Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

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Ishii S, Saito T, Ise K,Yamashita M, Sato Y, Saito T, Tsukada M, Oshibe I, Kenjo A, Kimura T, Anazawa T, Suzuki S, Gotoh M.	Preservation of pancreatic islets in cold UW solution before transplantation.	Islets.	4(1)	32-39	2012
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Ⅳ. 研究成果の刊行物・別刷

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Preservation of pancreatic islets in cold UW solution before transplantation

Show Ishii,[1] Takuro Saito,[1] Kazuya Ise,[1] Michitoshi Yamashita,[1] Yoshihiro Sato,[1] Takaharu Saito,[1] Manabu Tsukada,[1] Ikuro Oshibe,[1] Akira Kenjo,[1] Takashi Kimura,[1] Takayuki Anazawa,[1] Shigeya Suzuki[2] and Mitsukazu Gotoh[1,*]

[1]Department of Surgery; Fukushima Medical University; Fukushima City, Fukushima, Japan; [2]Research and Development Division; Kikkoman Co., Ltd; Noda City, Chiba, Japan

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Culture of islets prior to transplantation needs to be revisited for maintaining functional islet capacity. This study was conducted to compare cold UW (University of Wisconsin) preservation with conventional culture based on insulin secretory capacity in vitro and in vivo. Islets isolated from Wistar rats were either cultured for 24 h at 37°C in RPMI1640 medium or DMEM containing various concentrations of glucose or preserved for the same period in UW solution or in DMEM solution at 4°C. The islet yield in UW group, but not in other groups, was maintained as comparable with that of fresh islets. Insulin secretory capacity in response to glucose was maintained only in the islets of UW group, but not in other groups. SCID mice given 300 IEQ islets of UW group showed gradual restoration of normoglycemia as found in the mice given freshly isolated islets. Meanwhile, those mice given cultured islets for 24 h at 37°C in RPMI1640 medium showed rapid decrease of blood glucose levels on day 1 followed by relatively elevated levels on day 2, suggesting unstable insulin secretory capacity of islets.

Morphological staining with anti-HMGB1 (high mobility group B1) antibody revealed central damage of islets in all culture groups regardless of glucose concentration and in islets of cold DMEM group, whereas those in the UW group were quite intact. These results suggest that cold preservation in UW solution is simple and beneficial in protecting islets morphologically and functionally before transplantation.

Introduction

Islet transplantation has become a viable treatment option for type 1 diabetes mellitus after the Edmonton group published their encouraging clinical report.[1,2] Although insulin independence is maintained in 82% of patients at 1 y post-transplant, this ratio declines thereafter.[2,3] The suggested reasons for this decline include alloimmune rejection, autoimmune recurrence and the toxicity of immunosuppressive medications.[4,5] However, these factors might be ameliorated by refined immunosuppressive protocols.

Immunosuppression for islet transplantation in the Edmonton protocol consists of daclizumab for induction therapy and sirolimus plus low-dose tacrolimus for maintenance therapy.[1-3] However, both sirolimus and tacrolimus inhibit β-cell regeneration, presenting a potential post-transplant disadvantage.[5] Thus, a new immunosuppression protocol has been developed including T-cell directed antibodies for induction therapy with or without a tumor necrosis factor-α (TNF-α) blockade.[6-9] These studies showed significant effects, but several hours or days of induction therapy are required before islets can be transplanted.[6-9] Here, we examined the viability of islets after culture or cold preservation using insulin secretory capacity in vitro as a marker.

The effect of islet culture after isolation remains controversial. Short-term culture is considered disadvantageous for transplantation because the number of islets is reduced, and the expression of tissue factors that lead to an instant blood mediated inflammatory reaction (IBMIR) is enhanced.[10-13] However, reducing the immunogenicity of passenger leukocytes and the recovery of islet function might positively impact the outcome of islet transplantation.[11-16] On the other hand, Delfino[17] showed that rat islets stored in cold UW solution for up to 24 h could reverse experimental diabetes. Thereafter, Inui et al.[18] stressed that islets are particularly sensitive to damage by storage in UW solution under suboptimal temperatures.[18] Shirouzu et al.[19] reported that the recovery and insulin secretory capacity of islets preserved in UW solution for 7 d was better than those of islets cultured in media at 37°C, and that the loss of cultured islets caused by apoptosis was significantly suppressed by such preservation. Noguchi et al. demonstrated that cold storage in UW solution was superior to room temperature and 37°C culture up to 48 h using human islets.[20] They also mentioned their group used cold storage in clinical islet transplantation. Furthermore, Matsumoto et al. compared UW cold storage and various culture conditions using both human and non-human primate islets.[21] However, the mechanisms to prevent damage of isolated islets remain unclear. Here, we compared fresh islets with those cultured for 24 h at 4 or 37°C and those preserved in culture medium or UW solution at 4°C in terms of islet function in vitro and histology, to determine how to minimize islet damage during

*Correspondence to: Mitsukazu Gotoh; Email: mgotoh@fmu.ac.jp
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the first 24 h after isolation. We found that cold preservation in UW solution results in islet function and morphology that are comparable with those of fresh islets and that central necrosis of isolated islets could be avoided.

Results

Islet count. Islets were counted immediately after isolation or 24 h after incubation in culture medium (C groups) or in preservative solution (P groups). A ratio of the count after each incubation to that obtained before the incubation (control) was used as an index for survival rate of morphologically intact islets. The islet yield in the P-UW group was 552 ± 115/pancreas, which was not significantly different from 592 ± 66 /pancreas of freshly isolated islets. In addition, the ratio of islet count in the P-UW-group was significantly higher than that in the C-DMEM-0, C-RPMI-200 or P-DMEM-200 groups (87.9 ± 9.3% vs. 60.8 ± 5.8%, 53.4 ± 0.7%, 34.1 ± 2.6%, respectively, $p < 0.05$; **Fig. 1**).

In vitro and in vivo islet function. Islets were incubated for 60 min in RPMI 1640 medium containing either 3.3 or 16.7 mmol/l glucose to determine the glucose-stimulated insulin release in the medium after 24 h incubation in culture medium or cold preservative solution. Insulin secretion in response to 16.7 mmol/l glucose was significantly higher than in response to 3.3 mmol/l glucose in both freshly isolated islets and islets in P-UW group ($p < 0.05$). However, there was no such difference in C-DMEM-0, C-DMEM-100, C-DMEM-200, C-RMPI-200, or P-DMEM-200 (**Fig. 2**).The stimulation index in C-DMEM-0 (1.3 ± 1.2), C-DMEM-100 (1.2 ± 0.5), C-DMEM-200 (0.3 ± 0.7), C-RPMI-200 (0.7 ± 0.3), or P-DMEM-200 (0.6 ± 0.2) was lower than that in freshly isolated islets (8.3 ± 7.4, $p < 0.05$) or islets in P-UW group (6.3 ± 3.7, $p < 0.05$) (**Fig. 3**).

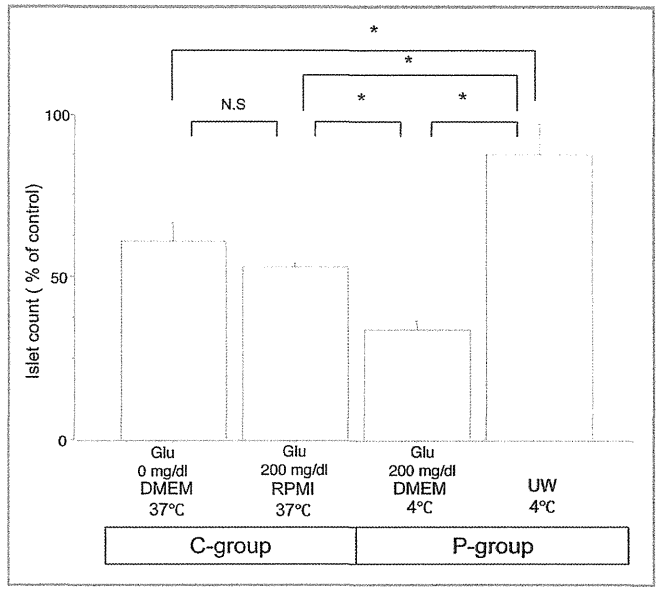

Figure 1. Islet counts in cultured and preserved groups after 24 h. Data are expressed as % average islet count immediately after isolation (n = 3; *$p < 0.05$). Islet counts are significantly higher in the P-UW group than in the C-DMEM-0, C-RPMI-200 and P-DMEM-200 groups (87.9 ± 9.3 vs. 60.8 ± 5.8, 53.4 ± 0.7 and 34.1 ± 2.6, respectively; $p < 0.05$).

The blood glucose levels of fresh group decreased gradually and reached normoglycemia on day 3 (**Fig. 4**). Those of UW group showed the same tendency and reached normoglycemia on day 4. In contrast, those of RPMI 1640 group, reached normoglycemia on day 4, and showed sharp decrease in blood glucose levels on day 1 (4 of 6: normoglycemia) and rebound levels on day 2 (1 of 6: normoglycemia), suggesting unstable insulin secretory capacity of the islet grafts.

