Abstract

Tissue factor (TF) and monocyte chemoattractant protein (MCP)-1 expressed on the islets have been identified as the main trigger of the instant blood-mediated inflammatory reaction (IBMIR), in islet transplantation. Since 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. Tissue factor 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 control group ($p < 0.01$). It may be suggested that BD is of great importance as an initiator of TF and MCP-1 induction in the isolated islets. Furthermore, up-regulation of crucial inflammatory mediators induced by BD could be exacerbated by warm ischemic damage during digestion procedure. In the present study, the islet yield and purity were affected by BD. However, almost no influences were observed in terms of islet viability, indicating that the expression of inflammatory mediators rather than islet viability is more susceptible to BD. According to the time course change of TF and MCP-1 expression in the isolated islets, it was revealed that the selected time point for islet

infusion in current clinical islet transplantation was at its worst level at least in terms of 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. To reduce the expression of crucial inflammatory mediators in the islet grafts, management of the pancreas from brain-dead donors with early anti-inflammatory treatments is warranted.

Key words: islets, transplantation, brain death, tissue factor, monocyte chemoattractant protein-1

Introduction

Islet transplantation is now becoming one option for clinical treatments for type 1 diabetic patients (22,23). Although the Edmonton protocol introduced various suggestions for improvements of islet transplantation, one of the most crucial messages is no doubt the necessity of multiple donor organs to make one diabetic patient insulin-independent. In other words, Edmonton protocol could be regarded as a refined dose-finding study for the amount of islets needed to cure diabetes. Therefore to make islet transplantation 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 has been well known that a large part of the transplanted islets is 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 (3). We and other groups have thus far showed that tissue factor (TF) and monocyte chemoattractant protein (MCP)-1 expressed on the grafted islets elicits an injurious IBMIR when the islets come in direct contact with the blood stream (4,9,15,18,19). 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) (26). In the field of islet transplantation, it was reported by Contreras et al. that BD up-regulated pro-inflammatory cytokines such as tumor necrosis factor (TNF) -α, interleukin (IL)-1β, and IL-6 in the serum and pancreatic tissues (5). 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 (27). However, the influence of BD on TF expression in the isolated islets remains uncertain.

Furthermore in islet transplantation, unlike other organ transplantation, the islet grafts are particularly placed under hypoxic condition at 37°C during whole digestion procedure. This period is theoretically considered as one kind of severe warm ischemia (2,10,24).

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 isolated islets to dissect the key steps that induce crucial inflammatory mediators in the islet grafts.

Materials and methods

Rodent brain death model

All animals in the study were handled in accordance with *the Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health and *The Guidelines for Animal Experiment and Related Activities at Tohoku University*. Brain death 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 intra-cranially, as previously described (5,20). Briefly, anesthesia was induced with diethleter and maintained by intraperitoneal 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 1mm hole drilled through the skull 3mm lateral to the sagittal suture. For raising the intracranial pressure gradually, the balloon was inflated with 40 μl/min of distilled water until respiration ceased. The absence of refluxes, apnea, and 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 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 Dynascope (Fukuda Denshi, Tokyo, Japan). To avoid ischemic effects, mean arterial pressure (MAP) was kept over 80 mmHg. When MAP became under 80 mmHg during maintenance of BD, 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 received intravenously. The control rats were anesthetized and tracheotomized with the same method. Then a Fogarty catheter was inserted without ballooning. The control rats weren't mechanically respirated because sustained anesthesia was needed for 6-h ventilation.

Islet isolation and culture

Before removal of the pancreas, the cannulated bile duct was injected with 10ml of cold Hanks' Balanced Salt Solutions (HBSS) containing 1 g/L collagenase (Sigma type V; Sigma Chemicals, St. Louis, MO, USA). After addition of 10 ml HBSS, the pancreas was digested at 37°C for 14 minutes. Thereafter, the density-gradient centrifugation was performed using Histopaque-1119 (Sigma Diagnostics, St. Louis, MO, USA) and Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway) to isolate 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. 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

Adenosine triphosphate (ATP)/deoxyribonucleic acid (DNA) ratio was measured to evaluate the energy status of isolated islets after 3-h (BD; n=4, control; n=4) and overnight culture (BD; n=6, control; n=5). 80 IEQs of islets were used in both groups. The ApoGlow™ kit (Lonza Rockland Inc, Rockland, ME, USA) was used for ATP measurement as described previously (8). Using the same sample, the DNA content was measured using DNA Quantify kit (Primary cell, Sapporo, Japan) as described previously (28). We have evaluated the respiratory activity of isolated islets after 3-h (BD; n=4, control; n=4) and 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 high glucose (16.7 mmol/L) against that in 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 (7).

