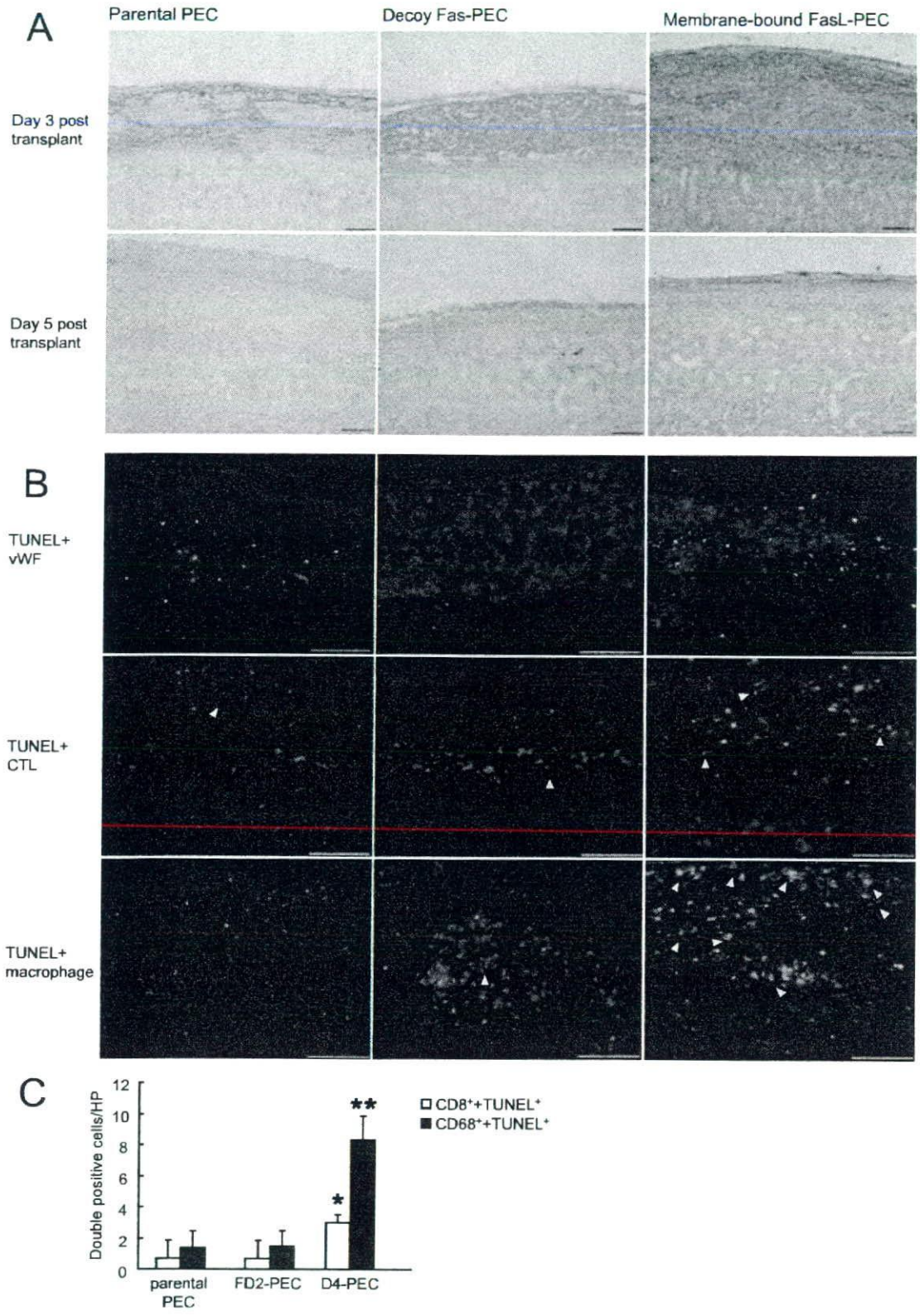

Fig. 6. Histologic analysis of grafts from rats transplanted with parental PEC, FD2-, or D4-transfected PEC. (A) Immunohistochemistry for vWF of a representative section of a parental PEC graft, decoy Fas transfected PEC transplant, or membrane-bound FasL transfected PEC harvested at 3 days (upper panels) or 5 days (lower panels) post-transplantation. Bars indicate 100 μm. (B) Dual labelings with TUNEL and either vWF (upper panels), CD8 (middle panels), or CD68 (lower panels) are shown. (C) Data represent the mean ± SD of double positive cells (green circled by red) in high-power fields of three individual mice/group. A significant difference between parental PEC-transplanted group and D4-PEC-transplanted group is indicated by asterisks ($^{*}P < 0.05$ for $CD8^+$ cytotoxic T lymphocytes, $^{**}P < 0.01$ for $CD68^+$ macrophages). White bars indicate 100 μm.

(Fig. 5B). As described above, the cytotoxicity of $CD8^+$ CTL against pig xenograft cells, including pig islets, is strong, and overexpression of either human decoy Fas or membrane-bound FasL can effectively prevent this strong killing of $CD8^+$ CTL. However, large numbers of macrophages infiltrated into pig xenografts secrete cytokines and may contribute to xenograft rejection. As shown in Fig. 6B, membrane-bound FasL overexpression in pig xenograft cells induced apoptosis for infiltrated macrophages. Therefore, membrane-bound FasL, which we developed, is effective not only for inhibition of $CD8^+$ CTL cytotoxicity, but also for prevention of macrophage killing.

The adenovirus-mediated expression of human decoy Fas or membrane-bound human FasL in pig islets effectively prevented human $CD8^+$ CTL-mediated xenocytotoxicity in vitro. Furthermore, prolonged survival of pig islet xenografts was elicited by the overexpression of these molecules in an in vivo study. Moreover, we monitored post-transplant blood glucose level. The post-transplant blood glucose levels in rats receiving both Ad-EGFP-FD2- and Ad-DsRed-D4-transfected islets was significantly lower than the blood glucose