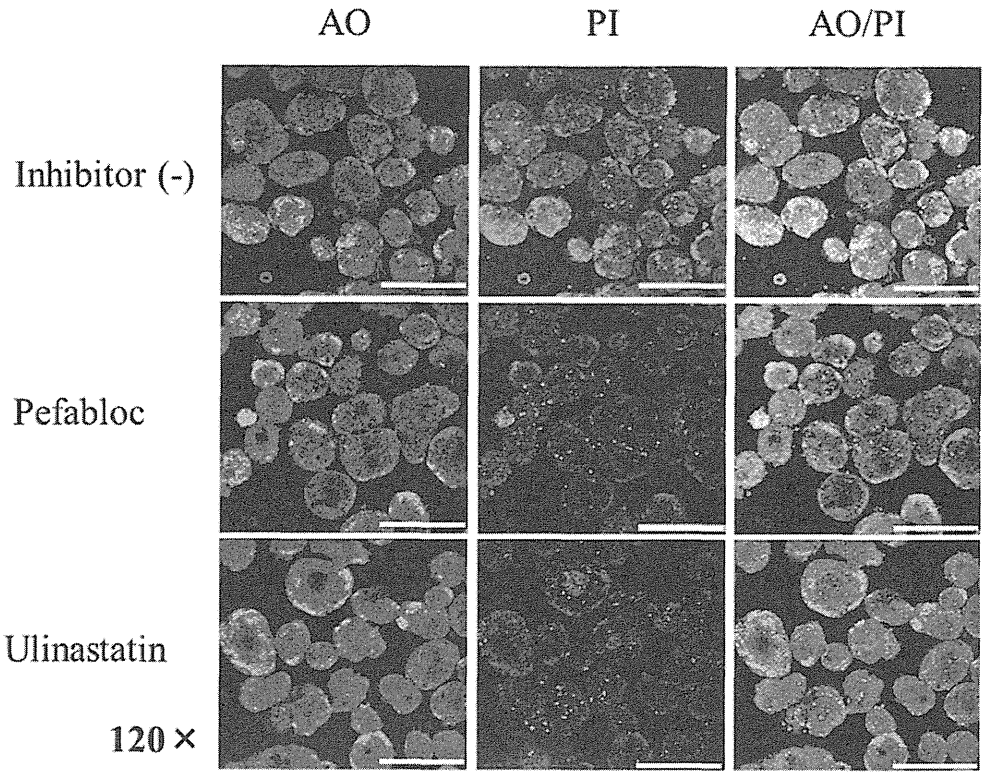


Figure 7. Histological findings of AO/PI staining observed by confocal microscopy for isolated islets cultured with digestion supernatants during collagenase digestion for 30 min, with or without serine protease inhibitors. The islets were cultured overnight and cultured in the supernatant for 30 min at 37°C in a culture dish. A green color after acridine orange (AO) staining means viable cells, while a red color of propidium iodide (PI) staining means dead cells. A yellow color after AO/PI indicates the presence of damaged and dead cells. The observation of the islets using confocal microscopy with AO/PI revealed the protective effects of Pefabloc and ulinastatin against lethal damage. Scale bars: 200 μm.

islets (3,6–8). However, little is known about the ATP or adenosine levels during the digestion phase. This study showed that the ATP and TAN contents of pancreatic tissue significantly decreased during collagenase digestion from 15 min after starting the stationary digestion, when histological damage to the exocrine tissue was evident, but such damage to the islets was limited.

Histological damage to the exocrine tissue and islets was evident at 60 min after starting digestion. Therefore, ATP and TAN contents in digesting pancreatic grafts could be more sensitive and quantitative markers than the histological findings.

Isolated islets were cultured with supernatants obtained from various digestion periods. The histology of islets cultured with these fluids clearly showed a time-dependent disruption of the cellular integrity of islets. Furthermore, the levels of ATP of islets cultured for 30 min in the digestion supernatants were decreased with the length of digestion periods, indicating the detrimental effects of endogenous enzymes to the isolated islets.