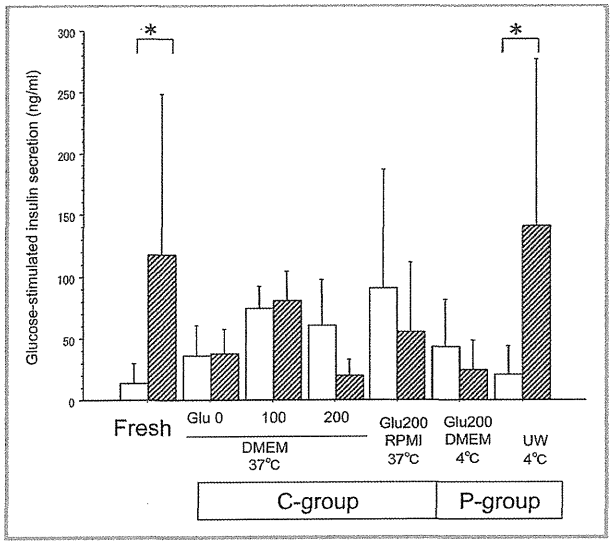

Figure 2. Glucose-stimulated insulin secretion in low and high glucose. "Fresh islets" means islets immediately after isolation. Open and dashed bars indicate low (3.3 mM) and high (16.7 mM) glucose, respectively (*$p < 0.05$).

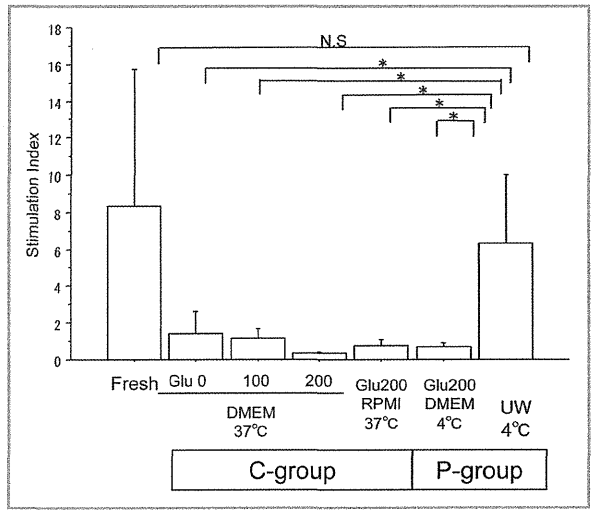

Figure 3. Stimulation index calculated by dividing amount of insulin released during incubation in high glucose (16.7 mmol/l) by that released in low (3.3 mmol/l) glucose (*$p < 0.05$).

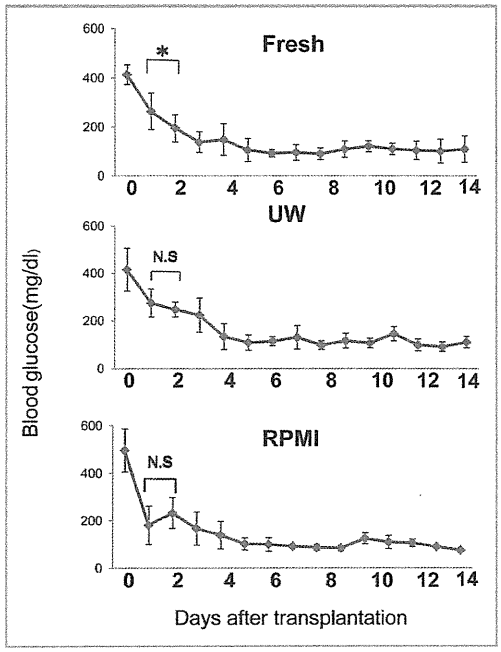

Figure 4. Blood glucose levels after transplantation of 300 IEQ in SCID mice. The groups consists of fresh islets, islets preserved in UW solution at 4°C for 24 h, and islets cultured in RPMI 1640 at 37°C for 24 h. The hyperglycemic rebounds were observed on day 2 as compared with blood glucose levels on day 1 in RPMI 1640 group.

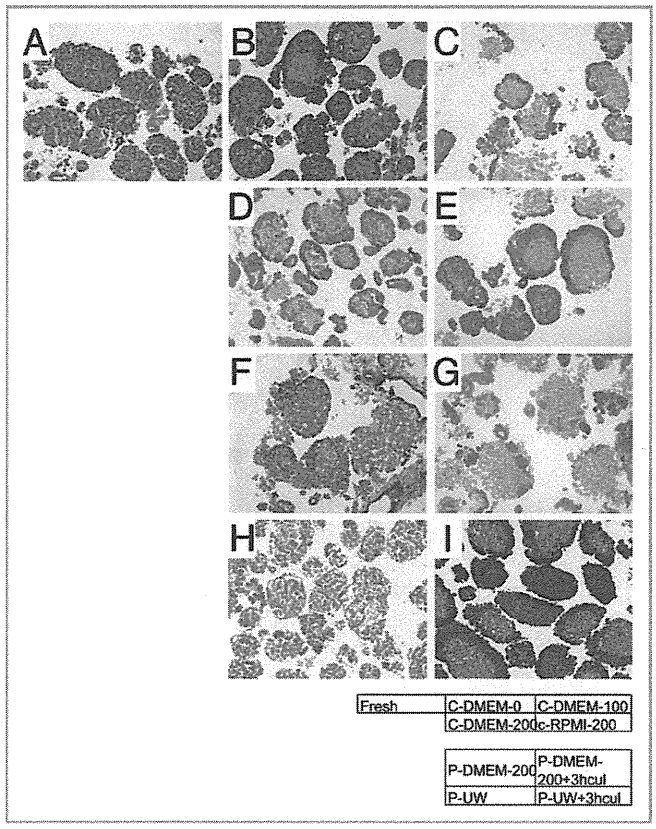

Figure 5. Histological findings of islets stained with HE. Magnification × 100. (A) Islets immediately after isolation; (B) C-DMEM-0; (C) C-DMEM-100; (D) C-DMEM-200; (E) C-RPMI-200; (F) islets preserved in DMEM for 24 h at 4°C with 200 mg/dl of glucose; (G) P-DMEM, islets preserved in DMEM at 4°C with 200 mg/dl of glucose for 24 h then cultured in DMEM at 37°C for 3 h; (H) P-UW, islets preserved in UW solution for 24 h at 4°C; (I) P-UW, islets preserved in UW solution at 4°C for 24 h then cultured in RPMI at 37°C for 3 h. Twenty-four hours after the culture at 37°C, the central damage of islets became easily distinguishable from the surrounding intact area by its poor staining for eosin regardless of the glucose concentration or of the culture medium used (B–E). Islets incubated in DMEM with 200 mg/dl glucose for 24 h at 4°C became swollen (F) and most of them tended to be dispersed after additional culture (G). In contrast, islets preserved in UW solution (P-UW) appeared to be intact with slight shrinkage (H) and quite intact with a relatively smaller portion of central damage even after additional culture (I).

Histological analysis of islets. The islets were essentially intact immediately after isolation, although smooth outer continuity was lost (**Fig. 5A**). Twenty-four hours after the culture at 37°C in RPMI 1640 medium (C-DMEM-0), the central damage of islets became easily distinguishable from the surrounding intact area by its poor staining for eosin (**Fig. 5B**). This histological alteration was similarly observed regardless of the glucose concentration or of the culture medium used (**Fig. 5B–E**).

Islets incubated in DMEM with 200 mg/dl glucose for 24 h at 4°C (P-DMEM-200) became swollen (**Fig. 5F**). When the islets were further cultured for 3 h at 37°C in RPMI 1640 medium with 200 mg/dl glucose, most of them tended to be dispersed, and those morphologically recognized as islets were stained poorly for eosin indicating that significant damage occurred (**Fig. 5G**). In contrast, islets preserved in UW solution (P-UW) appeared to be intact with slight shrinkage (**Fig. 5H**). After the additional culture for 3 h in RPMI 1640 medium, the central damage appeared, but the area appeared to be smaller than those observed in the C- and P-DMEM-200 groups (**Fig. 5I**)

Next, insulin-, glucagon- and HMGB1-positive cells within the islets were compared between groups (**Figs. 6–8**). Insulin-positive cells were scattered in the islets immediately after isolation (**Fig. 6A**). The central damaged areas of islets in the C-groups were stained positive, but the peripheral regions hardly showed clear-cut signal. This staining pattern in C-groups did not differ regardless of the glucose concentration or of the culture medium used (**Fig. 6B–E**). A whole area of some islets, and most of the central areas of other islets in the P-DMEM group, were positively stained after 24 h of preservation (**Fig. 6F**). It was the case with the islets after a further 3 h of culture at 37°C (**Fig. 6G**). In contrast, insulin-positive cells in the islets in P-UW group after 24 h of preservation were scattered (**Fig. 6H**) as was similarly observed in freshly isolated islets. After an additional 3 h culture at 37°C, peripheral as well as the central damaged area contained insulin-positive cells. Moreover, the signal in the peripheral region appeared to be stronger than those in other experimental groups (**Fig. 6I**).

Glucagon-positive cells were found in the rim of the islet, or the outer layer of the peripheral region in the freshly prepared islets, which was constantly observed in every experimental group (**Fig. 7**).