values obtained after the transplant of either non-transfected or Ad-EGFP-transfected pig islets at 12 and 18 hr post-transplant (data not shown). However, the improvement of blood glucose levels in transplanted rats was transient. This discrepancy between glycemic control and histological survival is seen often in islet transplant models. At the 5-year follow-up of the Edmonton protocol, EA Ryan and colleagues observed that the majority (~80%) of islet transplant recipients have C-peptide present (insulin staining positive), but only a minority (~10%) maintain insulin independence (keep normal blood glucose level) [31]. These results indicate that sufficient numbers of islets are required to maintain normal blood glucose levels. We judged the effectiveness of gene modification by histological survival. Another possible reason is that β-cells may be directly destroyed by adenovirus-induced cytolysis, or alternatively, the transfection process may induce a non-specific inflammatory reaction [33]. Additionally, the reason that the DNA fragments transfected by adenovirus were not integrated into the genome of pig islets may be that adenoviral gene expression is transient. Therefore, our final goal is to generate a transgenic pig that expresses these inhibitory molecules.

When measured by in vitro cytotoxicity assay, membrane-bound human FasL was more effective than human decoy Fas in the inhibition of CTL killing. However, the viability of pig islets expressing membrane-bound FasL was downregulated, as judged by TMRE staining. The reasons for this finding are as follows: (i) membrane-bound FasL-expressing pig islets grafts may kill other pig islets via endogenous Fas antigen in pig islets [34]; and (ii) FasL expression in islets may provoke inflammation and destructive insulitis [35]. These findings indicate that overexpression of FasL in islets plays a dual role as a cytoprotective molecule for $CD8^+$ CTL and as a mediator for islets graft injury [36]. Therefore, high expression of membrane-bound FasL in pig islets may not be beneficial for the protection of pig islets grafts.

