concentrations were determined before and at 5, 10, 20, 30, 60, 90, and 120 min after the glucose injection.

Insulin Amount in the Liver of the Recipients

Recipient livers were retrieved and homogenized in 5 mL of deionized water at 4°C. After adding 25 mL of deionized water and 75 mL of 0.18 M HCl in 96% ethanol, the homogenate was stored at 4°C for 24 h and was then centrifuged at 2150 *g* for 10 min. The resulting supernatant was stored at −80°C. The insulin concentration in a supernatant was evaluated using a commercial ELISA kit (Mercodia, Uppsala, Sweden).

Single Cell Preparation of Pancreatic Islets

The isolated islets were dispersed into individual cells by treatment with Accutase (Innovative Cell Technologies, San Diego, CA) at 37°C for 4 min.

Preparations of Hepatic Leukocytes

Rat hepatic leukocytes were prepared by anesthetizing the animals with isoflurane. They were killed by total bleeding from the incised axillary artery and vein. The liver was excised, cut into small pieces, pressed through a cell strainer mesh with the diameter of 70 μm (Becton Dickinson, Mountain View, CA), and suspended in phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA). Leukocytes were separated from parenchymal and nonparenchymal cells by 36% Percoll gradient centrifugation.

Flow Cytometry Analysis

Single cells (2×10^5) suspended in PBS with 0.5% BSA were incubated with antibody for 30 min at 4°C on ice in the dark and subsequently washed and suspended in PBS with 0.5% BSA. The expression of C3aR, C5aR, and C5L2 on the rat islet surfaces was detected by FACSCalibur flow cytometry analysis (Becton Dickinson) using a monoclonal antibody to rat C3a receptor (Hycult biotech, Uden, Netherlands) followed by a goat anti-mouse IgG1 (Beckman Coulter, Miami, FL), a complement component 5a Receptor 1/CD88 mouse monoclonal antibody (Lifespan Biosciences, Atlanta, GA) followed by a goat anti-mouse IgG1, and a polyclonal antibody to rat C5L2 (Hycult biotech) followed by a donkey anti-rabbit IgG (Beckman Coulter). As a negative control of C3aR and C5aR, a mouse IgG1 isotype control (Beckman Coulter) was used. As a negative control of C5L2, a rabbit IgG isotype control (Beckman Coulter) was used.

Freshly isolated islets were cultured with or without 250 pg/mL mTNF-α (Roche Diagnostics, Indianapolis, IN), 100 pg/mL hIL1-β (Roche Diagnostics), and 20 ng/mL mIFN-γ (Roche Diagnostics) for approximately 12 h at 37°C. Likewise, to investigate the effect of C5a, syngeneic serum, and C5aIP, the islets were incubated with the medium alone, with the medium containing 10 μg/mL recombinant C5a (Recombinant Mouse Complement Component C5a, R&D Systems, Inc., MN), with syngeneic serum, and with serum containing 700 nM C5aIP for 1 h at 37°C. Furthermore, the expression of CD11b and CD31 of C5aR-positive cells was examined using mouse anti-rat CD11b antibody (LifeSpan Biosciences) and mouse anti-rat CD31 antibody (LifeSpan Biosciences), respectively.

The expression of TF on leukocytes in recipient livers was detected by flow cytometry analysis using Rabbit Anti-Mouse TF IgG (American Diagnostica, Greenwich, CT), followed by secondary phycoerythrin-labeled Donkey Anti-Rabbit IgG (Beckman Coulter). The clinical relevant graft dose (10 IEQs/g) was applied because the rate of TF-positive cells in whole liver cells could be expected rather low.

Immunohistochemical Staining

Pieces of pancreas were collected and snap-frozen in liquid nitrogen. The samples were sectioned and stained with a monoclonal antibody to rat C5a receptor (Hycult biotech), followed by staining using the EnVision kit (Dako, Denmark).

The recipient livers with islet grafts (10 IEQs/g) were retrieved 3 h after the islet infusion. The clinical relevant graft dose was applied to raise the possibility of finding the grafts in the host livers. Tissue samples were snap-frozen in liquid nitrogen and stored at −80°C. Immunohistochemical staining was performed using mouse anti-rat C5b-9.(Santa Cruz Biotechnology, Santa Cruz, CA) and EnVision kit (Dako) for C5b-9 and mouse anti-rat CD11b (Lifespan Biosciences) and Streptavidin-APC (eBioscience, San Diego, CA) for CD11b. The deposits of C5b-9 and the infiltration of CD11b-positive cells on the islet grafts were divided semiquantitatively into five categories (Fig. 4A). More than 70 sections from the each experimental group were evaluated and scored by double-blind evaluations.

Statistical Analysis

All data are expressed as the mean ± SD. Statistical significance was determined using Student's *t* test, Mann-Whitney test, and one-factor analysis of variance with Bonferroni post hoc test. *P* value less than 0.05 was considered significant. Analysis of euglycemic conversion was performed by Kaplan-Meier method with a log-rank test.

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ELSEVIER

The Impact of Ischemic Stress on the Quality of Isolated Pancreatic Islets

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ABSTRACT

Background. Although the ischemic stress of donated organs has been shown to have strong negative effects on islet recovery, the impact on islet quality remains uncertain. In the present study, therefore, we examined the influence of ischemic stress on the expression of inflammatory mediators among isolated islets.

Materials and methods. Islets were isolated from adult porcine pancreata subjected to 16-hour cold ischemia time (CIT) in addition to 40-minute warm ischemia time (WIT). We evaluated the islet yield, islet loss during the first 24 hours in culture, adenosine diphosphate (ADP)/adenosine triphosphate (ATP) ratio, ATP/DNA ratio, glucose-stimulated respiratory activity, in vivo bioassay, and the expression of inflammatory mediators (tissue factor [TF], [MCP-1], macrophage migration inhibitory factor) on the isolated islets. We also analyzed ATP/DNA ratios of the exocrine tissues during isolation procedures.

Results. The islet yield, survival rate during culture, and glucose-stimulated respiratory activity were significantly lower in cases of 16-hour CIT plus 40-minute WIT compared with the control group ($P < .0001$, .0006, and .002, respectively). In contrast, ADP/ATP ratio as well as TF and MCP-1 expressions on the isolated islets were higher among the ischemic group ($P = .005$, .16, and .005, respectively). During isolation procedures, the ATP/DNA of the exocrine tissues was extremely lower in the ischemic compared to the control group ($P < .0001$). Notably, however, both ATP/DNA and ADP/ATP ratio of isolated islets were well preserved even in the ischemic group ($P = .45$ and .40).

Discussion. These data suggest that ischemic stress during the preservation period negatively affects the energy status of exocrine tissues. Destruction of the exocrine tissues, in combination with warm ischemic stress during the isolation procedures, subsequently decreases isolated islet activity, inducing the expression of inflammatory mediators.

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ALTHOUGH THE ISCHEMIC STRESS of the donated organs has a strong negative effect on islet recovery,[1–4] its impact on islet quality remains uncertain. In the present study, therefore, we examined the influence of ischemic stress on the expression of inflammatory mediators in isolated islets. Furthermore, we evaluated the impact of ischemic stress on the energy status of the pancreatic tissues. We employed scanning electrochemical microscopy, a technique in which the tip of a microelectrode monitors the local distribution of electroactive species near the sample surface, to assess islet viability and examine the potency of insulin release.