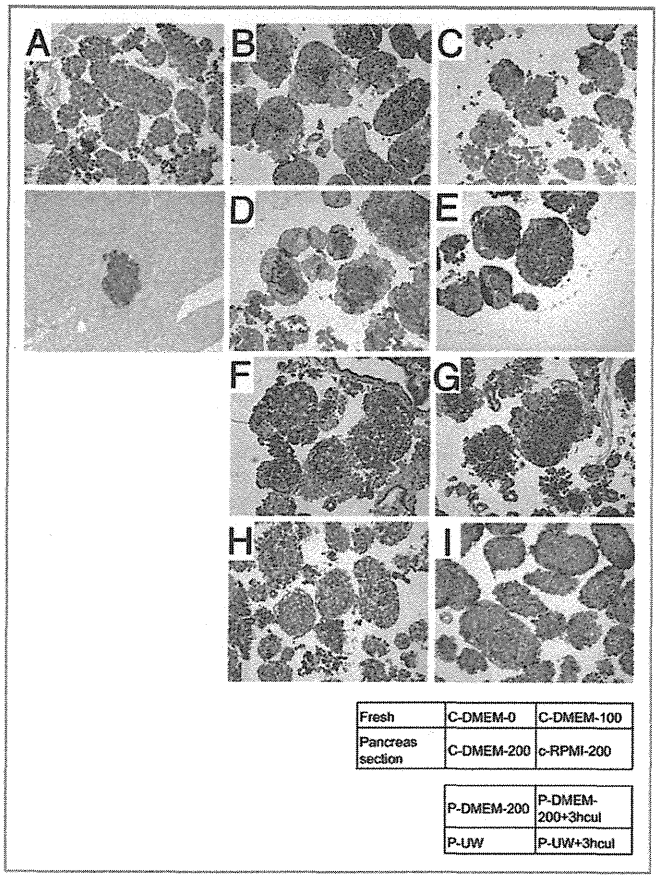

Figure 6. Immunohistological findings of islets using anti-insulin antibody. Magnification × 100. (A) Islets immediately after isolation; (B) C-DMEM-0; (C) C-DMEM-100; (D) C-DMEM-200; (E) C-RPMI-200; (F) islets preserved in DMEM for 24 h at 4°C with 200 mg/dl of glucose; (G) P-DMEM, islets preserved in DMEM at 4°C with 200 mg/dl of glucose for 24 h then cultured in DMEM at 37°C for 3 h; (H) P-UW, islets preserved in UW solution for 24 h at 4°C; (I) P-UW, islets preserved in UW solution at 4°C for 24 h then cultured in RPMI at 37°C for 3 h. The signal in the peripheral region of islets preserved in UW solution (I) appeared to be stronger than those in other experimental groups (B–E and G).

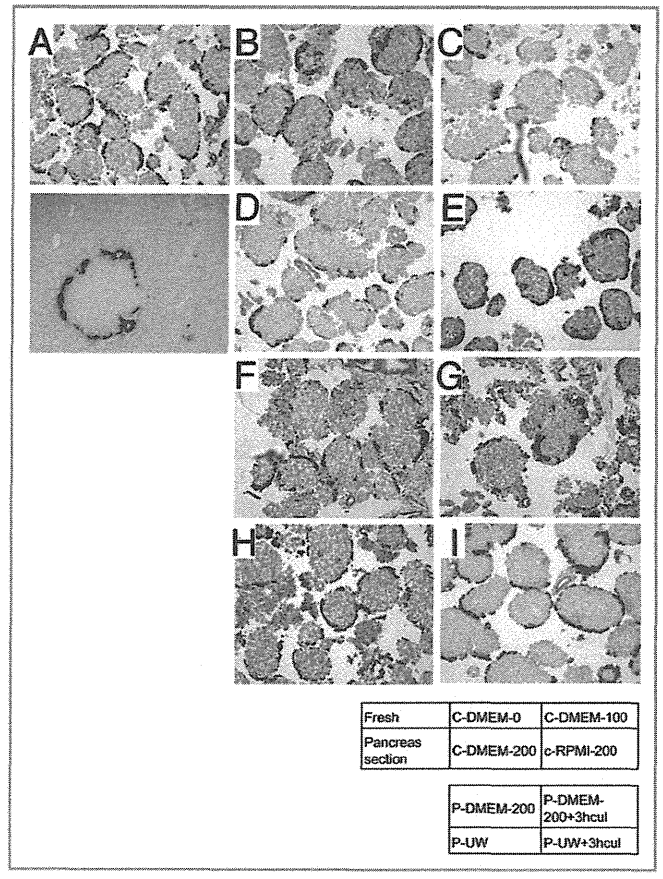

Figure 7. Immunohistological findings of islets using anti-glucagon antibody Magnification × 100. (A) Islets immediately after isolation; (B) C-DMEM-0; (C) C-DMEM-100; (D) C-DMEM-200; (E) C-RPMI-200; (F) islets preserved in DMEM for 24 h at 4°C with 200 mg/dl of glucose; (G) P-DMEM, islets preserved in DMEM at 4°C with 200 mg/dl of glucose for 24 h then cultured in DMEM at 37°C for 3 h; (H) P-UW, islets preserved in UW solution for 24 h at 4°C; (I) P-UW, islets preserved in UW solution at 4°C for 24 h then cultured in RPMI at 37°C for 3 h. Glucagon-positive cells were found in the rim of the islet, or the outer layer of the peripheral region in the freshly prepared islets, which was constantly observed in every experimental group.

Strong HMGB1-signal was found in the cytoplasm of some islet cells located in the peripheral as well as central area of the islets (**Fig. 8A**). After 24 h of culture, some peripheral cells as well as many cells in the central area of islets showed HMGB1-signal in the cytoplasm. These changes in C-groups were not different regardless of culture media, DMEM or RPMI, or of glucose concentration (**Fig. 8B–E**).

HMGB1-signal was found similarly in the P-DMEM as well as P-UW-groups after 24 h of preservation (**Fig. 8F**). However, after a further 3 h of culture at 37°C, almost all cells in remained islets in the P-DMEM group were positive for HMGB1-signal (**Fig. 8G**). Meanwhile, after a further 3 h of culture at 37°C in UW-group, the HMGB1-signal was more frequently found in the nuclei, but lower cytoplasmic signal compared with those in P-DMEM group (**Fig. 8I**).

To obtain quantitative analysis of HMGB1 positive cells in the cytoplasm of islets in each group, numbers of these cells in central and peripheral areas were counted separately and compared between the groups (**Fig. 9a and b**). HMGB1 positive cells in experimental groups except for cold P-UW group were significantly increased in number as compared with control group (**Fig. 9a**). Although numbers appeared to be not so different, when HMGB1 positive cells in the peripheral area were compared, significant increase in number was only observed in C-RPMI-200 and P-DEMEM 3 h after culture over control. When HMGB1 positive cells in the central area were compared, islets of P-UW group even after additional 3 h culture at 37°C showed significant small number of positive cells as compared with other experimental groups, suggesting superiority of this method (**Fig. 9b**). The TUNEL assay revealed scattering positive cells in central or peripheral areas in both C- and P-group (**Fig. 10**).

These findings showed that the central damaged area was mostly comprised of insulin-positive cells that were vulnerable to

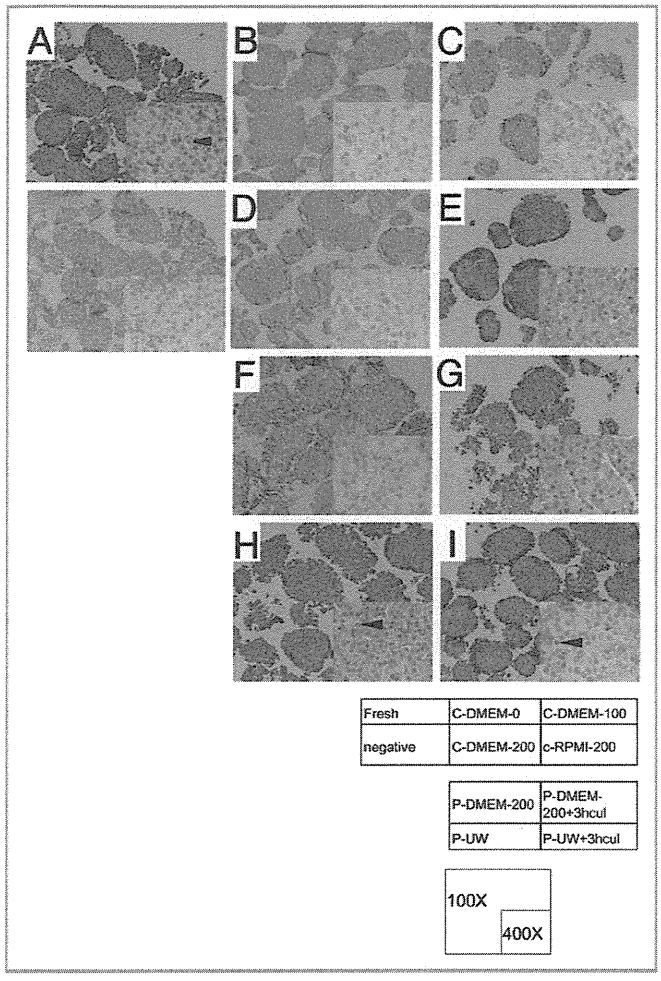

Figure 8. Immunohistological findings of islets using anti-HMGB1 antibody Magnification × 100 (high-power field × 400). (A) Islets immediately after isolation; (B) C-DMEM-0; (C) C-DMEM-100; (D) C-DMEM-200; (E) C-RPMI-200; (F) islets preserved in DMEM for 24 h at 4°C with 200 mg/dl of glucose; (G) P-DMEM, islets preserved in DMEM at 4°C with 200 mg/dl of glucose for 24 h then cultured in DMEM at 37°C for 3 h; (H) P-UW, islets preserved in UW solution for 24 h at 4°C; (I) P-UW, islets preserved in UW solution at 4°C for 24 h then cultured in RPMI at 37°C for 3 h. Strong HMGB1-signal was found immediately after isolation in the cytoplasm of some islet cells located in the peripheral as well as central area of the islets (A) After 24 h of culture, some peripheral cells as well as many cells in the central area of islets showed HMGB1-signal in the cytoplasm regardless of culture media or of glucose concentration (B–E). In contrast, HMGB1-signal in islets preserved in UW solution even after culture (I) was more frequently found in the nuclei, but lower cytoplasmic signal compared with other groups. Arrows indicate HMGB1 positive cells only in nucleus.

procedures of isolation and culture. Glucagon-positive cells were rather intact compared with insulin-positive cells. HMGB1-signal was induced in nuclei as well as the cytoplasm of some islets following isolation and subsequent culture. Most of the cells in the central damaged area in the C-group were positive for HMGB1-signal. In contrast, very few islets were TUNEL-positive in all experimental groups. Therefore, these results suggest that preservation of islets in UW solution prevents the development of central damage and loss of insulin-positive granule in surviving cells.