These findings lead us to hypothesize that the double expression of both decoy Fas and membrane-bound FasL in pig islets may be more effective for the inhibition of CTL cytotoxicity than high expression of FasL in pig islets. Our previous data demonstrated that strong inhibition of $CD8^+$ CTL cytotoxicity can be elicited by the stable double expression of these molecules in PEC [11]. Others have also reported that the adenoviral-mediated double expression of vascular endothelial growth factor and interleukin-1 receptor antagonist improved the function of human islets [37]. Unfortunately, we cannot demonstrate the synergistic effects of double expression in pig islets in this study because of adenovirus-mediated toxicity against pig islets themselves (data not shown). In the future, double transgenic expression of these molecules in pigs may be useful for the long-term survival of pig islets.

Acknowledgments

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In Vivo Controlling of Cellular Response to Pig Islet Xenografts by Adenovirus-Mediated Expression of Either Membrane-Bound Human FasL or Human Decoy Fas

K. Kawamoto, M. Tanemura, T. Deguchi, T. Machida, T. Nishida, Y. Sawa, Y. Doki, M. Mori, and T. Ito

ABSTRACT

The critical problem with clinical islet transplantation for patients with type 1 diabetes is the severe shortage of human donors. Pig islet xenotransplantation has the potential to provide a virtually unlimited source of donor pancreata. However, our previous studies demonstrated that cell-mediated rejection, especially human $CD8^+$ cytotoxic T lymphocyte (CTL)–mediated cytotoxicity, remains a major obstacle for long-term islet xenograft survival. Moreover, we have demonstrated that the overexpression of either membrane-bound human FasL (mFasL) or human decoy Fas antigen (decoy Fas) in pig islets not only prevented CTL xenocytotoxicity in vitro, but also prolonged histological survival of pig islet xenografts in vivo. Therefore, the aim of the present study was to determine whether adenoviral transfer of these genes into pig islets ex vivo prior to transplantation had a beneficial effect on posttransplantation glycemic control of diabetic recipients. Isolated pig islets were transfected with adenovirus vector carrying complementary DNA (cDNA) of either mFasL or decoy Fas. The transfected islets were transplanted under the kidney capsule of diabetic recipient rats. Rats transplanted with either mFasL- or decoy Fas-transfected pig islet grafts showed significantly suppressed blood glucose levels from 12 hours to 18 hours posttransplantation compared with control groups transplanted with empty vector-transfected pig islets. Unfortunately, blood glucose levels of these groups were increased, with no significant difference observed at 24 hours posttransplantation. However, transgenic expression of these molecules with clinically tolerable amount of immunosuppressants may be more effective to achieve islet xenograft survival in the future.

PIG ORGANS are considered to be an attractive, promising alternative for the severe shortage of human donors. However, both humoral and cellular immune responses remain formidable barriers preventing vascularized xenograft survival, with the former predominantly consisting of the interactions between natural anti-Gal antibody and the α-gal epitopes on pig cells, and the latter including $CD4^+$ T cells, $CD8^+$ cytotoxic T lymphocytes (CTLs), macrophages, and natural killer (NK) cells. Fortunately, the humoral immunologic barrier may not occur in the case of pig-to-human islet xenotransplantation, because pig islets fail to express these α-Gal epitopes. However, pig islet xenografts are rejected by cellular immunity. Therefore, it is rational to target cellular immunity for successful pig islet xenotransplantation. Recently, several groups have shown that prolonged xenograft survival of functional adult pig islets may be achieved in immunosuppressed nonhuman primate recipients.[1,2] However, the high level of immunosuppression in these studies may be difficult to use clinically for human diabetic patients. Therefore, specific immunosuppression, consisting of local expression of cytoprotective molecules on pig islets, may be required to realize pig-to-human islet xenotransplantation. Our group previously demonstrated that cell-mediated xenocytotoxicity, especially human $CD8^+$ CTL, is highly detrimental to pig cells.[3] We have explored methods to prevent killing by overexpression of either membrane-bound human FasL (mFasL) or

From the Osaka University Graduate School of Medicine, Osaka, Japan.

Address reprint requests to Masahiro Tanemura, MD, PhD, Osaka University Graduate School of Medicine, Department of Surgery (E1), 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: mtanemura@gesurg.med.osaka-u.ac.jp

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human decoy Fas antigen (decoy Fas).[4] In this study, we determined whether overexpression of these molecules into pig islets ex vivo prior to transplantation showed a beneficial effect to improve islet xenograft survival and function.