MATERIALS AND METHODS

Islets were isolated from adult porcine pancreata subjected to 16-hour cold ischemia time (CIT) in addition to 40-minute warm ischemia time (WIT). We evaluated on the isolated islets the yield, islet loss during the first 24 hours in culture, adenosine diphosphate (ADP)/adenosine triphosphate (ATP) and ATP/DNA ratios, glucose-stimulated respiratory activity, in vivo bioassay, and the expression of inflammatory mediators—tissue factor (TF), MCP-1, macrophage migration inhibitory factor. For the respiratory activity assay, we used scanning electrochemical microscopy, which automatically measured the reduced current of far and near points of the samples based upon spherical diffusion theory. The respiratory activity of 10 islets in each group was calculated by detecting the difference in the reduction current around the samples using 2- to 4-μm platinum-coated microelectrodes as described previously.[5] Glucose-stimulated respiratory activity was indicated by the stimulation index of respiratory activity, defined as the ratio of the activity in high (16.7 mmol/L) versus that in basal glucose concentrations (1.67 mmol/L). The ATP/DNA of exocrine tissues was also analyzed during isolation procedures. The ADP/ATP assays, levels of insulin/DNA, and Trypan blue exclusion tests were performed as previously described.[6,7]

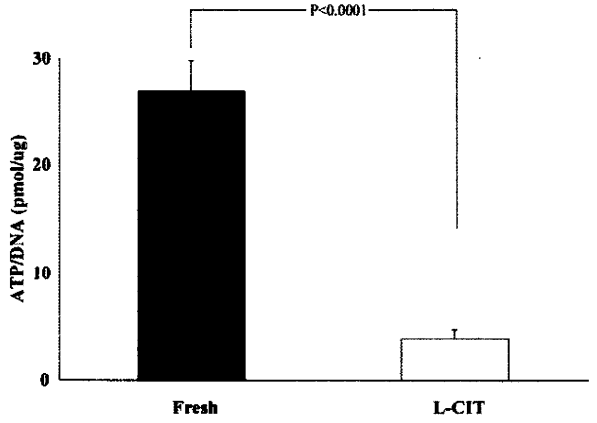

Fig 1. Energy status of the exocrine tissues during isolation procedures is shown. The y-axis indicates the adenosine triphosphate (ATP) amount per DNA in the exocrine tissues. The black bar shows fresh islets (Fresh), and the white bar shows 16-hour cold ischemia times (L-CIT). During isolation procedures, the ATP/DNA of the exocrine tissues was extremely lower in the ischemic group compared to the control group ($P <$ .0001).

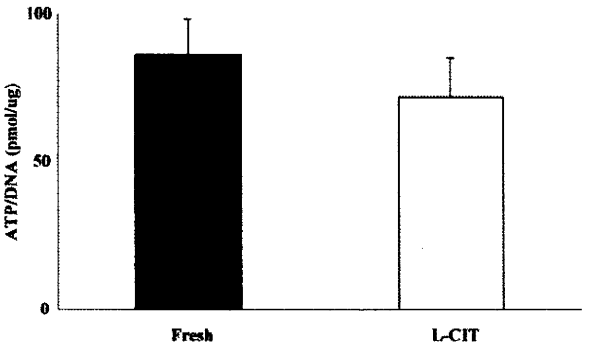

Fig 2. Energy status of the pancreatic islets during isolation procedures is shown. The y-axis indicates the adenosine triphosphate (ATP) amount per DNA in the pancreatic islets. The black bar shows fresh islets (Fresh), and the white bar shows 16-hour cold ischemia times (L-CIT). Energy status of the isolated islets was well preserved even in the ischemic group.

RESULTS

The islet yield, survival rate during culture, and glucose-stimulated respiratory activity were significantly lower among the 16-hour CIT and 40-minute WIT compared with the control group (P = .0001, .0006, and .002, respectively). In contrast, the ADP/ATP ratio as well as TF and MCP-1 expression on the isolated islets were higher in the ischemic group (P = .005, .16, and .005, respectively). During isolation procedures, the ATP/DNA in exocrine tissues was extremely less in the ischemic compared with the control group ($P <$ .0001; Fig 1). Notably, however, both the ATP/DNA (Fig 2) and ADP/ATP ratios of isolated islets were well preserved even among the ischemic group (P = .45 and .40).

DISCUSSION

The success of clinical islet isolations is less than 50% due to several reasons.[8] Ischemic stress is one of the crucial factors affecting the recovery of isolated islets. In the present study, we have shown that ischemic stress during the preservation period negatively affects the energy status of the exocrine tissues, but not of the isolated islets. Furthermore, we have also demonstrated that the expression of inflammatory mediators, such as TF and MCP-1, on isolated islets was remarkably induced in the ischemic compared with the fresh group. These data suggested that the destruction of exocrine tissues during cold preservation, in combination with warm ischemic stress during isolation procedures, could contribute to decrease islet activity possibly due to the release of proteases from exocrine tissues and subsequently induction of the expression of inflammatory mediators on the isolated islets.

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Brain Death in Combination With Warm Ischemic Stress During Isolation Procedures Induces the Expression of Crucial Inflammatory Mediators in the Isolated Islets

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Tissue factor (TF) and monocyte chemoattractant protein-1 (MCP-1) expressed on the islets have been identified as the main trigger of the instant blood-mediated inflammatory reaction (IBMIR) in islet transplantation. Because the key steps that directly induce TF and MCP-1 remain to be determined, we focused on the influence of brain death (BD) on TF and MCP-1 expression in the pancreatic tissues and isolated islets using a rodent model. TF and MCP-1 mRNA levels in the pancreatic tissues were similar between the BD and the control group. However, TF and MCP-1 mRNA in the fresh islets of the BD group were significantly higher than that of the control group ($p < 0.01$). BD may thus be suggested to be of great importance as an initiator of TF and MCP-1 induction in the isolated islets. Furthermore, the upregulation of crucial inflammatory mediators induced by BD could be exacerbated by warm ischemic damage during digestion procedures. In the present study, the islet yield and purity were affected by BD. However, almost no influences were observed with respect to islet viability, indicating that the expression of inflammatory mediators rather than islet viability is more susceptible to BD. According to the change in time course of TF and MCP-1 expression in the isolated islets, the selected time point for islet infusion in current clinical islet transplantation was thus shown to be at its worst level, at least with respect to the damage caused by BD and ischemic stress. In conclusion, BD in combination with warm ischemic stress during isolation procedures induces a high expression of TF and MCP-1 in the isolated islets. In order to reduce the expression of crucial inflammatory mediators in the islet grafts, the management of the pancreas from brain-dead donors with early anti-inflammatory treatments is thus warranted.

Key words: Islets; Transplantation; Brain death; Tissue factor (TF); Monocyte chemoattractant protein-1 (MCP-1)

INTRODUCTION

Islet transplantation is now becoming a viable option for the clinical treatment of type 1 diabetic patients (26,27,32). Although the Edmonton protocol introduced various suggestions for the improvement of islet transplantation, one of the most crucial messages was undoubtedly the necessity for multiple donor organs to render diabetic patient insulin independent. In other words, the Edmonton protocol could be regarded as a refined dose-finding study for the amount of islets needed to cure diabetes. Therefore, in order for islet transplantation to become a widespread standard therapy, diabetes reversal must be achieved with a single donor to reduce the risks and costs, and to increase the availability of transplantation.

However, it is well known that a large part of the transplanted islets tend to be destroyed immediately after transplantation. One of the possible explanations for the poor outcome is the instant blood-mediated inflammatory reaction (IBMIR), which is an innate immune response during islet engraftment (4). Our group, as well as others, have showed that tissue factor (TF) and monocyte chemoattractant protein-1 (MCP-1) expressed on the grafted islets elicit an injurious IBMIR when the islets come into direct contact with the blood

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stream (5,11,17,21,23). Therefore, in order to improve the outcome of clinical islet transplantation, the expression of these crucial inflammatory mediators in the isolated islets should be reduced prior to transplantation.

It is well known that the result of organ transplantation is highly influenced by brain death (BD) (31). In the field of islet transplantation, it was reported by Contreras et al. that BD upregulated the proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α, interleukin-1β (IL-1β), and IL-6 in the serum and pancreatic tissues (6). Toyama et al. also demonstrated that TNF-α, IL-1β, IL-6, and MCP-1 were activated in the isolated islets from rodent BD donors (33). However, the influence of BD on the TF expression in the isolated islets still remains uncertain.

Furthermore, in islet transplantation, unlike other organ transplantation, the islet grafts are placed under hypoxic condition at 37°C during the whole digestion procedure. This period is theoretically considered as one kind of severe warm ischemia (2,12,28).

In the present study, we therefore analyzed the influence of BD on the expression of TF and MCP-1 in both the pancreatic tissues and the isolated islets, in order to understand the key steps that induce crucial inflammatory mediators in the islet grafts.