Discussion

Islets are immediately transplanted after isolation in the Edmonton protocol, which offers an 82% likelihood of insulin independence for 1 y thereafter.[1-3] However, several immunosuppression protocols have been developed, including induction therapies to improve outcomes that require various periods before islet transplantation.[6-9] Thus, a viable and functional mass of islets must be maintained for up to 24 h to ensure successful islet transplantation. Here, we compared the quality of fresh control with cultured and cold-preserved islets. We found that islet counts and insulin secretory capacity in vitro were better after preservation in UW solution at 4°C than those of islets incubated in culture medium at 4 and 37°C. Moreover, our findings suggested that cold preservation in UW solution inhibited the central damage to islets that is usually encountered as a result of several hours of culture at 37°C after isolation.

The UW solution has been used for cold preservation in clinical organ transplantation because it prevents the cold-induced necrotic injury caused by impermeable and osmotically active endogenous factors.[23] Recent studies have shown that islets recover well from preservation in UW solution and that integrity and function is maintained, which results in improved transplant outcomes.[17-19] Superiority of the cold preservation in UW-solution to conventional culture for shorter period was also reported by others,[20,21] however, the mechanisms of its damage and protection remained unclear. Here, we examined islet counts and histological findings and assessed islet function in vitro after culture under various conditions. Temperature, glucose concentration and type of medium did not affect islet counts or integrity and function after 24 h of culture. We also showed that islet integrity and function were better maintained after preservation in cold UW solution than after culture under various conditions at 37°C or after cold preservation at 4°C in conventional culture media for 24 h. Our histological studies suggested that one of the underlying mechanisms for these findings is the avoidance of central damage to the isolated islets through preventing necrosis.

The contribution of necrosis or apoptosis to the reduced function of the islet mass islet culture remains controversial. The apoptotic index (15–45%) is high among islets obtained from canine or human pancreases cultured for three days.[24-26] In contrast, only a few apoptotic cells are evident among human islets cultured for 24 h.[27] On the other hand, Annexin V/PI staining of model canine islets revealed more necrotic than apoptotic cells among islets cultured for 12–72 h.[28] PARP cleavage assays of a cultured human islet model developed to test the effects of antioxidants showed ongoing necrotic and apoptotic events after isolation and culture.[29]

We found that the area of central damage was mainly composed of HMGB1-positive necrotic cells, with a few apoptotic cells determined by the TUNEL assay. HMGB1 was originally identified as a chromosomal protein facilitating the binding of

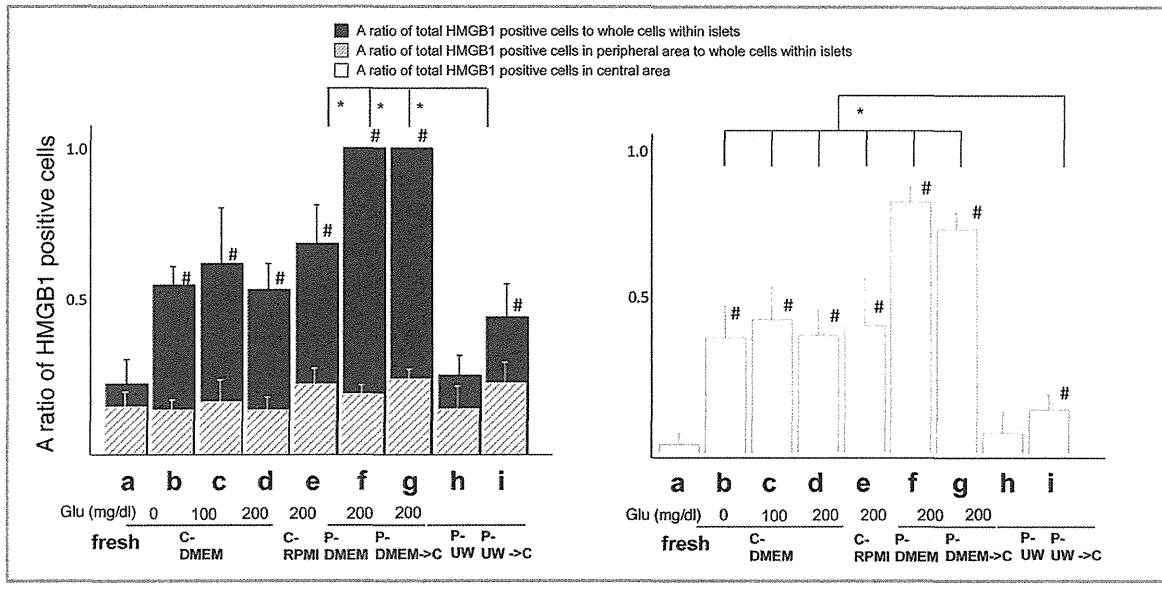

Figure 9. A ratio of HMGB1 positive cells in peripheral and central area of islets to whole cells within islets. HMGB1 positive cells in peripheral and central area were calculated to whole cells within islets or to the cells in the central area. The islets preserved in UW solution even after culture showed significant small number of positive cells as compared with other experimental groups, suggesting superiority of this method. #p < 0.05 vs Fresh (a) *p < 0.05 vs UW 3 h after culture (i).

transcription factors to their cognate DNA sequences.[30] Scaffidi et al. recently reported that HMGB1 is released from necrotic but not apoptotic cells and that it induces inflammatory responses in vivo.[31] Furthermore, interleukin-1 stimulates β-cell necrosis and the release of the immunological adjuvant HMGB1.[32] Matsuoka et al. have assayed HMGB1 of transplantated islet and inflammatory cytokines like interleukin-12 or interferon-γ, and described that HMGB1 could be a marker of islet damage and potential target to improve efficiency of islet transplantation.[33] Our results indicated that preservation in UW solution at 4°C reduced the central damage to islets. Less HMGB1 was expressed in the central damaged area of these islets than in those cultured for 24 h in DMEM or RPMI at 37°C regardless of the glucose concentration.

Based on these findings, we postulate that central damage to islets during culture is composed of necrotic cells releasing HMGB1 and that preservation in UW solution reduces this damage, which results in maintained islet function. This modality could be applicable in the clinical setting when an interval is required for induction therapy before islet transplantation.

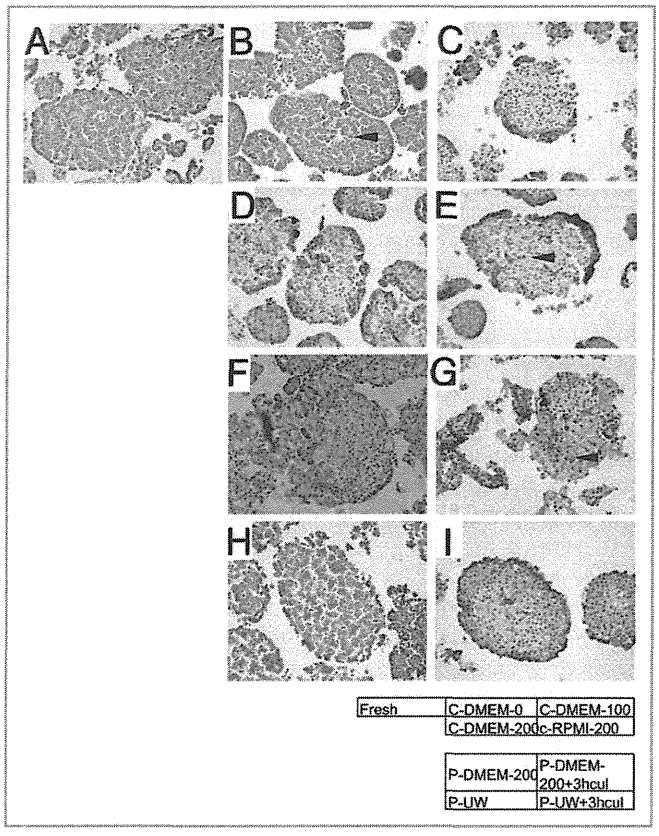

Figure 10. Immunohistological findings of islets by TUNEL assay. Magnification x 100. (A) Islets immediately after isolation; (B) C-DMEM-0; (C) C-DMEM-100; (D) C-DMEM-200; (E) C-RPMI-200; (F) islets preserved in DMEM for 24 h at 4°C with 200 mg/dl of glucose; (G) P-DMEM, islets preserved in DMEM at 4°C with 200 mg/dl of glucose for 24 h then cultured in DMEM at 37°C for 3 h; (H) P-UW, islets preserved in UW solution for 24 h at 4°C; (I) P-UW, islets preserved in UW solution at 4°C for 24 h then cultured in RPMI at 37°C for 3 h. Arrows indicate positive cells, suggesting very few positive cells in all experimental groups.