MATERIALS AND METHODS

Preparation of Pig Islets

Pig pancreata were harvested from a slaughterhouse that handles young market weight pigs (Large White/Landrace×Duroc, 200–300 kg). The gland was shipped to our laboratory using 2-layer methods. Pig islets were isolated by the modified Ricordi method.[5] Briefly, the pig pancreas distended with Liberase HI solution (Roche Diagnostics, Indianapolis, Ind, United States) was cut into several pieces, which were placed into a sterile chamber for clinical islet isolation (Umihira, Kyoto, Japan) by digestion as previously described.[5] The pig islets were purified with a continuous density gradient of iodixianol-based solution in an apheresis system (COBE2991 cell processor, Gambro Laboratory, Denver, Cdo, United States), which was cooled with special equipment (Umihira).

Pig Endothelial Cell Culture

A pig endothelial cell (PEC) line, MYP-30,[6] was cultured in Dulbecco's Modified Eagle's Medium (DMEM), including 10% fetal bovine serum (FBS), 100 U/mL Penicillin, 100 μ g/mL streptomycin, and 0.1 mmol/L nonessential amino acids (Invitrogen, Carlsbad, Calif, United States).

Construction of Adenoviral Vectors and Gene Expression in Pig Islets

cDNAs encoding either mFasL gene[7] or decoy Fas[8] were subcloned into the SwaI cloning site of the cytomegalovirus (CMV) promoter-containing adenovirus-based cosmid vector (Ad-), pAxcwit (Takara Bio, Otsu, Japan).[9] All virus stocks were purified using cesium chloride density gradient centrifugation. Nonfunctioning enhanced green fluorescent protein (eGFP)–adenovirus vector was used as a vehicle control (ie, MOCK). Freshly isolated pig islets were exposed to these adenovirus vectors at a multiplicity of infection of 30 for 2 hours. The expression of these molecules in pig islets was assessed using FACS analysis.

In Vitro Cytotoxicity Assay

The cytotoxicity of cultured human $CD8^+$ CTL toward pig islets was assessed with a ^{51}Cr release assay, as previously described.[4]

Transplant Studies and Immunohistochemical Analysis

Lewis rats of 8 to 10 weeks old were purchased from Oriental Yeast (Tokyo, Japan). Recipient rats were preimmunized with 250 mg of pig kidney membranes as previously described.[10] Then, the recipients were rendered diabetic by a single intraperitoneal injection of streptozotocin (60 mg/kg) at day 7 prior to islet transplantation. Diabetes was confirmed by blood glucose levels >300 mg/dL on 2 individual days. We transplanted 3000 islet equivalents (IEQ) of adenoviral transfected pig islets under the left kidney capsule of the recipient rats and monitored blood glucose levels. The grafted kidney was retrieved at day 3 or 5 posttransplantation for immunohistochemical analysis (IHC), using anti-pig insulin antibody (Dako, Glostrup, Denmark).[11] Briefly, deparaffinized and rehydrated tissue sections had endogenous peroxidase blocked with 3% H_2O_2 methanol. After blocking with 10% BSA-Tris buffered saline containing 0.1% Tween 20, the sections were incubated with either guinea pig anti-pig insulin polyclonal antibody (Dako). Then the sections were visualized using Dako LSAB+/HRP kit (Dako) with 0.02% diaminobenzidine as the chromogen. After washing, the sections were counterstained with hematoxylin. The specificity of the primary antibodies was verified by control sections omitting the primary antibody. The apoptotic cells were visualized using TUNEL staining at day 3 posttransplantation as previously described.[11]

RESULTS

Expression of these molecules in pig islet transfectants was detected as judged by mean fluorescence intensity of FACS analysis (Fig 1). The expression levels of transfected molecules in islets are summarized in Table 1. The histological survival of transplanted pig islets was evaluated using insulin staining. The prolongation of xenograft survival with either Ad-decoy Fas pig islets or Ad-mFasL pig islets was shown compared with Ad-eGFP or parental islet groups (Table 1). Apoptotic cells were clearly detected in parental pig islet xenografts at day 3 posttransplantation. In contrast, a smaller number of apoptotic cells was observed in decoy Fas islet xenografts at day 3 posttransplantation. In membrane-bound FasL islets grafts, many infiltrating cells were apoptotic (data not shown). Glycemic control among experimental groups was improved compared with the control groups through 12 to 18 hours posttransplantation. Unfortunately, blood glucose levels gradually increased in the experimental groups; no significant difference in glucose levels could be observed at 24 hours posttransplantation. This short-term improvement of glucose levels might be further prolonged by the use of immunosuppressive drugs.