MATERIALS AND METHODS

Rodent Brain Death Model

All the animals in this study were handled in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (3) and the guidelines for animal experiment and related activities at Tohoku University. BD was induced in male Lewis rats weighing 250–300 g by inflation of a No. 3 Fogarty catheter (Edwards Lifesciences Corporation, Irvine, CA, USA) placed intracranially, as previously described (6,24). Briefly, anesthesia was induced with diethleter and maintained by the IP administration of pentobarbital sodium, Nembutal (Abbott Laboratories, Abbott Park, IL, USA) at a dose of 30 mg/kg. A No. 3 Fogarty catheter was inserted through a 1-mm hole drilled through the skull at 3 mm lateral to the sagittal suture. For the gradual rise in the intracranial pressure, the balloon was inflated with 40 μl/min of distilled water until respiration ceased. The absence of refluxes, apnea, and the maximally dilated and fixed pupils confirmed the condition. The average balloon volume for making BD was 210 μl. The rats were tracheotomized for intubation and mechanically respirated (respiratory rate: 60/min, tidal volume: 10 ml/kg) with SAR-830 Ventilator (CWE, Inc., Ardmore, PA, USA) for 6 h until the removal of the pancreas. The arterial blood pressure was monitored continuously via 24G SURFLO I.V. Catheters (TERUMO, Tokyo, Japan) placed into the right femoral artery and attached to a Dynascope (Fukuda Denshi, Tokyo, Japan). In order to avoid the ischemic effects, the mean arterial pressure (MAP) was maintained over 80 mmHg. When the MAP fell under 80 mmHg during the maintenance of BD, the balloon volume was reduced by 10 μl/min until the animal became normotensive. During the 6-h period, 6 ml/kg/h of normal saline solution was administered IV. The control rats were anesthetized and tracheotomized using the same method. Thereafter, a Fogarty catheter was inserted without ballooning. The control rats were not mechanically respirated because sustained anesthesia was needed for the 6-h ventilation.

Islet Isolation and Culture

Before the removal of the pancreas, the cannulated bile duct was injected with 10 ml of cold Hanks' balanced salt solutions (HBSS) containing 1 g/L collagenase (Sigma type V; Sigma Chemicals, St. Louis, MO, USA). After the addition of 10 ml HBSS, the pancreas was digested at 37°C for 14 min. Thereafter, density-gradient centrifugation was performed using Histopaque-1119 (Sigma Diagnostics, St. Louis, MO, USA) and Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway) to isolate the pancreatic islets. The islet count was performed as islet equivalents (IEQs) under a scaled microscope using diphenylthiocarbazone (Wako, Osaka, Japan) staining (BD, $n = 8$; control, $n = 7$). One IEQ was the islet mass equivalent to a spherical islet of 150 μm in diameter. The islets were cultured in RPMI-1640 containing 5.5 mmol/L glucose and 10% fetal bovine serum at 37°C in 5% CO_2 and humidified air before examination.

Islet Viability and Function

The adenosine triphosphate (ATP)/deoxyribonucleic acid (DNA) ratio was measured to evaluate both the energy status of the isolated islets after 3 h (BD, $n = 4$; control, $n = 4$) and the overnight culture (BD, $n = 6$; control, $n = 5$). A total of 80 IEQs of the islets were used in both groups. The ApoGlow™ kit (Lonza Rockland Inc., Rockland, ME, USA) was used for the ATP measurements as described previously (10). Using the same sample, the DNA content was measured using the DNA Quantify kit (Primary cell, Sapporo, Japan) as described previously (34). We have evaluated the respiratory activity of the isolated islets after 3 h (BD, $n = 4$; control, $n = 4$) and of the overnight culture (BD, $n = 6$; control, $n = 5$) using scanning electrochemical microscopy. The stimulation index of the respiratory activity, defined as the ratio of the respiratory activity in the high glucose (16.7 mmol/L) to that in the basal glucose (1.67 mmol/L), is a novel marker that could be applied as a

rapid and potent predictor for the outcome of clinical islet transplantation (8).

Determination of TF and MCP-1 mRNA in the Pancreatic Tissues

The pieces of the pancreatic tissues from the BD and the control groups were snap-frozen in liquid nitrogen and stored at −80°C until further use ($n = 6$ and $n = 4$, respectively). The total RNA was extracted using the RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The RNA concentration was estimated from the absorbance at 260 nm. The first-strand complementary DNA (cDNA) was synthesized from 2500 ng of total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, IN, USA). The cDNAs were amplified by the polymerase chain reaction (PCR) using a rat TF primer and a probe, rat MCP-1 primer probe set (Nihon Gene Research Laboratories Inc., Sendai, Japan), and rat glyceraldehydes-3-phosphate dehydrogenase (GAPDH) primer probe set (Nihon Gene Research Laboratories Inc.) with a Lightcycler (Roche Diagnostics). The primer sequences of the rat TF from the 5′ to 3′ direction were as follows: forward, AGC TAC TGC TTC TTC GTA CA; reverse, AAA GAC AGT GAC CAG GAA CA. The hybridization (FRET) probe sequences from 5′ to 3′ direction were as follows: TCC CAG GAC ACT CTT CCA TTG CTC AGT G-Fluorescein; LC Red 640-ACT TGG TGA TGC TTT CTG GGC TCT TGT G-phosphorylation. In order to perform the PCR for TF and GAPDH, an initial denaturation step of 10 min at 95°C was followed by 40 cycles of 10 s at 95°C, an annealing of 15 s at 60°C, and extension of 7 s at 72°C. For MCP-1, an initial denaturation step of 10 min at 95°C was followed by 40 cycles of 10 s at 95°C, an annealing of 15 s at 62°C, and extension of 6 s at 72°C.

Determination of TF and MCP-1 mRNA in the Fresh Isolated Islets

The total RNA extracted from 300 IEQs of islets with a 3-h culture was prepared using the RNeasy Micro Kit (Qiagen) according to the manufacturer's protocol (BD, $n = 3$; control, $n = 3$). The RNA concentration was estimated from the absorbance at 260 nm. The first-strand cDNA was synthesized from 100 ng of total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). The cDNAs were amplified by PCR using the rat TF primer and a probe, rat MCP-1

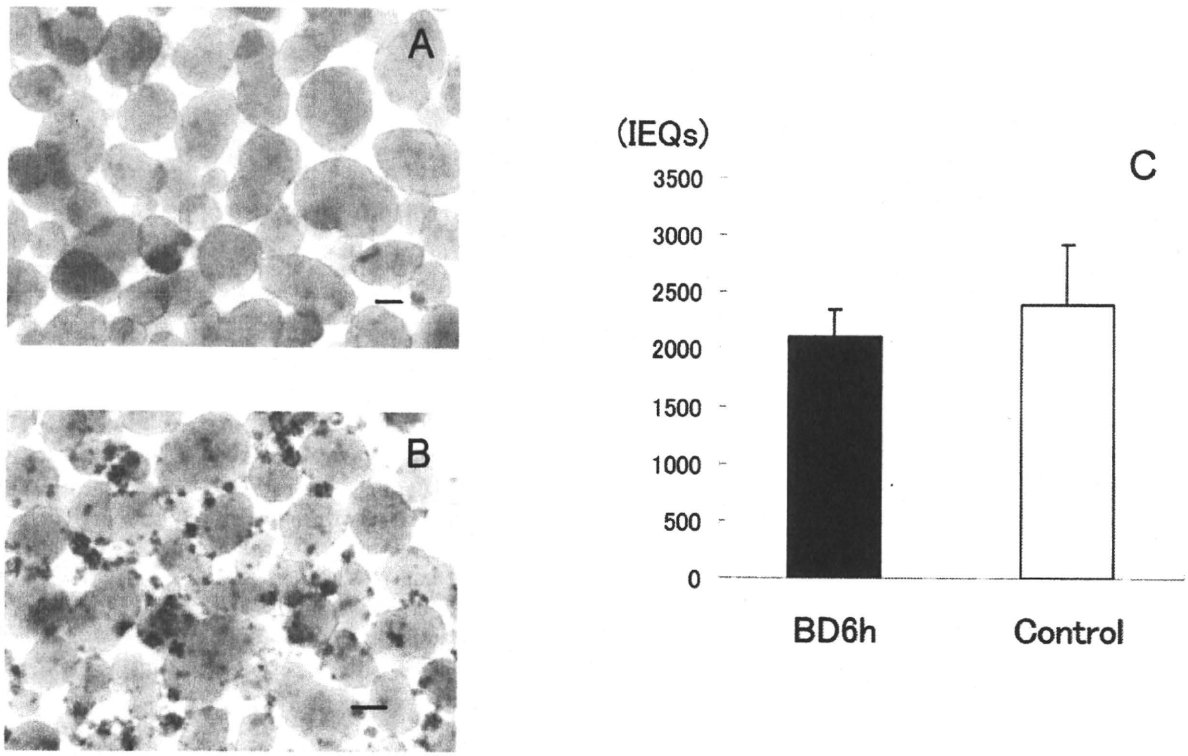

Figure 1. Islet recovery and purity after isolation. The appearance of the isolated islets without brain death (A) and with brain death (B). Scale bar: 100 μm. (C) The isolated islet yield from brain-dead donors (black bar) and control donors (white bar).