Materials and Methods

Animals. Islets were isolated from 8-week-old male Wistar rats (CLEA Japan Inc.) under ether anesthesia. The Ethics Review Committee for Animal Experimentation of Fukushima Medical

University approved the experimental protocol, which proceeded according to the guidelines of the National Research Council's *Guide for the Care and Use of Laboratory Animals.*

Isolation of rat islets. Islets were isolated using a protocol similar to that reported.[22] Briefly, we injected 12 ml of Hank's balanced salt solution (Nissui) containing 2 mg/ml of collagenase S-1 (Nitta Gelatin) into the common bile duct. The distended pancreas was removed and incubated at 37°C for 35 min. Islets separated by collagenase digestion were purified by density gradient (1.12, 1.09 and 1.05) centrifugation on Ficoll (Type 400, Sigma-Aldrich Co.) and then washed with Hanks solution.

Experimental groups. Islets were isolated from six animals in each group. The culture group (C-group) comprised islets that were suspended for 24 h at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 medium (with L-glutamine and without glucose; Sigma Chemical Co.) or DMEM (Dulbecco's modified Eagle's medium without glucose; Invitrogen Co.) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in 90 × 90 mm non-treated dishes to which islets do not adhere. Glucose concentrations were: DMEM with 0 (C-DMEM-0), 100 (C-DMEM-100) and 200 (C-DMEM-200) mg/dl glucose and RPMI 1640 medium with 200 mg/dl glucose (C-RPMI-200 group). Thus, the C group comprised four subgroups. The Preservation group (P group) comprised islets that were suspended for 24 h at 4°C in DMEM containing 200 mg/dl glucose (P-DMEM) or UW solution (ViaSpan; Bristol-Myers Squibb Co.) (P-UW group) in 90 × 90 mm non-treated dishes. Islets in all groups were functionally evaluated after 3 h culture at 37°C under a humidified 5% CO_2 atmosphere in RPMI 1640 medium with 200 mg/dl glucose. The UW solution contained potassium lactobionate (100 mM) KH_2PO_4 (25 mM), $MgSO_4$ (5 mM), raffinose (30 mM) adenosine (5 mM), glutathione (3 mM), allopurinol (1 mM) and hydroxyethyl starch (50 g/L), and the pH was adjusted to 7.4 with KOH (final values: Na, 25 ± 5 mM; K, 120 ± 5 mM; mOsm/L, 320 ± 10).

In vitro and in vivo islet function. For in vitro islet function, islets in each group were first cultured for 3 h at 37°C in RPMI 1640 medium containing 5.5 mmol/l glucose. Then after the preincubation for 60 min at 37°C in RPMI 1640 medium containing 3.3 mmol/l glucose, 100 islets from each group were further incubated for 60 min at 37°C in 1.0 ml RPMI 1640 medium supplemented with 0.1% bovine serum albumin containing 3.3 mmol/l glucose and then for 60 min in the same medium containing 16.7 mmol/l glucose. The amount of insulin released in the medium during static incubation was determined using an ELISA (Rat Insulin ELISA kit; Shibayagi Co., Ltd.) according to the manufacturer's protocols. Stimulation indices were calculated by dividing the amount of insulin released during incubation under high (16.7 mmol/l) by that under low (3.3 mmol/l) glucose. Static incubation comprised islets obtained from six animals per group.

To examine in vivo function of islet, 300 IEQ islets were transplanted into diabetic 8 week-old male SCID Mice (C.B-17/Icr-scid;CLEA Japan Inc.) under the capsule of left kidney. Diabetes was induced by intraperitoneal injection of Streptozotocin (STZ) (Sigma) (0.24mg/g). Immediately after isolation, 300 IEQ islet/mice were transplanted (Fresh: n = 5). Three hundred IEQ islet/dish were cultured in RPMI1640 (n = 6) at 37°C in a humidified 5% CO_2 atmosphere, or preserved in UW solution (n = 6) at 4°C for 24 h before transplantation. After transplantation, blood glucose levels were measured for 2 weeks. Mice were considered diabetic when their blood glucose levels were > 350 mg/dl, and normoglycemic < 150 mg/dl.

Islet count. Morphologically intact islets with 50–200 μm in diameter were counted immediately after the procedure in each experimental group under a microscope, and a ratio (%) of the islet count to that obtained immediately after isolation was used to compare the effect of each procedure on islet yield.

Histological analysis of islets. Islets for histological studies were transferred into 1.5 ml Falcon micro tubes and collected by centrifugation at 500 rpm for 1 min. These islets were then fixed in 4% paraformaldehyde for 1 d, dehydrated, embedded in paraffin and stained with hematoxylin-eosin (HE). Insulin, glucagon and HMGB1 were immunohistologically stained using purified rabbit anti-insulin (H-86) (Santa Cruz Biotechnology, Inc.), anti-glucagon (Progen; Cat. No. 11184; Biotechnik), and anti-HMGB1 (Product No 326052219; Shino-test Corporation) polyclonal antibodies. Sections were stained using a peroxidase-labeled biotin-avidin detection system Histofine® Simple Stain Rat MAX PO(R) (Nichirei Biosciences Inc.) according to the manufacturer's instructions. To obtain quantitative analysis of HMGB1 positive cells in the cytoplasm of islets (n = 6) in each group, numbers of positive and negative cells in central and peripheral areas were counted separately and expressed as a ratio to whole cells within islets following cold preservation or conventional culture.

The fragmentation of DNA in situ was detected in 4 μm thick paraffin sections of islets using the In Situ Apoptosis Detection Kit (Takara Bio Inc.). Deparaffinized sections were digested with proteinase K and then endogenous peroxidase activity was blocked with 3% H_2O_2. The TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) was added for 60 min at 37°C. Islet cells containing damaged DNA were visualized as dark brown areas in the nucleus using an anti-FITC antibody conjugated with HRP and DAB substrate. The sections were counterstained with hematoxylin to identify regions of central damage in the islets.

Statistical analysis. Data are expressed as means ± SD. Insulin release and stimulation indices were compared using ANOVA. Values of $p < 0.05$ were considered statistically significant. All data were statistically analyzed using Stat-view J 4.58 for Windows software (Abacus Concepts Inc.).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

Acknowledgments

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Adenine Nucleotide Levels in a Closed Enzymatic Digestion System for Porcine Islet Isolation

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

*Department of Surgery I, Fukushima Medical University, Fukushima City, Japan
†Biochemical Department, Kikkoman Food Products Company, Noda-shi, Chiba, Japan
‡Department of Biochemistry, Fukushima Medical University, Fukushima City, Japan

Obtaining viable islets is a crucial step for successful islet transplantation. Adenosine triphosphate (ATP) is a marker of cell viability. However, little is known about any changes in the energy status of the tissues that are being digested during the digestion phase. We herein examined whether the ATP content in serially digested pancreatic tissue samples could be specific objective parameters that signal the optimal point to stop the digestion process. We obtained partial pancreata (body to tail) from 4- to 5-year-old pigs from a slaughterhouse. The tissue samples were preserved in M-Kyoto solution for less than 3 h. They were digested using an automated enzymatic and mechanical dissociation system at 37°C for 90 min following intraductal injection of Liberase HI. Samples were collected from the digestive circuit every 5 or 10 min to determine the ATP level, total adenine nucleotide (TAN) level, islet count (count/g), and yield of islet equivalent (IEQ) in the serial digestive fluids. The ATP and TAN levels, IEQ and islet count were increased and then decreased during digestion process. The profile of these parameters differed from case to case. However, when ATP changing ratio (respective value/precedent value) was compared with IEQ changing ratio, a greater than threefold increase in the ATP changing ratio followed by an increase in the islet count changing ratio within 5 min was consistently observed, indicating the optimal time to stop the digestion. The ATP levels of the handpicked islets in the digested samples were lower in the overdigested phase in comparison to those in the earlier digested phase. These results indicate that the ATP level in digested fluid could be an effective indicator to estimate the viability of cells as well as determine the optimal time to terminate the digestion process in order to obtain viable islets.

Key words: Islet isolation; Enzymatic digestion; Adenine nucleotide

INTRODUCTION

During the past few decades, pancreatic islet transplantation has become a treatment option for patients with type 1 diabetes mellitus (7,25). However, it is still difficult to recover the entire quantity of islets contained in a pancreas (16). Given that islet isolation is crucial for the success of this transplantation procedure, many attempts to obtain more viable islets have been reported in the literature (1,12,18). In rodent models, collagenase digestion can be performed for a specific period of time to obtain a peak islet yield; however, in humans, the timing of dilution is empirically determined by human expertise and experience (1,16–18). If the timing is delayed, then poor results are inevitable. Various factors can affect the quantity and quality of islets during the digestion phase (17).

The adenosine content is known to reflect viability and is considered useful for outcome prediction in islet transplantation (5,6). We previously reported a new method for the determination of adenosine levels for isolated islets (8,9). This methodology, using the bioluminescent enzymatic cycling assay, produces rapid measurements with only a small sample volume (8,9), thus enabling us to determine the adenosine levels from a small volume of samples collected during the collagenase digestion phase of islet isolation.

The collagenase digestion phase results in the release of endogenous pancreatic enzymes from the exocrine tissues (3,15,20–23). A strong increase in such enzyme

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Address correspondence to Mitsukazu Gotoh, M.D., Department of Surgery I, Fukushima Medical University, 1 Hikarigaoka Fukushima, 960-1295, Japan. Tel: +81-24-547-1252; Fax: +81-24-548-2735; E-mail: mgotoh@fmu.ac.jp

activity involved in the digestion process has been shown to be detrimental to the islet isolation process (3,15,20–23). However, little is known about any changes in the energy status of the tissues that are being digested during the digestion phase.

The goal of this study was to examine the serial changes in the energy status of digesting fluid containing exocrine and endocrine cells of the pancreas in relation to the islet count and yield. To address this issue, pancreatic tissue samples from retired breeder pigs were subjected to continuous mechanical and enzymatic digestion using a Ricordi chamber with Liberase-HI until all pancreatic tissue disappeared (this process is termed the "overdigestion model"). Using this model, we then tried to determine the specific objective parameters that signal the optimal point to stop the digestion process in relation to the adenosine triphosphate (ATP) content of the digested fluid, islet count, and islet yield.