DISCUSSION

We demonstrated that both mFasL and decoy Fas were effective to prolong pig islet xenograft survival in an in vivo transplantation model. Moreover, these molecules exerted

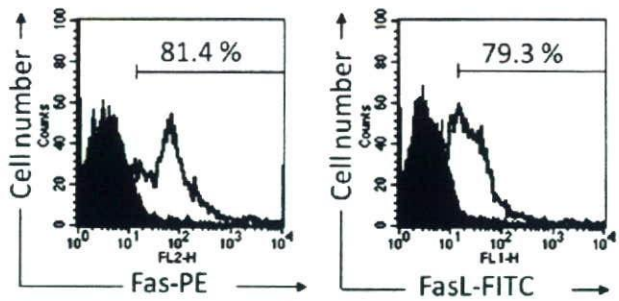

Fig 1. Adenovirus-mediated overexpression of either human decoy Fas or membrane-bound human FasL in transfected pig islets: (closed histogram) unstained islets; (open histogram) stained islets with anti-human Fas mAb (DX2) or anti-human FasL mAb (4H9). The percentages of transfected islets displaying fluorescence in channels greater than 10 in each of the preparations are indicated in the upper right of each histogram.

Table 1. Adenoviral Expression in Pig Islets and Xenograft Survival of Pig Islets in Pig-to-Rat Transplantation Model

	Mean Florescence Intensity (Transduction Efficiency by Adenovirus [%])		% Cytotoxicity by ^{51}Cr Release Assay	Xenograft Survival	Blood Glucose Levels of Recipients (mg/mL)		
Pig Islets	FasL	Fas Antigen	E:T Ratio = 50:1	d 3	12 h	18 h	24 h
Parental pig islets	Not determined	25 (endogenous)	36.7 ± 3.9	Rejected	430.5 ± 38.8	462.2 ± 52.4	478.6 ± 42.1
Mock islets	Not determined	26 (endogenous)	47.8 ± 1.0	Rejected	447.8 ± 45.0	450.3 ± 44.3	469.5 ± 33.3
Decay Fas islets	Not determined	198 (81.4)	18.8 ± 1.0	++ (survived)	186.6 ± 18.7*	272.5 ± 56.5*	458.3 ± 36.9
mFasL islets	75 (79.3%)	30 (endogenous)	5.7 ± 1.8	++ (survived)	196.4 ± 44.3*	304.3 ± 42.9*	490.0 ± 38.5

Note: The expression levels of pig islet transfectants, the glycemic control of the recipient rats, and histological islet xenograft survival were summarized. Each value is expressed as mean ± SD from 4 independent experiments.
*$P < .05$ compared with mock.

their cytoprotective effects for the metabolic outcome of recipients for the first 24 hours after transplantation. These results suggested that adenoviral transduction of cytoprotective genes into pig islets is a promising method to prolong xenograft survival. However, there were only transient positive effects on glycemic control in recipient rats. We speculated that this may be explained by several factors. Although adenovirus vectors are powerful weapons to transduce genes of interest in most tissues, including pig islets, adenoviral gene delivery may have deleterious effects upon pig islets, otherwise their chemotactic properties may restrict the efficiency of modified genes. We did not use immunosuppressants seeking to verify the pure effect of gene modification; therefore, islets might be damaged by multiple factors, including non-gal-dependent antibody-mediated, nonimmunological or immunological rejections. We expect synergistic effects between gene transduction and immunosuppressants. Therefore, our future projects seek to assess the effectiveness of transgenic pig islets bearing these molecules.