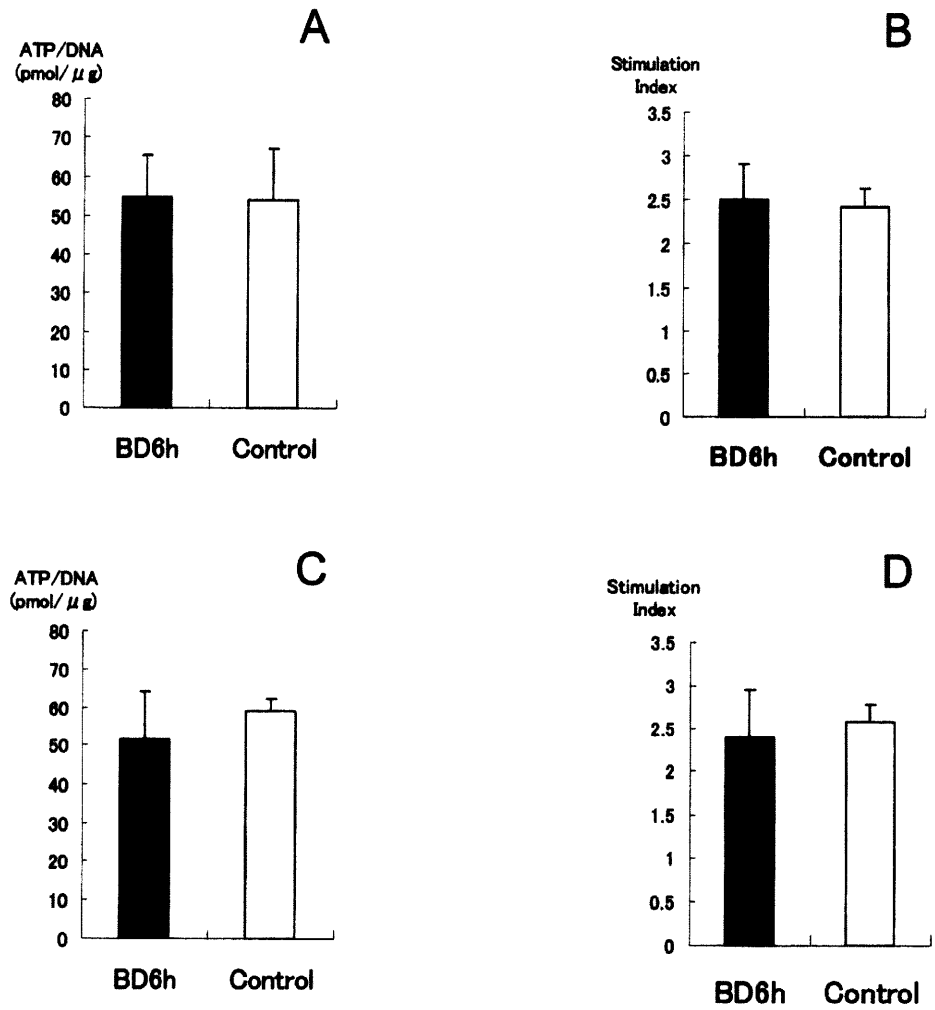

Figure 2. Islet viability and function after isolation. The ATP/DNA ratio in the fresh isolated islets (A) and in the overnight-cultured islets (C). The stimulation index of the respiratory activity in the fresh isolated islets using scanning electrochemical microscopy (B) and in the overnight-cultured islets (D). The black bar represents the brain-dead donors and the white bar represents the control donors.

primer probe set, and rat GAPDH primer probe set with a Lightcycler. The primer and probe sequences and the PCR conditions were the same as above.

Time Course Change of TF and MCP-1 mRNA Expression in the Isolated Islets

The isolated islets from one donor were divided equally into seven groups (BD, $n = 3$; control, $n = 4$). Each group was cultured for 3, 6, 12, 18, 24, 48, and 72 h, respectively. Just before examination, the cultured islets were handpicked for preparation. The TF and MCP-1 mRNA in each group were analyzed using the same procedure as above.

Statistical Analyses

All the data are expressed as the mean ± SD. The comparisons between two groups were performed by using the Student *t*-test. One-factor ANOVA with Bonferroni-Dunn post hoc test was used to determine the time course effect of the TF and MCP-1 expression in the isolated islets. Statistical significance was established at $p < 0.05$.

RESULTS

Islet Recovery and Purity After Isolation

The islet yield was considerably lower (BD, 2110 ± 231 IEQs; control, 2390 ± 528 IEQs; $p = 0.19$), and the

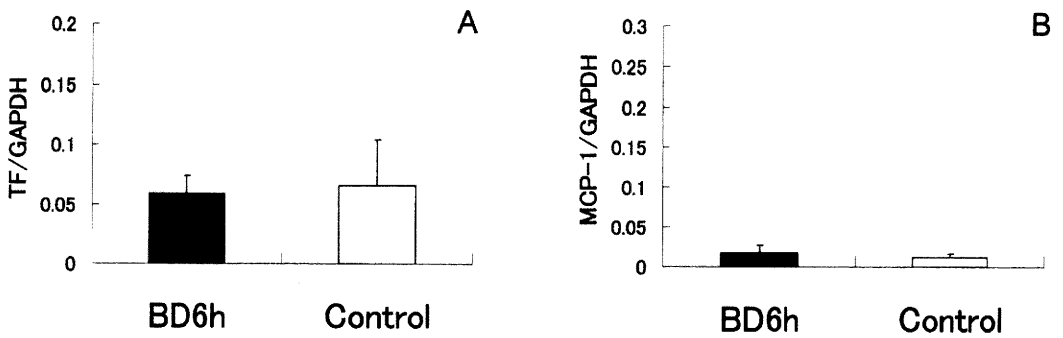

Figure 3. The mRNA expression of TF and MCP-1 in the pancreatic tissues. The mRNA expression of TF (A) and MCP-1 (B) in the pancreatic tissues from the donors with/without brain death was analyzed using a real-time PCR assay. The black bar represents the brain-dead donors and the white bar represents the control donors.

purity was significantly lower in the BD group in comparison to the control group (BD 87.7 ± 7.5%; control 97.0 ± 2.6%; $p = 0.002$) (Fig. 1).

Islet Viability and Function After Isolation

Unexpectedly, the ATP/DNA ratio and the respiratory activity were comparable between the groups, irrespective of the time point (ATP/DNA 3-h BD 54.7 ± 10.7; control 54.0 ± 13.3, $p = 0.94$; ATP/DNA overnight BD 51.6 ± 12.8; control 59.1 ± 3.47, $p = 0.20$; respiratory activity 3-h BD 2.50 ± 0.41; control 2.42 ± 0.21, $p = 0.74$; respiratory activity overnight BD 2.39 ± 0.55; control 2.58 ± 0.19, $p = 0.45$) (Fig. 2).

mRNA Expression of TF and MCP-1 in the Pancreatic Tissues

The TF and MCP-1 mRNA levels in the pancreatic tissues prior to the isolation procedures were similar between the BD and control groups (TF/GAPDH BD 0.059 ± 0.015; control 0.066 ± 0.038, $p = 0.67$, MCP-1/GAPDH BD 0.018 ± 0.0098; control 0.012 ± 0.0040, $p = 0.40$) (Fig. 3).

mRNA Expression of TF and MCP-1 in the Fresh Isolated Islets

The TF mRNA levels in the fresh isolated islets of the BD group was significantly higher in comparison to the control group (TF/GAPDH BD 0.148 ± 0.010; control 0.061 ± 0.0096, $p = 0.0004$). The MCP-1 mRNA levels in the fresh isolated islets of the BD group was also significantly higher in comparison to the control group (MCP-1/GAPDH BD 0.240 ± 0.035; control 0.140 ± 0.0070, $p = 0.008$) (Fig. 4).