MATERIALS AND METHODS

Animals and Isolation Methods

Partial pancreata (body to tail) from retired breeder pigs were obtained from a local slaughterhouse and preserved using cold M-Kyoto solution. M-Kyoto solution is an extracellular-type organ preservation solution developed at Kyoto University (19). It contains the cytoprotectives disaccharide, trehalose, and ulinastatin (Mochida Pharmaceutical. Co. Ltd., Tokyo, Japan). Trehalose has a cytoprotective effect against stress, and ulinastatin inhibits trypsin. All the pancreata were procured within 17 min of warm ischemic time (15.4 ± 1.1 min). The partial pancreata were carefully handled and a cannula was inserted into the main pancreatic duct and fixed with a purse suture. Thereafter, 0.5 ml/g of cold M-Kyoto solution was carefully infused into the pancreatic duct (21). The pancreas was preserved in cold M-Kyoto solution and transported to our laboratory within 3 h. In the laboratory, the connective tissue surrounding the pancreas was removed and the pancreas was distended for 15 min with cold Hank's balanced salt solution (HBSS) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 0.5 mg/ml of collagenase (Liberase HI; Roche Diagnostics Corporation, Indianapolis, IN, USA). After distention, the pancreas was cut into seven pieces and then they were placed in a Ricordi chamber. The pieces were subsequently digested using an enzymatic and mechanical dissociation system at 37°C for 60–90 min until all pancreatic tissue disappeared. The mechanical dissociation procedures were performed by the same one person in cases 1 to 4, and case 5 was performed by another.

We took samples from the digestive circuit every 5 or 10 min. We analyzed the samples to determine the values of ATP, total adenine nucleotides (TAN), islet equivalent (IEQ), an index based on the size of islets (1 IEQ equals an islet of 150 μm diameter), and islet yield in serial digestive fluids. For ATP and TAN measurements, we took 1 ml of digestive fluid from the digestive circuit and immediately treated it with 100 μl of 5 N perchloric acid ($HClO_4$) to inactivate various enzymes (including ATPase) before measurement. The fluid was frozen at −80°C until it was assayed.

Measurement of Adenosine Nucleotides

We performed a bioluminescent enzymatic cycling assay using synthetic firefly luciferase, pyruvate kinase

Table 1. Basic Information of the Pancreas Grafts and Outcome of Isolation in Five Cases

	Case 1	Case 2	Case 3	Case 4	Case 5	Average ± SD
Weight of pancreas, warm/cold ischemic time						
Digested pancreas weight (g)	31.8	56.4	33.3	41.1	51.3	42.8 ± 10.8
Warm ischemic time (min)	17	15	16	14	15	15.4 ± 1.1
Cold ischemic time (min)	149	135	180	183	190	167.4 ± 24.0
Peak values of ATP, TAN, yield, and IEQ, and time to reach the respective peak value						
ATP (nmol/μl)	90	38	107	360	873	293.5 ± 347.1
Time (min)	40	50	30	20	25	33.0 ± 12.0
TAN (nmol/μl)	493	203	741	1,284	5,354	1,615.2 ± 2,128.3
Time (min)	60	45	70	30	20	45.0 ± 20.6
Yield (nmol/μl)	19,310	6,920	18,619	13,693	83,942	28,496 ± 31,388
Time (min)	80	35	50	25	20	44.0 ± 22.7
IEQ (/g)	5,528	2,846	5,567	9,262	26,984	10,037 ± 9,744
Time (min)	70	40	50	35	20	43.0 ± 18.6

ATP, adenosine triphosphate; TAN, total adenine nucleotide; IEQ, islet equivalent.

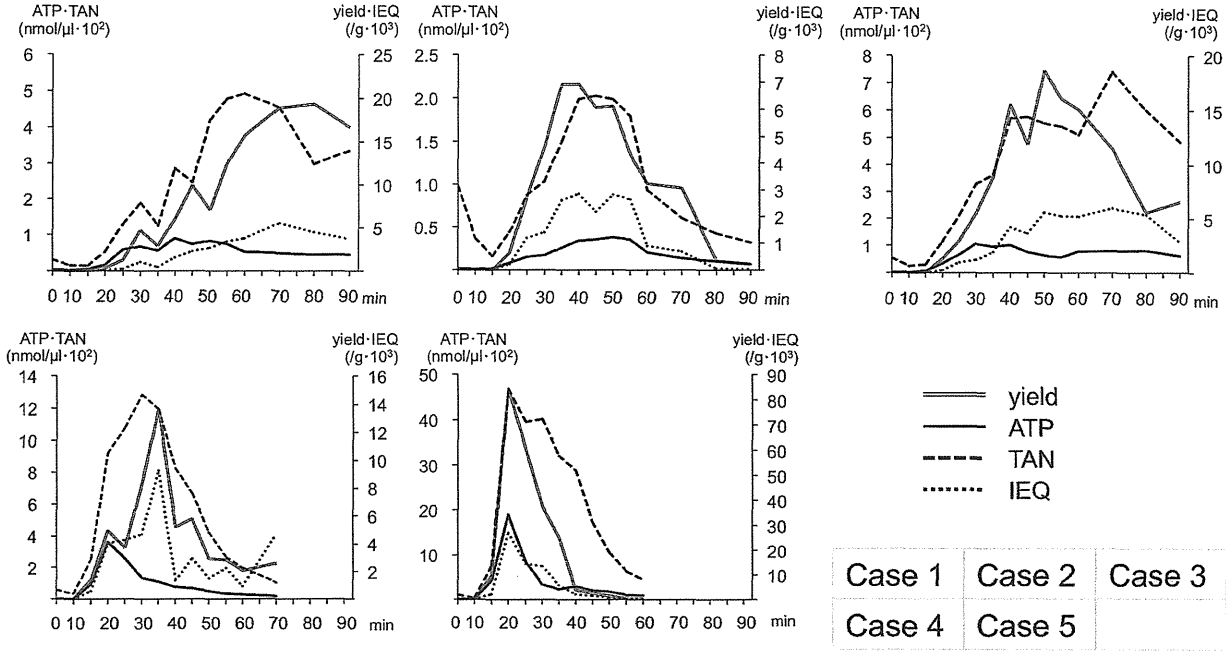

Figure 1. Chronological course of the ATP/TAN level, islet count, and IEQ during pancreas digestion in five cases. The solid line, bold dashed line, thin dashed line, and double line indicate the adenosine triphosphate (ATP) content, total adenine nucleotide (TAN) content, islet equivalent (IEQ)/g pancreas, and islet count/g pancreas, respectively. The correspondence table is in the bottom-right corner. These parameters differed from case to case, and the duration to reach the peaks of these parameters was also different.

(PK) and pyruvate orthophosphate dikinase (PPDK) to measure the levels of ATP and TAN in the samples (25). This method makes it possible to obtain results quickly and it also has a high sensitivity for detecting the ATP content in even one islet cell (8,9). We mixed 20 μl of a 5 N $HClO_4$-treated sample with 80 μl of Tris-HCl buffer (pH 8.4) and 100 μl of reagent containing firefly luciferase (Kikkoman Corp., Chiba, Japan). The reagent reacted with ATP or TAN and emitted light in proportion to the quantities of ATP or TAN in the samples. The time required to complete the reaction between ATP or TAN and luciferase was 90 s. The light generated from the samples was measured with a Mithras LB 940 luminometer (Berthold Technologies KmBH & Co. KG, Germany).

To determine the appropriate condition to assay the adenine nucleotide levels, we measured dilution rates of samples at various concentrations of $HClO_4$. Finally, we adopted the concentration of $HClO_4$ at 5 N and a dilution rate at 100 times for the measurement of adenosines.

Measurement of Islet Equivalent and Yield

To measure the IEQ and the islet yield, we took 200-μl samples from the circuit with a syringe. These samples were placed in a dish filled with 1 ml of HBSS, and 200 μl of dithizone was added to stain any islets present in the sample. In all five exams, one experienced person analyzed the islet yield, IEQ, purity, and morphology. The values of the islet yield and IEQ/g were calculated based on the values of the islet yield or IEQ of a 200-μl sample and the dilution factor (3,250×).

Measurement of ATP Content in Islets

We also measured the ATP content of the islets picked up from the digestive fluid. Zinquin (10,26), a specific probe for zinc, enabled us to identify the islets from the digestive fluid. A 1-ml sample of digestive fluid was taken from the digestive circuit and then it was washed two times in HBSS. We subsequently added 20 μl of zinquin to the fluid. We next picked up 10 islets, 100–150 μm in diameter, from the digestive fluid at various points. They were placed in 900 μl of HBSS with 100 μl of 5 N $HClO_4$ and frozen until assayed.