Determination of TF and MCP-1 mRNA in the pancreatic tissues

Pieces of pancreatic tissues from BD and control groups snap-frozen in liquid nitrogen, were stored at -80°C until use (n=6 and n=4, respectively). Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. RNA concentration was estimated from absorbance at 260 nm. First-strand complementary DNA (cDNA) was synthesized from 2500 ng of total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, IN, USA). The cDNAs were amplified by polymerase chain reaction (PCR) using rat TF primer and probe (as follows), 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, Indianapolis, IN, USA). Primer sequences of rat TF are given from 5' to 3' as follows: forward AGC TAC TGC TTC TTC GTA CA and reverse AAA GAC AGT GAC CAG GAA CA. A hybridization (FRET) probe sequences are given from 5' to 3' 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. 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

Total RNA extracted from 300 IEQs of islets with 3-hour culture was prepared using RNeasy Micro Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol (BD; n=3, control; n=3). RNA concentration was estimated from absorbance at 260nm. First-strand cDNA was synthesized from 100 ng of total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, IN, USA). The cDNAs were amplified by PCR using rat TF primer and probe, rat MCP-1 primer probe set, and rat GAPDH primer probe set with a Lightcycler. Primer and probe sequences and 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 under 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, cultured islets were hand picked for preparing. TF and MCP-1 mRNA in each group were analyzed using the same procedure as above.

Statistical analyses

All data are expressed as means ± standard deviation. Comparisons between two groups were performed by student t test. One-factor ANOVA was used to determine time course effect of TF and MCP-1 expression in isolated islets. Statistical significance was established at $P < 0.05$.

Results

Islet recovery and purity after isolation (Fig. 1)

Islet yield was considerably lower (BD 2110±231 IEQs; control 2390±528 IEQs; p=0.19), and purity was significantly lower in BD group than control group (BD 87.7±7.5%; control 97.0±2.6%; p=0.002).

Islet viability and function after isolation (Fig. 2)

Unexpectedly, ATP/DNA ratio and respiratory activity were comparable between the groups irrespective of 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).

mRNA expression of TF and MCP-1 in the pancreatic tissues (Fig. 3)

TF and MCP-1 mRNA levels in the pancreatic tissues prior to isolation procedures were similar between BD and control group (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±0.004; p=0.40).

mRNA expression of TF and MCP-1 in the fresh isolated islets (Fig. 4)

TF mRNA levels in the fresh isolated islets of the BD group was significantly higher than that of the control group (TF/GAPDH BD 0.148±0.01; control 0.061±0.0096; p=0.0004). MCP-1 mRNA levels in the fresh isolated islets of the BD group was also significantly higher than that of the control group (MCP-1/GAPDH BD 0.240±0.035; control 0.140±0.007; p=0.008).

Time course change of TF and MCP-1 mRNA expression in the isolated islets (Fig. 5)

In the fresh islets, a sharp difference was observed between BD and the control group in terms of TF and MCP-1 mRNA expression. In both groups, TF and MCP-1 mRNA levels decreased time-dependently during culture period. Between fresh and 48 h culture, a significant difference was seen in TF expression in the islets from BD donors.

Discussion

This study demonstrated that BD influences TF and MCP-1 expression in the isolated islets but not in the pancreatic tissues prior to the digestion procedure. It may be speculated that the difference is attributed to the warm ischemic stress during digestion procedure. However, TF and MCP-1 were not upregulated in the isolated islets from the donors without BD, suggesting that the warm ischemic damage *per se* during digestion procedure is not enough for inducing crucial inflammatory mediators in the islet grafts. We therefore believe that up-regulation of crucial inflammatory mediators induced by BD could be further exacerbated by warm ischemic damage during digestion procedure.

In the present study, the islet yield and purity were certainly affected by BD. However, the difference was extremely low compared to that of the previous report (5), moreover almost no influences were observed in terms of 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 except during the digestion phase. Furthermore, at the density-gradient centrifugation phase, we applied Histopaque-1119 and Lymphoprep^{TM}, on the other hand, dextran gradient separation was performed in the previous report (5). Hence, the important message from our present study is that the expression of inflammatory mediators rather than islet viability is more susceptible to BD. In other words, it seems more likely that the current standard methods of islet quality assessment are not suitable tools to detect the graft damage in the early phase of islet transplantation.

Although islet culture modulated the inflammatory status of human pancreatic islets (16,17), the effect is still controversial. We therefore investigated the time course change of TF and MCP-1 expression in the isolated islets with/without BD. As shown in Fig. 5, the influence of BD and ischemic stress during isolation procedure was most pronounced after 3-h culture. Clinical islet transplantation is currently performed at this time period, in most institutions, using fresh islets according to the Edmonton protocol

(22,23). Notably, in most clinical cases, the islet grafts with 3-h culture are used in fresh islet transplantation since 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 in terms of damage due to BD and ischemic stress during isolation procedure.

One way to avoid the adverse effect of BD and ischemic stress is short-term culture of the islet grafts. This might be one of the possible explanations for the outstanding result of clinical islet transplantation reported by Froud T. et al. (6) and Hering BJ. et al. (12). In support of our previous findings (25), the present study also implied that TF expression in the islets without BD was substantially upregulated during overnight culture. The reason is uncertain but the hypoxic condition during culturing might at least in part be responsible for TF induction (1,11,21). Taking into account this finding, most likely 48-h culture introduced in the previous reports (6,12) is reasonable. However, it is also reported that the number and function of isolated islets could be decreased after short-term culture (13,14). This is consistent with our previous findings that the isolated islets without culture is more beneficial to the transplant outcome under a current style of culture (25). Moreover, it may be difficult to maintain adequate number of islets from the marginal donors after substantial periods of culture, 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 cultured islets.

The other way to avoid the adverse effect of BD and ischemic stress is to establish effective anti-inflammatory treatments through whole steps from intensive care unit to digestion procedure.

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

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