Time Course Change of TF and MCP-1 mRNA Expression in the Isolated Islets

In the fresh islets, a sharp difference was observed between the BD and the control groups with respect to

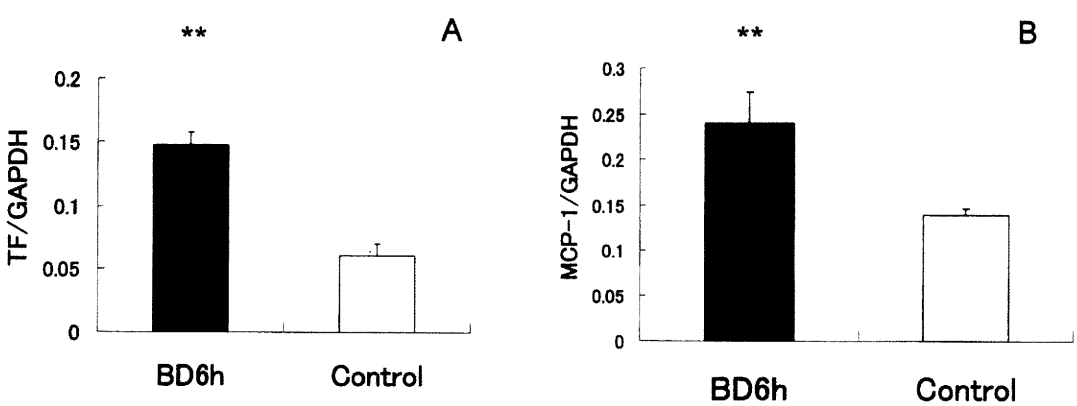

Figure 4. The mRNA expression of TF and MCP-1 in the fresh isolated islets. The mRNA expression of TF (A) and MCP-1 (B) in the fresh isolated islets from the donors with/without brain death was analyzed using a real-time PCR assay (**$p < 0.01$ vs. control). The black bar represents the brain-dead donors and the white bar represents the control donors.

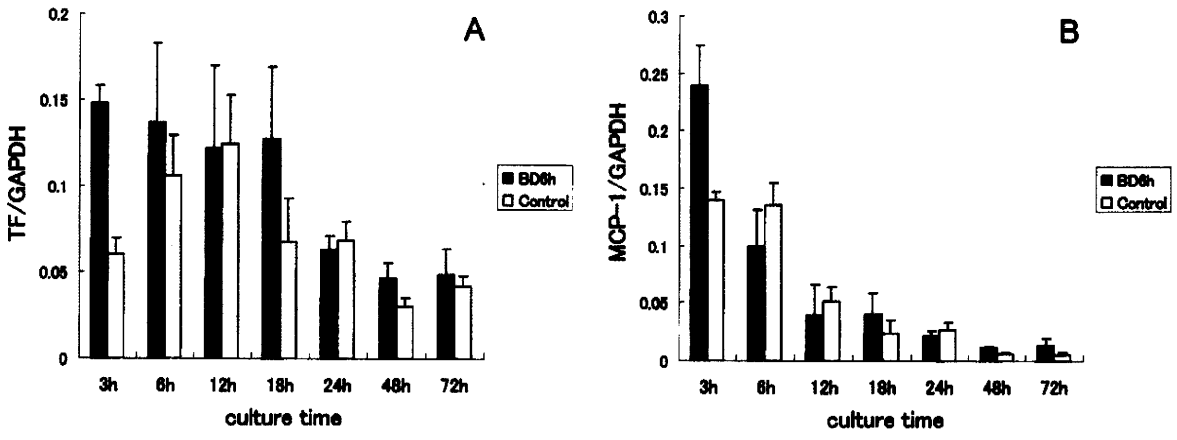

Figure 5. The change in time course of the TF and MCP-1 mRNA expression in the isolated islets. The change in time course of the TF (A) and MCP-1 (B) mRNA expression in the isolated islets from the donors with/without brain death. The black bar represents the brain-dead donors and the white bar represents the control donors. A significant difference was observed in the TF expression in the islets from the brain-dead donors between the 3- and 48-h cultures ($p < 0.05$).

the TF and MCP-1 mRNA expression. In both groups, the TF and MCP-1 mRNA levels decreased time dependently during the culture period. Between the fresh and the 48-h culture, a significant difference was seen in the TF expression in the islets from the BD donors ($p < 0.05$) (Fig. 5).

DISCUSSION

This study demonstrated that BD influences the TF and MCP-1 expressions in the isolated islets but not in the pancreatic tissues prior to the digestion procedure. It is possible that the difference may be attributed to the warm ischemic stress during the digestion procedures. However, TF and MCP-1 were not upregulated in the isolated islets from the donors without BD, thus suggesting that the warm ischemic damage per se during the digestion procedure was not sufficient to induce crucial inflammatory mediators in the islet grafts. We therefore believe that upregulation of the crucial inflammatory mediators induced by BD could be further exacerbated by warm ischemic damage during the digestion procedure.

In the present study, the islet yield and purity were certainly affected by BD. However, the difference was extremely low in comparison to the previous report (6). Moreover, almost no influences were observed in terms of the islet viability. One of the possible explanations for this discrepancy is the difference of the isolation procedure. In our methods, the pancreatic tissues were kept on ice during the whole procedure, with the exception of the digestion phase. Furthermore, at the density-gradient centrifugation phase, we applied Histopaque-1119 and Lymphoprep™. However, a dextran gradient separation was performed in the previous report (6). Therefore, the important message from our present study is that the expression of the inflammatory mediators, rather than the islet viability, is more susceptible to BD. In other words, it seems more likely that the current standard methods for the islet quality assessment are not suitable tools for detecting graft damage in the early phase of islet transplantation.

Although the islet culture modulated the inflammatory status of the human pancreatic islets (19,20), the effect remains controversial. We therefore investigated the change in time course of the TF and MCP-1 expressions in the isolated islets with/without BD. As shown in Figure 5, the influence of BD and ischemic stress during the isolation procedure was most pronounced after a 3-h culture. Clinical islet transplantation is currently being performed in most institutions, using fresh islets according to the Edmonton protocol (26,27). Notably, in most clinical cases, the islet grafts with a 3-h culture are used in fresh islet transplantation because several quality tests and preparation for graft injections are needed. Therefore, the present study clearly showed that the worst time point was selected for islet infusion in current clinical islet transplantation, at least with respect to the damage due to BD and ischemic stress during the isolation procedure.

One way to avoid the adverse effect of BD and ischemic stress is by performing short-term culture of the islet grafts. This may be one of the possible explanations for the outstanding result of the clinical islet transplantation reported by Froud et al. (7) and by Hering et al. (14). In support of our previous findings (30), the present study also implied that the TF expression in the islets without BD was substantially upregulated during overnight culturing. The reason for this is uncertain but the hypoxic

condition during culturing may at least be partially responsible for the TF induction (1,13,25). Taking into account this finding, it is most likely that the 48-h culture introduced in the previous reports (7,14) was reasonable. However, it was also reported that the number and function of the isolated islets may decreased after a short-term culture (9,15,16,18,22). This is consistent with our previous findings that isolated islets without culture are more beneficial to the transplant outcome under a current style of culture (29). Moreover, it may be difficult to maintain an adequate number of islets from the marginal donors after substantial periods of culturing, especially in Japan, where only non-heart-beating donors are available for islet transplantation. Therefore, further improvements are required to maintain the number and function of the cultured islets.Another way to avoid the adverse effect of BD and ischemic stress is to establish effective anti-inflammatory treatments through whole steps from the intensive care unit to the digestion procedure.

In conclusion, BD in combination with warm ischemic stress during the isolation procedures induces high expression of TF and MCP-1 in the isolated islets. In order to reduce the expression of the crucial inflammatory mediators in the islet grafts, the management of the pancreas from brain-dead donors with early anti-inflammatory treatments is warranted.

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ELSEVIER

Superiority of Fresh Islets Compared With Cultured Islets

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ABSTRACT

Introduction. It has recently been reported that the outcomes of islet transplantation with short periods of culture are comparable with those of freshly isolated islets. To clarify the influence of culture, fresh islets were compared with cultured islets in terms of quality.

Materials and Methods. The quality of freshly isolated islets was compared with that of cultured islets with CMRL 1066 including 10% allogeneic serum, CMRL 1066 including 0.5% human serum albumin, or Miami medium. We evaluated static glucose stimulation tests, insulin/DNA contents, ADP/ATP ratios, and an intraportal transplantation model into syngeneic diabetic rats. The expression of inflammatory mediators in the islets was examined using Western blotting for tissue factor (TF), which is the initiator of detrimental instant, blood-mediated, inflammatory reactions (IBMIR).