The trypsin activity of the supernatants obtained from collagenase digested tissue was observed to increase with the progression of digestion, thus indicating the leakage of various endogenous enzymes including serine proteases from the digested exocrine tissue. An increase of such enzyme activity is harmful for islet isolation (2,10,12,17,21–23). However, the effect of inhibition of serine proteases on islet isolation still remains controversial (12,17,21–23).

The protease inhibitors, Pefabloc and ulinastatin, were added to islets cultured with supernatants obtained from collagenase digested tissue at various digestion periods, and these inhibitors clearly prevented the occurrence of histological damage to the islets. Moreover, these inhibitors maintained the energy levels of islets under such conditions. Finally, the assessment of the viability using confocal microscopy and staining of AO/PI indicated the maintenance of membrane integrities in the presence of these inhibitors. Therefore, the protective effect of Pefabloc and ulinastatin during the digestion

phase was evident. This islet culture system might therefore be useful for evaluating the direct effect of drugs on islets during the digestion phase in the isolation of islet cells.

ACKNOWLEDGMENTS: This work was supported in part by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology and in part by a Grant-in-Aid for Research on Human Genome, Tissue Engineering Food Biotechnology, Health Sciences Research Grants, Ministry of Health, Labor and Welfare of Japan and in part by a research grant from Fukushima Medical University. The reagents of adenosine nucleotides luciferase were provided by Kikkoman Corp. The authors also wish to thank Dr. Yuichi Endo (Immunology), and Dr. Masaya Yamamoto and Dr. Satoshi Waguri (Histology) for their valuable technical advice. The authors declare no conflict of interest.