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 5–20% acrylamide gels (SuperSep; Wako Pure Chemical Industries, Ltd., Osaka, Japan) as described by Laemmli (14). Protease inhibitor cocktail (Complete; Roche

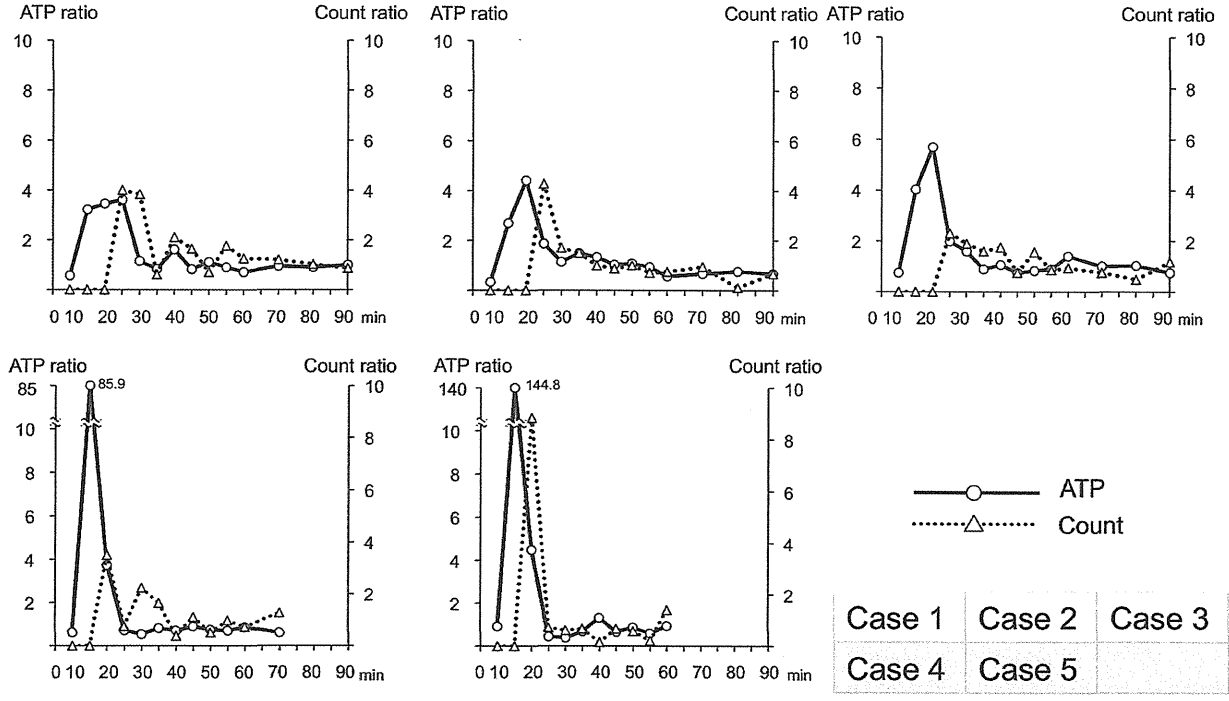

Figure 2. Chronological changes in the islet count changing ratio and the ATP changing ratio. The solid line with open circles indicates the ATP changing ratios. The dashed line with open triangles indicates the islet count changing ratio. The stop points of five separate digestions were matched with the peaks of the islet count changing ratios, and these peaks were preceded by the peak of the ATP level.

Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) was added immediately when samples were taken from digestive circuit to prevent proteolysis. Samples were divided into supernatants and pellets by centrifugation and then supernatants were analyzed. Supernatants were mixed with SDS buffer, containing of 0.35 mM Tris-HCl, pH 6.8, 10% SDS, 9.3% dithiothreitol (DTT), and 30% glycerol, and heated at 95°C for 5 min. Then the gels were run at 300 V until the prestained molecular marker running through the gel. Samples were made visible with silver staining reagent (2D silver stain II; Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan).

Statistical Analysis

Data are expressed as the mean ± SD. The ATP and TAN contents are expressed as the amount of luminescence. The ATP increment of the sample was determined by comparing the values between the serial samples. The islet increment (count and IEQ) was also determined in this manner. The "ATP changing ratio" and the "islet count changing ratio" were calculated to determine the changing ratios of the ATP and the islet count by the following equations: ATP changing ratio = ATP content in 1 ml sample/precedent ATP content in 1-ml sample; islet count changing ratio = islet count in 200-μl sample/precedent islet count in 200-μl sample. The ATP contents and ATP/TAN ratio were compared using the unpaired Student's *t*-test with Bonferroni correction. Values of $p < 0.05$ were considered to be statistically significant. The relationship between the TAN level and IEQ was compared using Pearson's correlation coefficient. All statistical analyses were performed using the Stat-view J 4.58 for Windows software program (Abacus Concepts Inc., Berkeley, CA, USA).

RESULTS

Changes in the ATP Level, TAN Level, Islet Count, and IEQ During the Digestion of Pancreatic Tissue

Five separate experiments were performed, with the ATP and TAN contents, islet count, and IEQ analyzed in the digestive fluid. The digestion time was 90 min in three experiments, 70 min in one experiment, and 60 min in another. Table 1 shows the basic information of the digested pancreata. The average weight of the digested pancreata was 42.8 ± 10.8 g. The average warm ischemic time was 15.4 ± 1.1 min, and the average cold ischemic time was 167.4 ± 24.0 min.

Table 1 also shows the four parameters measured in this study. The time required to reach the peak ATP value ranged from 20 to 50 min, with an average time of 33.0 ± 12.0 min. The required time to reach the peak TAN value ranged from 20 to 70 min, with an average time of 45.0 ± 20.6 min. The islet yield peaked at 20–80 min (44.0 ± 22.7 min), and the IEQ/g peaked at 20–70 min (43.0 ± 18.6 min) from the beginning of digestion.

The peak values are shown in Table 1 as well. The peak values of the amount of luminescence of ATP ranged from 38 to 873 nmol/μl, with an average of 293.5 ± 347.1 nmol/μl. The peak values of the TAN luminescence ranged from 203 to 5,354 nmol/μl, with an average value of 1615.2 ± 2128.3 nmol/μl. The maximum values of the islet yield ranged from 6,920 to 83,942/g of pancreas digested, with an average value of 28,496 ± 31,388/g. The maximum values of the islet yield ranged from 2,846 to 26,984 IE/g, with an average value of 12,684 ± 9,992 IE/g.

The chronological course of the ATP level, TAN level, islet count, and IEQ during pancreatic digestion in five cases are shown in Figure 1. The ATP levels, TAN levels, islet counts, and IEQ differed from case to case. The time required to reach the peaks of these parameters also differed from case to case.

Appropriate Timing to Stop the Digestion

One experienced person performed serial observations of dithizone-stained samples under a microscope. We determined the point in time at which the digestion process should be stopped according to conventional criteria, including the presence of a number of individual acinar cells and the emergence of at least 10 islets free from the surrounding exocrine tissue within one visual field (40×). The stop points of five separate digestions were matched with the peaks of the islet count changing ratios, as shown in Figure 2. These peaks were preceded by the peak of the ATP level. Therefore, a greater than threefold increase in the ATP changing ratio followed by an increase in the islet count changing ratio within 5 min indicated the optimal time to stop the digestion.

The relationship between TAN (nmol/μl) and IEQ ($\times 10^2$/ml) in five digestions is shown in Figure 3. It indicates a significant correlation between these two parameters (Pearson's correlation coefficient, $r^2 = 0.71$, $p < 0.01$). The ATP/TAN ratio of the digestion fluid

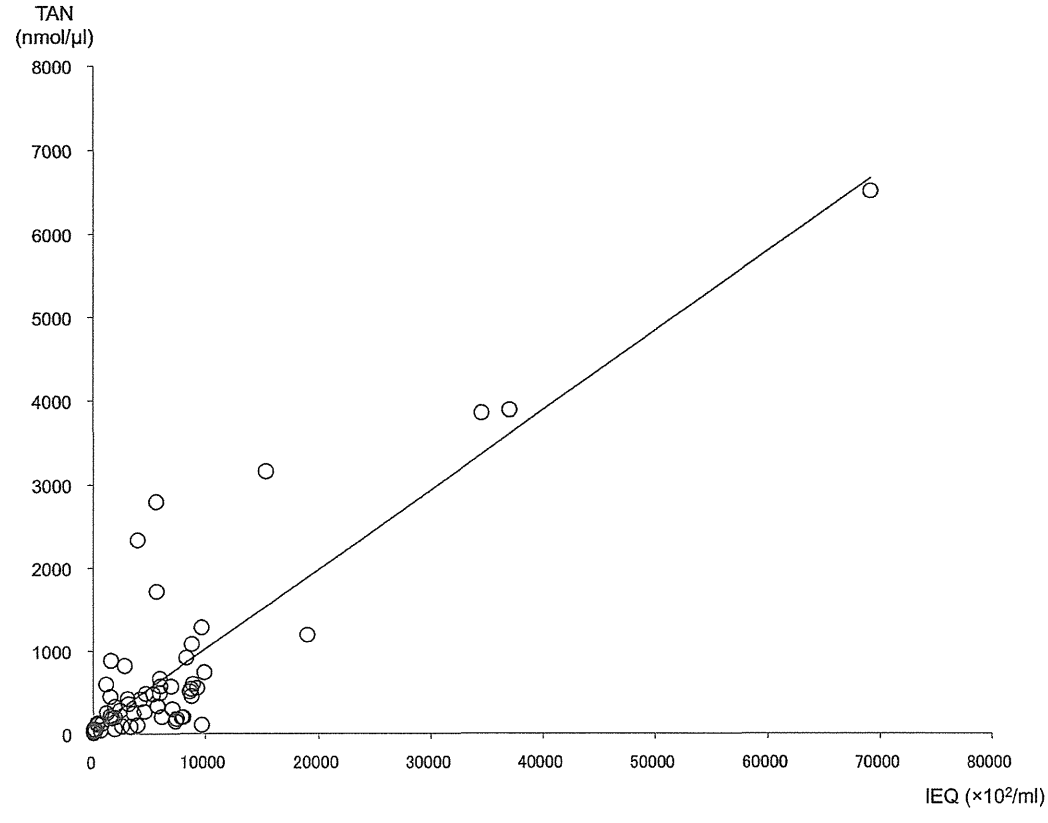

Figure 3. The relationship between the TAN level and IEQ in different five digestions. A significant correlation was observed between these two parameters (Pearson's correlation coefficient, $r^2 = 0.71$, $p < 0.01$).