Results. Although the survival rate was similar in all groups, the stimulation index upon glucose challenge and the insulin/DNA ratio were significantly higher among fresh islets. Most importantly, the expression of TF on islets was significantly lower in fresh islets, suggesting that culture enhanced TF-dependent IBMIR after transplantation. In an in vivo transplantation model, the curative rate and insulin production by the recipient liver was considerably greater in the fresh islet group.

Conclusions. Isolated islets without prior culture showed results superior to cultured islets.

ALTHOUGH one of the key factors of the Edmonton protocol is transplantation of fresh islets just after isolation, comparable outcomes of islet transplantation have recently been reported with a short-period culture. To clarify the influence of culture on isolated pancreatic islets, we compared fresh islets with those cultured using several current techniques, in terms of islet quality, including not only viability but also inflammatory mediator expressed on the islets. Also in this study, we examined the effects of correcting the islet dose just prior to rat islet transplantation.

MATERIALS AND METHODS

The quality of freshly isolated islets was compared with that of islets cultured using CMRL 1066 plus 10% allogeneic serum, CMRL 1066 plus 0.5% human serum albumin (HAS), or Miami medium (Miami). The evaluation used islets survival rates, visual scoring, static glucose stimulation tests,[1] insulin/DNA contents,[1] ADP/ATP ratios,[2] and intraportal transplantation models into syngeneic Streptozotocin-induced diabetic rats. The influence of culture on the expression of inflammatory mediators in the islets was examined using Western blotting assay for tissue factor (TF), which is the initiator of detrimental instant blood-mediated inflammatory reactions (IBMIR).[3,4] Statistical analyses were performed using analysis of variance (ANOVA).

RESULTS

Although the survival rate was similar in all groups, the visual scoring was lower among the Miami group. Stimulation index on glucose challenge tests was higher in the fresh group: fresh, 17.89 ± 4.93; serum, 13.69 ± 5.44; HSA, 5.36 ± 1.60; and Miami, 2.69 ± 0.82 (P = .008). Insulin/DNA ratios revealed a similar tendency as the glucose challenge tests: fresh, 1.02 ± 0.07; serum, 0.83 ± 0.11; HSA, 0.52 ± 0.07; and Miami, 0.37 ± 0.08 (P = .0001). The ADP/ATP ratios were lower for both the fresh and serum groups than the others: fresh, 0.047 ± 0.021; serum, 0.054 ±

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0.017; HSA, 0.086 ± 0.004; and Miami, 0.084 ± 0.026 (P = .38), suggesting a relatively lower number of apoptotic islets in both the fresh and the serum groups. Most importantly, the expression of TF on the islets was significantly lower in fresh islets (P = .01), suggesting that a current culture method could enhance TF-dependent IBMIR after transplantation. In an in vivo transplantation model, the curative rate and insulin amount in the recipient liver were considerably higher in the fresh islet than the other groups (Fig 1). Intravenous glucose tolerance was also ameliorated in the fresh and serum groups rather than the nonserum group: AUC of fresh = 25,376.71 ± 973.9; serum, 24,691.43 ± 1233.1; and Miami, 30,654.86 ± 2706.1 (P = .06) and the glucose disappearance rate (Kg) of fresh = 1.231 ± 0.101; serum, 1.203 ± 0.12, and Miami, 0.851 ± 0.174 (P = .114). Notably, the disadvantage of the nonserum culture groups was recovered by augmenting the graft amount just prior to transplantation (data not shown).

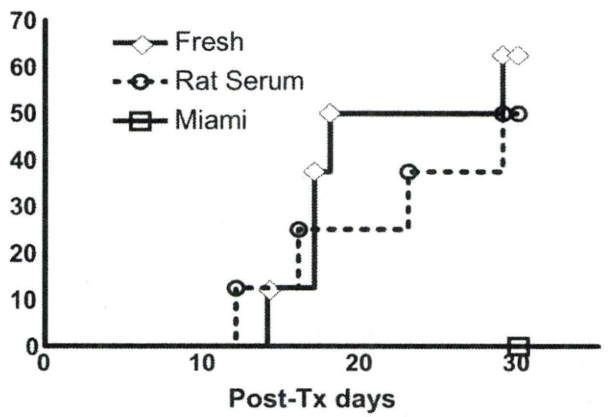

Fig 1. The influence of culture on islet quality was evaluated by intraportal transplantation of syngeneic islets into streptozotocin-induced diabetic rats. In an in vivo transplantation model, the curative rate was higher in the fresh islet group than in the others.

DISCUSSION

Isolated islets without prior culture showed results beneficial to transplantation compared with current culture methods. Further improvements are required to optimize a substitute for serum supplements using a clinically available model.

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Dissecting the instant blood-mediated inflammatory reaction in islet xenotransplantation

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Abstract: Background: A massive destruction of transplanted tissue occurs immediately following transplantation of pancreatic islets from pig to non-human primates. The detrimental instant blood-mediated inflammatory reaction (IBMIR), triggered by the porcine islets, is a likely explanation for this tissue loss. This reaction may also be responsible for mediating an adaptive immune response in the recipient that requires a heavy immunosuppressive regimen.
Materials and methods: Low molecular weight dextran sulfate (LMW-DS) and the complement inhibitor Compstatin were used in a combination of in vitro and in vivo studies designed to dissect the xenogeneic IBMIR in a non-human primate model of pancreatic islet transplantation. Adult porcine islets (10 000 IEQs/kg) were transplanted intraportally into three pairs of cynomolgus monkeys that had been treated with LMW-DS or heparin (control), and the effects on the IBMIR were characterized. Porcine islets were also incubated in human blood plasma in vitro to assess complement inhibition by LMW-DS and Compstatin.
Results: Morphological scoring and immunohistochemical staining revealed that the severe islet destruction and macrophage, neutrophilic granulocyte, and T-cell infiltration observed in the control (heparin-treated) animals were abrogated in the LMW-DS-treated monkeys. Both coagulation and complement activation were significantly reduced in monkeys treated with LMW-DS, but IgM and complement fragments were still found on the islet surface. This residual complement activation could be inhibited by Compstatin in vitro.
Conclusions: The xenogeneic IBMIR in this non-human primate model is characterized by an immediate binding of antibodies that triggers deleterious complement activation and a subsequent clotting reaction that leads to further complement activation. The effectiveness of LMW-DS (in vivo and in vitro) and Compstatin (in vitro) in inhibiting this IBMIR provides the basis for a protocol that can be used to abrogate the IBMIR in pig-human clinical islet transplantation.

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Introduction

Clinical islet transplantation is a promising treatment for type I diabetic patients. The improved protocol introduced by Shapiro et al. in 2000 [1] has greatly improved the results of this approach, but despite these advances, islets derived from more than one donor pancreas are still generally required to cure an individual diabetic patient. This requirement has drawn

attention to the limited availability of human islets for transplantation and sparked interest in the use of islets from alternative sources, particularly the pig [2].

One obstacle to be surmounted before porcine islets can be used in clinical islet xenotransplantation is the injurious instant blood-mediated inflammatory reaction (IBMIR) that elicits massive cell destruction when porcine islets are exposed to fresh human blood [3]. The xenogeneic IBMIR is characterized by activation of platelets and the coagulation and complement systems. This activation is accompanied by infiltration of the islets by polymorphonuclear lymphocytes (PMNs) [3].

The occurrence of this deleterious IBMIR is supported by studies demonstrating that porcine islets are immediately destroyed when transplanted intraportally into the liver of non-human primates [4,5]. Kirchhof et al. [6] reported that most of their porcine islet xenografts (22–73%) were substantially damaged after 24 h when transplanted into non-immunosuppressed monkeys. The grafts exhibited cell destruction, with deposition of coagulation and complement components and platelets, supporting the contention that the IBMIR contributes to the islet damage in this model. Further support for the importance of the IBMIR comes from the observation that although porcine islets can successfully survive in the liver of diabetic monkeys for more than 100 days [7,8], very high quantities of islets (25 000 and 50 000 IEQs/kg BW, respectively) are needed to produce normoglycemia in the monkeys, indicating that there is a substantial loss of transplanted tissue.