REFERENCES

1. Brandhorst, D.; Brandhorst, H.; Hering, B. J.; Federlin, K.; Bretzel, R. G. Islet isolation from the pancreas of large mammals and humans: 10 years of experience. Exp. Clin. Endocrinol. Diabetes 103(Suppl. 2):3–14; 1995.
2. Brandhorst, H.; Friberg, A.; Andersson, H. H.; Felldin, M.; Foss, A.; Salmela, K.; Lundgren, T.; Tibell, A.; Tufveson, G.; Korsgren, O.; Brandhorst, D. The importance of tryptic-like activity in purified enzyme blends for efficient islet isolation. Transplantation 87:370–375; 2009.
3. Goto, M.; Holgersson, J.; Kumagai-Braesch, M.; Korsgren, O. The ADP/ATP ratio: A novel predictive assay for quality assessment of isolated pancreatic islets. Am. J. Transplant. 6:2483–2487; 2006.
4. Gotoh, M.; Maki, T.; Kiyoizumi, T.; Satomi, S.; Monaco, A. P. An improved method for isolation of mouse pancreatic islets. Transplantation 40:437–438; 1985.
5. Gunji, T.; Saito, T.; Sato, Y.; Matsuyama, S.; Ise, K.; Kimura, T.; Anazawa, T.; Gotoh, M. Mitomycin-C treatment followed by culture produces long-term survival of islet xenografts in a rat-to mouse model. Cell Transplant. 17:619–629; 2008.
6. Ishii, S.; Saito, T.; Ise, K.; Sato, Y.; Tsutiya, T.; Kenjo, A.; Kimura, T.; Anazawa, T.; Suzuki, S.; Terashima, M.; Gotoh, M. Evaluation of energy state of islet independent of size using a newly developed ATP bioluminescence assay. Transplant. Proc. 37:3499–3500; 2005.
7. Ishii, S.; Sato, Y.; Terashima, M.; Saito, T.; Suzuki, S.; Murakami, S.; Gotoh, M. A novel method for determination of ATP, ADP, and AMP contents of a single pancreatic islet before transplantation. Transplant. Proc. 36: 1191–1193; 2004.
8. Kim, J. H.; Park, S. G.; Lee, H. N.; Lee, Y. Y.; Park, H. S.; Kim, H. I.; Yu, J. E.; Kim, S. H.; Park, C. G.; Ha, J.; Kim, S. J.; Park, K. S. ATP measurement predicts porcine islet transplantation outcome in nude mice. Transplantation 87:166–169; 2009.
9. Kim, S. C.; Han, D. J.; Kang, C. H.; We, Y. M.; Back, J. H.; Kim, Y. H.; Lim, D. G. Experimental islet isolation in porcine pancreas with new enzyme Liberase PI. Transplant. Proc. 36:2197–2199; 2004.
10. 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.
11. Lake, S. P.; Bassett, P. D.; Larkins, A.; Revell, J.; Walczak, K.; Chamberlain, J.; Rumford, G. M.; London, N. J.; Veitch, P. S.; Bell, P. R.; James, R. F. L. Large-scale purification of human islets utilizing discontinuous albumin gradient on IBM 2991 cell separator. Diabetes 38(Suppl. 1):143–145; 1989.
12. Lakey, J. R.; Helms, 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.
13. Mahler, R.; Franke, F. E.; Hering, B. J.; Brandhorst, D.; Brandhorst, H.; Brendel, M. D.; Federlin, K.; Schulz, A.; Bretzel, R. G. Evidence for a significant correlation of donor pancreas morphology and the yield of isolated purified human islets. J. Mol. Med. 77:87–89; 1999.
14. 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.
15. Nano, R.; Clissi, B.; Melzi, R.; Calori, G.; Maffi, P.; Antonioli, B.; Marzorati, S.; Aldrighetti, L.; Freschi, M.; Grochowiecki, T.; Socci, C.; Secchi, A.; Di Carlo, V.; Bonifacio, E.; Bertuzzi, F. Islet isolation for allotransplantation: Variables associated with successful islet yield and graft function. Diabetologia 48:906–912; 2005.
16. 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.
17. Noguchi, H.; Ueda, M.; Hayashi, S.; Kobayashi, N.; Nagata, H.; Iwanaga, Y.; Okitsu, T.; Matsumoto, S. Comparison of M-Kyoto solution and histidine-tryptophan-ketoglutarate solution with a trypsin inhibitor for pancreas preservation in islet transplantation. Transplantation 84: 655–658; 2007.
18. O'Gorman, D.; Kin, T.; Murdoch, T.; Richer, B.; McGhee-Wilson, D.; Ryan, E. A.; Shapiro, J. A.; Lakey, J. R. The standardization of pancreatic donors for islet isolations. Transplantation 80:801–806; 2005.
19. Ponte, G. M.; Pileggi, A.; Messinger, S.; Alejandro, A.; Ichii, H.; Baidal, D. A.; Khan, A.; Ricordi, C.; Goss, J. A.; Alejandro, R. Toward maximizing the success rates of human islet isolation: Influence of donor and isolation factors. Cell Transplant. 16:595–607; 2007.
20. Ricordi, C.; Lacy, P. E.; Scharp, D. W. Automated islet isolation from human pancreas. Diabetes 38(Suppl. 1): 140–142; 1989.
21. Rose, N. L.; Palcic, M. M.; Helms, L. M.; Lakey, J. R. Evaluation of Pefabloc as a serine protease inhibitor during human-islet isolation. Transplantation 75:462–466; 2003.
22. Rose, N. L.; Palcic, M. M.; Lakey, J. R. Evaluating the effect of serine proteases on collagenase activity during human islet isolation. Cell Transplant. 11:821–826; 2002.
23. Rose, N. L.; Palcic, M. M.; Shapiro, A. M.; Lakey, J. R. Endogenous pancreatic enzyme activity levels show no significant effect on human islet isolation yield. Cell Transplant. 13:153–160; 2004.
24. Sakakibara, T.; Murakami, S.; Imai, K. Enumeration of bacterial cell numbers by amplified firefly bioluminescence without cultivation. Anal. Biochem. 312:48–56; 2003.
25. Schwartz, B. D.; Traverso, L. W. Morphological changes in pancreatic fragments prepared for transplantation by collagenase treatment. Transplantation 38:273–280; 1984.