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Superiority of Fresh Islets Compared With Cultured Islets

H. Takahashi, M. Goto, N. Ogawa, Y. Saito, K. Fujimori, Y. Kurokawa, H. Doi, and S. Satomi

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 ±

From Tohoku University International Advanced Research and Education Organization, Sendai, Japan.

Address reprint requests to Masafumi Goto, MD, PhD, Tohoku University, International Advanced Research and Education Organization, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan. E-mail: gotokichi@aol.com

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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).

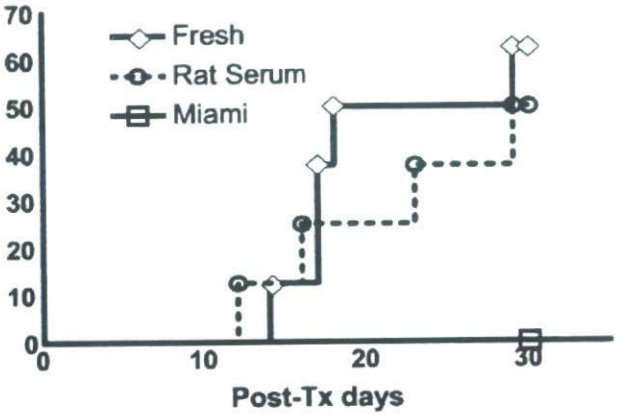

Fig 1. The influence of culture on islet quality was evaluated by intraportal transplantation of syngeneic islets into streptozotocin-induced diabetic rats. In an in vivo transplantation model, the curative rate was higher in the fresh islet group than in the others.

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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ISLETS
Human

A Novel Predictive Method for Assessing the Quality of Isolated Pancreatic Islets Using Scanning Electrochemical Microscopy

M. Goto, H. Abe, T. Ito-Sasaki, M. Goto, A. Inagaki, N. Ogawa, K. Fujimori, Y. Kurokawa, T. Matsue, and S. Satomi

ABSTRACT

Introduction. The current methods for evaluating islet potency are not useful in clinical transplantation. Therefore, we need reliable, rapid methods enabling accurate prediction of islet quality.

Materials and Methods. We evaluated respiratory activity using scanning electrochemical microscopy (SECM), glucose-stimulated respiratory activity, glucose-stimulated insulin release, ADP/ATP assays, insulin/DNA levels, and Trypan blue exclusion tests as predictive methods for the ability of isolated rat islets to cure syngeneic diabetic rats.

Results. Although glucose-stimulated respiratory activity, basal respiratory activity, ADP/ATP ratio, and glucose-stimulated insulin release were significantly correlated with the outcome of transplantation into diabetic rats, there was no correlation between outcomes, insulin/DNA ratios, and Trypan blue exclusion tests. The glucose-stimulated respiratory activity in islet preparations that could cure diabetic rats was significantly greater than those unable to cure diabetes. Rat islets with >1.5-fold glucose-stimulated respiratory activity consistently cured diabetic rats, whereas those with a value <1.5 hardly cured any rats.

Conclusion. Measurement of the glucose-stimulated respiratory activity using SECM technique is a novel method that may be useful as a rapid, potent predictor of the outcome of clinical islet transplantation.

From the Tohoku University International Advanced Research and Education Organization (Ma.G., Me.G., A.I.), Tohoku University, Sendai; Division of Advanced Surgical Science and Technology (Ma.G., N.O., S.S.), Tohoku University, Sendai; Faculty of Engineering (H.A., T.I.-S.), Yamagata University, Yonezawa; Division of Surgical Oncology (K.F.), Tohoku University, Sendai; Tohoku University Innovation of New Biomedical Engineering Center (Y.K.), Tohoku University, Sendai; and Graduate School Environmental Studies (T.M.), Tohoku University, Sendai, Japan.