Using in vitro and small-animal models, we have previously demonstrated that low molecular weight dextran sulfate (LMW-DS) effectively inhibits the activation of the coagulation and complement systems and the infiltration of leukocytes into the islets during xenogeneic islet transplantation [9]. In this study, we have used LMW-DS together with Compstatin, a new peptide complement inhibitor that is suitable for use in clinical islet xenotransplantation [10], to dissect the IBMIR in in vivo (LMW-DS) and in in vitro (LMW-DS and Compstatin) xenotransplantation models. The results of these studies have broadened our understanding of the innate immune events that might be expected to occur in clinical islet xenotransplantation and have provided the basis for a protocol for abrogating the IBMIR during clinical transplantation with porcine pancreatic islets.

Materials and methods

Animals

Retired breeder pigs, weighing approximately 200 kg, were used as donors for all experiments. Cynomolgus monkeys (*Macaca fascicularis;* 3- to 6-yr old; 4–6 kg) were used as recipients. All procedures using pigs were approved by the Swedish Council on Medical Ethics. Cynomolgus monkeys were housed according to the guidelines of the Belgian Ministry of Agriculture and Animal Care. All procedures using monkeys were approved by the local Ethical Committee for Animal Care of the Université Catholique de Louvain.

Islet isolation

Isolation of porcine islets was performed as previously described [11], with minimal modifications. Purified islet fractions were pooled and cultured at 37 °C in a humidified atmosphere with 5% CO_2 in CMRL 1066 medium (Biochrom, Berlin, Germany) supplemented with 20% heat inactivated porcine serum, 2 mM *N*-acetyl-L-alanyl-L-glutamine, 10 mM N-2-hydroxyethylpiperazine-N1-2-ethanesulfonic acid (HEPES), 100 IU/ml penicillin, 100 μg/ml streptomycin (Biochrom), and 20 μg/ml ciprofloxacin (Bayer, Leverkusen, Germany).

Evaluation of porcine islet quality

The in vitro function and viability of the porcine islets were assessed after overnight culture as described above. Islet viability determined by trypan blue exclusion assay and insulin release defined as the ratio of stimulated (16.5 mM glucose) to basal (1.65 mM glucose) insulin release, were performed as previously described [11]. For assays of islet insulin content, 1-ml samples were washed with distilled water, then sonicated (Labsonic; Braun, Melsungen, Germany) for 30 s. A 200-μl aliquot of each sample was subjected to acid–ethanol extraction (0.18 M HCl) and used for insulin measurement. Another 100-μl aliquot was dried at 60 °C overnight for consecutive fluorometric DNA assays [12], using calf thymus DNA type I (Sigma, Deisenhofen, Germany) as a standard; 24-h insulin secretion: Immediately after a medium change, 500-μl samples of the medium were taken in duplicate from the remaining Petri dishes for determination of insulin accumulation in the medium, to calculate the 24-h insulin secretion by the islets. Transplantation of islets into nude mice was performed as previously described [11].

Islet transplantation

Before each experiment, the monkeys were sedated with 6 mg/kg Zoletil® 100 (Virbac S.A., Carros, France) intramuscularly, and general anesthesia was maintained with inhalation of 1–3% enflurane. During the experiment, electrocardiogram, blood pressure, and pulse were continuously monitored. The pig islets were suspended in 10 ml of transplant medium (Ringer acetate; Braun) with 25% (w/v) human albumin and 5 mM glucose and injected slowly into the portal vein over the course of 5 min. The animals were treated in pairs, with each pair being given porcine islets from the same donor. One recipient in each pair received LMW-DS (monkeys M5, M7, and M9) and the other heparin as a control (monkeys M6, M8, and M10):

1. Intravenous infusion of LMW-DS (MW 5000; Sigma Chemicals, St Louis, MO, USA) was performed via an indwelling catheter placed in the jugular vein or via a catheter in the portal vein. In the LMW-DS-treated groups, dextran with a molecular weight of 1 kDa (Promiten, Pharmalink AB, Upplands Väsby, Sweden) was injected i.v. just before islet transplantations to avoid the risk of anaphylactoid reactions triggered by LMW-DS. After the injection of Promiten, the monkey received a bolus dose of LMW-DS (1.5 mg/kg) i.v. prior to islet infusion, followed by 3.0 mg/kg LMW-DS given together with the porcine islets (10 000 IEQs/kg of recipient BW). The transplantation was followed by a continuous i.v. infusion of LMW-DS (1.0–1.5 mg/kg/h) for up to 24 h.
2. In the heparin-treated groups, the monkeys received a continuous i.v. infusion of heparin (35 U/kg of BW, heparin LEO, 5000 U/ml; LEO Pharma Nordic, Malmö, Sweden) for 24 h, beginning immediately prior to islet infusion.

Blood samples

All blood samples from the monkeys were drawn from a femoral vein catheter at 0, 15, 30, 45, 60, 75, 90, 120 min and 24 h after transplantation. Blood was also drawn from healthy human blood donors into 7-ml tubes containing citrate, EDTA, or 500 μg of hirudin, a specific inhibitor of thrombin (Refludan; Pharmion Ltd, Cambridge, UK). To obtain plasma, the samples were centrifuged at 4500 ***g*** for 5 min. If not immediately analyzed for activated partial thromboplastin time (APTT), the samples were stored at 70 °C.

Analyses of blood and plasma samples

Activated partial thromboplastin time measurements were performed as previously described [13]. Platelet counts and differential leukocyte counts were obtained using a Coulter-AcT-diff analyzer (Beckman Coulter, Miami, FL, USA) and EDTA-treated blood. Plasma levels of thrombin–antithrombin (TAT) were quantified using commercially available EIA kits (TAT; Behringswerke, Marburg, Germany). C3a generation was measured in plasma according to the method of Nilsson Ekdahl et al. [14], and sC5b–9 was analyzed using a modification of the enzyme immunoassay described by Nilsson Ekdahl et al. [14] and Mollnes et al. [15].

Plasma interleukin-6 (IL-6), tumor necrosis factor-α (TNFα), IL-1β, and C-reactive protein (CRP) were measured using a commercial ELISA kit (Immulite IL-6, Immulite TNFα, Immulite IL-1β, and Immulite High Sensitivity CRP, respectively; Diagnostic Products Corporation, Los Angeles, CA, USA).

Histological and immunohistochemical staining

The monkey livers bearing transplanted adult porcine islet grafts were retrieved 24 h after transplantation, at a time when the major part of the IBMIR has generally occurred [3]. Some tissue samples were snap-frozen in isopentane and stored at −70 °C. Other samples were fixed with 4% *p*-formaldehyde overnight, and then embedded in paraffin. The samples were sectioned and subsequently used for morphological scoring after hematoxylin eosin staining.

Immunohistochemical staining was carried out using guinea pig anti-insulin (DAKO, Carpenteria, CA, USA), mouse anti-human neutrophil elastase (DAKO), mouse anti-human CD68 (DAKO), mouse anti-human MAC 387 (Abcam, Cambridge, UK), mouse anti-human CD56 (Monosan, Stockholm, Sweden), rabbit anti-human CD3 (DAKO), mouse anti-human CD20 (DAKO), rabbit anti-human IgG and IgM (DAKO), mouse anti-human CD41 (DAKO), mouse anti-human C3c (QUIDEL, San Diego, CA, USA), or goat anti-human C9 (Serotec Ltd Scandinavia, Oslo, Norway).

Treatment of porcine islets with human plasma

Approximately 1000 pig islets/40 μl of plasma (typically 5000 islets in 200 μl) were incubated in human hirudin-treated plasma in heparinized test tubes. Five different islet preparations and five

different plasma preparations were used in these experiments. In some experiments, hirudin-treated plasma was pre-incubated with 20 μM (final concentration) of the potent Compstatin analog, Ac-ICV(1-MeW)QDWGAHRCT-NH_2 [16], for 15 min at 37 °C before the islets were added. The mixture of islets and plasma was then incubated, with gentle shaking, at 37 °C for up to 30 min. After centrifugation, the islets were immediately prepared for complex object parametric analyzer and sorter (COPAS) analysis and confocal microscopy.

Preparation of islets for flow cytometry and confocal microscopy

Ten microliters of fluorescein isothiocyanate (FITC)-labeled antibody recognizing one of the following proteins was added to 5000 islets (corresponding to approximately 10×10^6 cells) in 100 μl of phosphate-buffered saline (PBS) according to the manufacturer's recommendations for single cells: C1q (1.0 g/l; AbCam), C3c (3.2 g/l, for detection of C3b and iC3b; DakoCytomation, Glostrup, Denmark), C4 (1.3 g/l; DakoCytomation), C9 (2.6 g/l; DakoCytomation), mannose-binding lectin (MBL) (0.7 g/l; DakoCytomation), IgG (2.6 g/l; DakoCytomation), or IgM (4.0 g/l; DakoCytomation). Irrelevant mouse IgG1 (0.1 g/l; DakoCytomation) was used as a negative control. For all immunostaining experiments, the islets were incubated, while gently rotating on ice, for 30 min in the presence of an individual antibody. After being washed with PBS, the islets were treated with 1% formaldehyde (Apoteket, Gothenburg, Sweden) and kept on ice until analyzed.