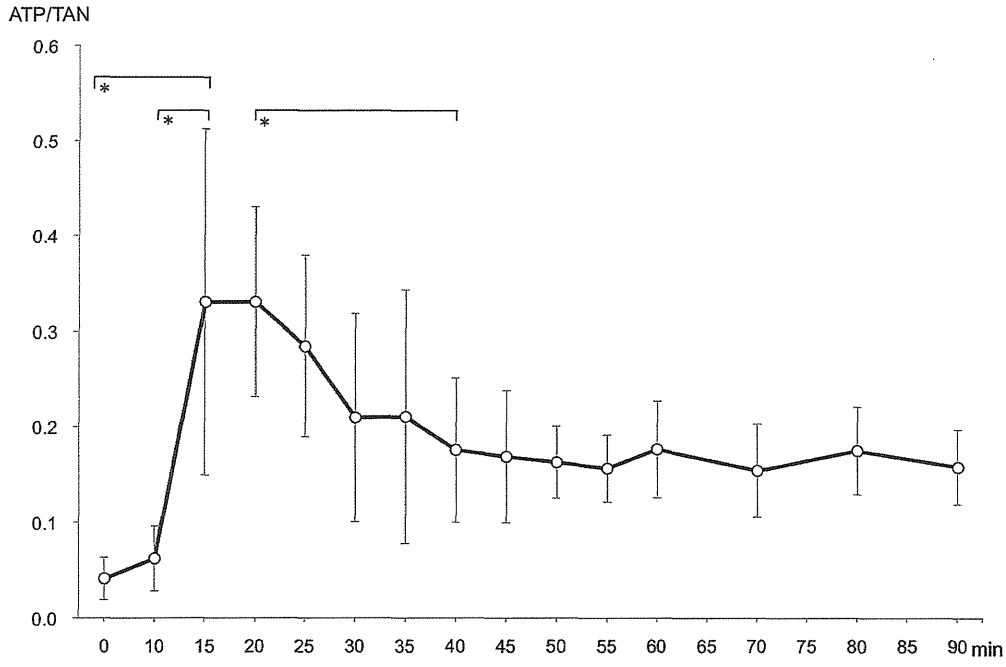

Figure 4. Chronological changes of ATP/TAN ratio. The ATP/TAN ratio indicates cell viability, and had increased significantly at 15 min in comparison to the beginning of digestion and also at 10 min ($p < 0.05$). Thereafter, it was observed to have significant decrease at 40 min.*Significant difference between the digestion periods, $p < 0.05$.

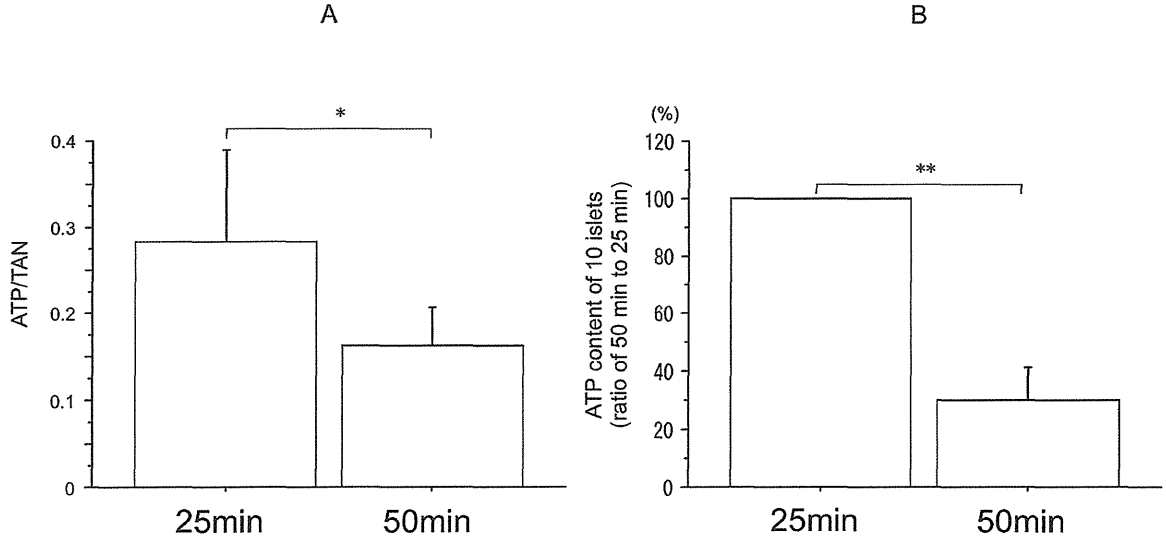

Figure 5. ATP/TAN ratio (A) and ATP content of 10 islets (B) at 25 and 50 min after the beginning of digestion. The ATP/TAN ratio had decreased significantly at 50 min ($p < 0.05$). ATP content of 10 islets at 50 min, which is expressed as a percentage to 25 min, was also significantly lower ($p < 0.01$). *Significant difference between the digestion periods, $p < 0.05$, **$p < 0.01$.

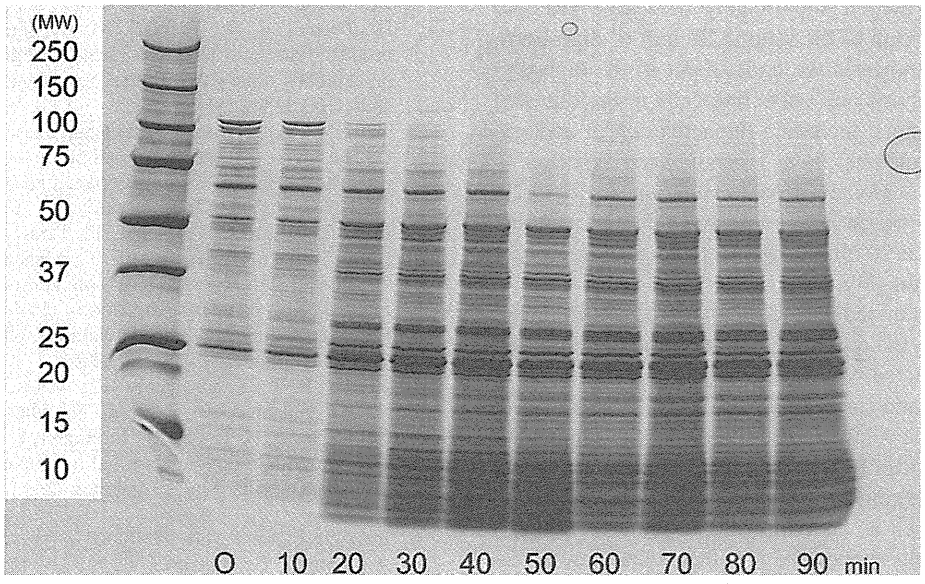

Figure 6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the digestive fluids of the porcine pancreas. The numbers below the columns indicate the times from the beginning of digestion. From 10 to 20 min a marked change was thus apparently observed in the digestive fluid.

increased rapidly as the temperature of the circuit rose. This increase lasted for 15 min until reaching a plateau. The levels declined significantly 40 min after the start of digestion ($p < 0.05$).

The Energy Levels of the Digesting Fluid and Islets During Appropriate Digestion and Overdigestion

To determine the energy levels of digesting fluid and islets during appropriate digestion and overdigestion periods, we compared the ATP/TAN ratio and the ATP content of 10 islets at 25 and 50 min after the start of digestion (Fig. 4). The ATP/TAN ratio, which indicates cell viability, decreased significantly at 50 min compared to the ratio at 25 min ($p < 0.05$) (Fig. 5A). The ATP content of 10 islets at 50 min, expressed as a percentage of 25 min, was significantly less than that at 25 min after the beginning of digestion ($p < 0.01$) (Fig. 5B).

Protein Expression

SDS-PAGE revealed that a marked change was thus apparently observed in the digestive fluid from 10 to 20 min, and degradations of large protein were observed as time passed (Fig. 6).

DISCUSSION

Obtaining a higher mean islet mass from a pancreatic graft is crucial for a successful outcome in the islet isolation procedure (7,25). Although several methods for obtaining a higher islet mass have been reported (12), the optimal timing at which to terminate the collagenase digestion and start dilution usually depends on the appearance and number of islets in the serially digested samples (4,13,25). Further objective parameters are therefore needed to determine the digestion termination point and promote successful outcomes (4). In this study, we measured the adenosine contents in relation to the islet count and islet yield using an overdigestion model. We observed the conventional termination of digestion to be consistent with the peak of the islet count changing ratio, which was preceded by the peak of the ATP changing ratio. The ATP changing ratio is also a useful objective indicator for terminating the digestion phase; within 5 min, a greater than threefold peak is always followed by an increase in the islet count changing ratio.

We previously reported the utility of the ATP bioluminescence assay for the evaluation of isolated islets. It enables us to measure the ATP content even in a small sample volume within several minutes (8,24). The ATP content has been identified as a marker for the viability of a pancreatic graft and isolated islets (2,5,8,9,11). We observed a rapid increase in the ATP level after 15–20 min of digestion, thus indicating the presence of a number of individual acinar cells and free islets with good viability. Furthermore, measuring the ATP level is more sensitive than conventional cell counting.

The TAN content in the digested fluids significantly correlated with the IEQ and it also displayed a peak value after 20 min of digestion. Meanwhile, the ratio of ATP/TAN gradually decreased with the progression of