Complex object parametric analyzer and sorter analysis

The fluorescence-stained islets were analyzed using a COPAS (Union Biometrica, Somerville, MA, USA), which is a large particle-based flow cytometry instrument [17]. For each experiment, 1000 islets were analyzed using a 488/514 multi-line laser, and positive cells were sorted out for further analysis by confocal microscopy. The COPAS flow cytometry data were analyzed using CellQuest Pro software (BD Biosciences Immunocytometry Systems, San Jose, CA, USA). Data were reported as mean fluorescent intensity (MFI).

Confocal microscopy

One to two hundred hand-picked, stained islets were contained in a drop of PBS in a small Petri dish and protected from light before examination in the confocal microscope (Zeiss 510 Meta confocal; Carl Zeiss, Jena, Germany). Examination of the stained islets was performed using the 488-nm laser at 10 times magnification. Counter staining with 4′,6-diamidino-2-phenylindole was used to visualize the nuclei of living islet cells.

Complement inhibition assay

One hundred microliters of 10% human serum (v/v), diluted in veronal buffer with 1 mM Ca^{2+}, 0.3 mM Mg^{2+}, 1% (w/v) bovine serum albumin, and 0.05% (v/v) Tween 20, was incubated in the presence of serially diluted LMW-DS and/or Compstatin in wells of microtiter plates for 30 min at 37 °C. The wells were then washed with PBS containing 0.05% (v/v) Tween 20, and the bound C3 fragments were detected using 100 μl of horseradish peroxidase-conjugated anti-C3c (Dako AS, Glostrup, Denmark).

Statistical analysis

All values are expressed as mean ± SEM and were compared using Student's unpaired *t*-test or using the Mann–Whitney test for unpaired samples. Values of $P < 0.05$ were considered statistically significant.

Results

Islet quality

The viability of the adult porcine islets (APIs) used in this study was 96, 100, and 97%, respectively. The stimulation index in the static glucose stimulation (SGS) test was 1.29, 1.84, and 1.40, and the mean insulin content was 613, 149, and 685 μU/IEQs, respectively. Adult porcine islets used in each experiment cured diabetic athymic mice. When we assessed the possible detrimental effect of LMW-DS by incubating APIs from three different pancreata in the presence (100, 1000, or 2500 mg/l) or absence of LMW-DS, we found no adverse effect of LMW-DS on insulin release at any of the concentrations tested (data not shown).

Influence of LMW-DS on blood cell counts, liver and renal function, and cytokine induction in transplanted monkeys

One of the transplanted control monkeys (M6) treated with heparin died 2 h after transplantation due to severe hypoglycemia. The platelet and leukocyte counts and the creatinine levels were kept within normal ranges throughout the experiments with one exception: The granulocyte count

tended to increase 2 h after transplantation in the heparin-treated group (3.9 ± 0.5 vs. 9.6 ± 1.6) compared to that of the LMW-DS-treated group (6.0 ± 0.9 vs. 7.3 ± 1.4). There was also a tendency towards an increase in the liver enzymes at 24 h after islet transplantation in the heparin-treated monkeys [heparin vs. LMW-DS: aspartate aminotransferase (AST), 434.7 ± 126.4 vs. 288.0 ± 130.4; alanine aminotransferase (ALT), 207.7 ± 68.7 vs. 116.8 ± 47.7]. No bleedings or other adverse reactions were observed.

Influence of LMW-DS on cytokine induction was examined using three healthy monkeys. Only a slight increase in the IL-6 levels was seen 24 h after administration of LMW-DS in two out of three healthy monkeys (maximum 27 μg/l). However, LMW-DS did not trigger an increase of plasma IL-1β, TNFα, or CRP (not shown).

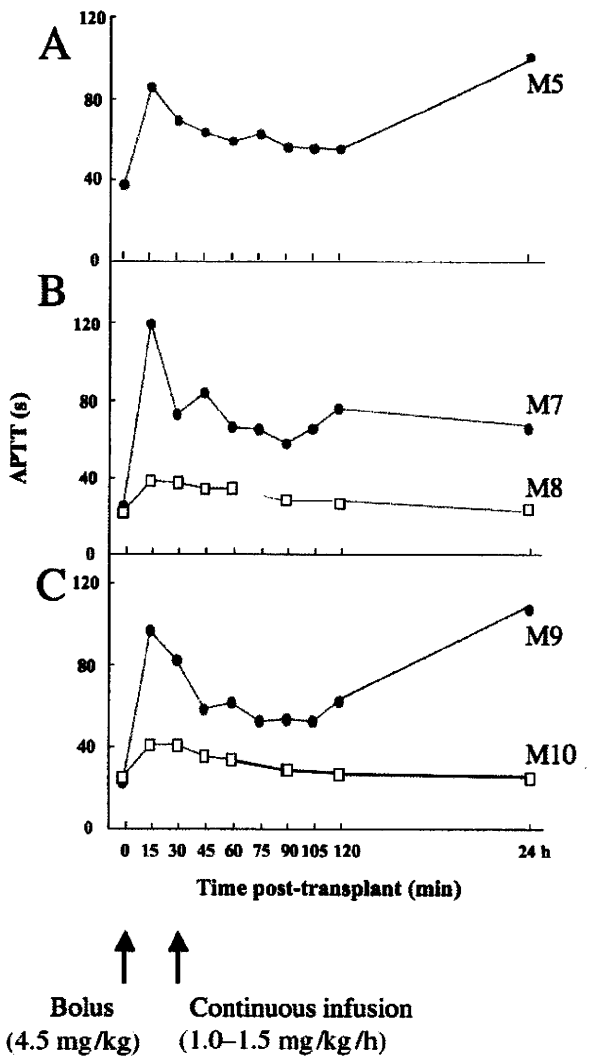

Fig. 1. Plasma APTT values in transplanted diabetic monkeys (M5 and M7–M10) treated with heparin (squares) or LMW-DS (circles).

LMW-DS concentrations in transplanted monkeys

Previous studies showed a strong correlation between APTT and the concentration of LMW-DS [13]. Plasma APTT was therefore used to follow the blood concentration of LMW-DS in the transplanted monkeys (Fig. 1). The APTT in monkeys treated with heparin at concentrations routinely used in clinical islet transplantation (i.e. 500–1000 IU/l) was kept constant at 25–40 s throughout the whole study period. The APTT in monkeys treated with LMW-DS reached around 100 s at 15 min after islet infusion, but gradually decreased during 2 h after islet transplantation. After 24 h, the APTTs in monkeys M5, M7, and M9 were 101, 66, and 107 s, respectively. Thus, both M5 and M9 had higher concentrations of LMW-DS compared with M7.

Inhibition of the IBMIR by LMW-DS during pig islet xenotransplantation

Low molecular weight dextran sulfate, unlike heparin, diminished both the coagulation and the complement cascade activation in two sets of monkeys. The increase of coagulation marker TAT was effectively inhibited by LMW-DS (Fig. 2). The complement activation parameters C3a and sC5b–9 were also suppressed by LMW-DS in both treated monkeys compared to the controls during the study period (Fig. 2). In M5, TAT was totally suppressed while C3a was more difficult to evaluate without the corresponding control (M6). In this animals, C5b–9 was not assessed due to an insufficient amount of plasma samples.

Histological evaluation of grafted pig islets after intraportal transplantation into monkeys treated with LMW-DS or heparin

Morphological aspects of islet grafts were scored semi-quantitatively according to the representative examples shown in Fig. 3. As summarized in Table 1, histology of the transplanted grafts were well kept in the monkeys treated with LMW-DS in both settings of experiments. However, the beneficial effects of LMW-DS were more pronounced in M5 and M9 compared with M7. Indeed, the completely preserved islets (score 0 in all categories) were encountered in 37.2 and 44% of the LMW-DS treated animals M5 and M9 (LMW-DS treated monkeys), respectively, but in only 22% of the control M10.