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Address reprint requests to Masafumi Goto, MD, PhD, Tohoku University International Advanced Research and Education Organization, Tohoku University, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi, 980-8575, Japan. E-mail: gotokichi@aol.com

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THE CURRENT standard methods to evaluate islet potency are not useful in clinical islet transplantation. Furthermore, most tests are relatively subjective and time-consuming.[1] We have thus far shown that the ADP/ATP ratio correlated with in vivo viability of isolated islets.[2] However, insulin release from isolated islets is not entirely related to the ADP/ATP ratio. Moreover, it is difficult to continuously measure the ADP/ATP ratio of the same islets. Therefore, we sought to establish a reliable, rapid method enabling accurate prediction of both islet viability and insulin release. Scanning electrochemical microscopy (SECM) is a technique in which the tip of a microelectrode monitors the local distribution of electro-active species near the sample surface. SECM has been used to investigate numerous biological molecules, including DNA,[3] enzymes,[4] and antigen-antibody interactions.[5] This technique noninvasively measures respiratory activity of isolated islets under physiological conditions. We have used SECM to examine islet viability and potency of insulin release.

MATERIALS AND METHODS

In the present study, we evaluated respiratory activity using SECM, glucose-stimulated respiratory activity, glucose-stimulated insulin release, ADP/ATP assays, insulin/DNA levels, and Trypan blue exclusion tests as predictive methods to evaluate the ability of isolated rat islets exposed to various degrees of heat shock stress (0, 40, 50, 60 or 80 seconds) to cure syngeneic Streptozotocin-induced diabetic rats (n = 7, 6, 6, 7, and 7, respectively). SECM was programmed to automatically measure the reduction current of far and near points of samples based on spherical diffusion theory.[6] The respiratory activity of 10 islets in each group was calculated by evaluating the difference of the reduction current around the samples using 2–4 μm platinum-coated microelectrode. The glucose-stimulated respiratory activity was indicated by the stimulation index of the respiratory activity, defined as the ratio of the respiratory activity in high-glucose concentration (16.7 mmol/L) against that in basal glucose concentration (1.67 mmol/L). The ADP/ATP assay, insulin/DNA levels, and Trypan blue exclusion tests were performed as previously described.[2,7] In islet transplantation, 6 islet equivalents/g of body weight were transplanted into recipient livers via the portal vein using a 24-gauge butterfly needle using the previously described method.[8] Heat shock stress was induced by placing the isolated islets at 60°C for 0, 40, 50, 60, or 80 seconds.

RESULTS

On the one hand, significant correlations with the outcome of transplantation into diabetic rats were observed for glucose-stimulated respiratory activity (heat shock stress; 0 seconds; 2.39 ± 0.08; 40 seconds, 1.85 ± 0.17; 50 seconds, 0.86 ± 0.08; 60 seconds, 0.49 ± 0.03; 80 seconds, 0.37 ± 0.07; cured group: 1.94 ± 0.18; noncured group: 0.57 ± 0.07, respectively), basal respiratory activity (heat shock stress: 0 seconds, 5.65 ± 0.15, 40 seconds, 5.31 ± 0.51, 50 seconds, 4.18 ± 0.58, 60 seconds, 1.83 ± 0.27, 80 seconds, 0.31 ± 0.05; cured group: 5.27 ± 0.26; noncured group: 1.98 ± 0.46, respectively), ADP/ATP ratio (heat shock stress; 0 seconds, 0.003 ± 0.003, 40 seconds, 0.05 ± 0.03, 50 seconds, 0.21 ± 0.05, 60 seconds, 0.30 ± 0.07, 80 seconds, 0.42 ± 0.05, cured group: 0.05 ± 0.03; noncured group: 0.30 ± 0.04, respectively), and glucose-stimulated insulin release (heat shock stress; 0 seconds, 11.0 ± 2.6, 40 seconds, 2.51 ± 0.76, 50 seconds, 1.12 ± 0.14, 60 seconds, 1.13 ± 0.21, 80 seconds, 1.40 ± 0.41; cured group: 6.59 ± 1.78; noncured group: 1.35 ± 0.18, respectively) $P < .0001$, $< .0001$, $< .0001$, and .002: $\rho = .80$, .71, −.66, and .53, respectively. On the other hand, there was no correlation between islet transplantation outcome and insulin/DNA ratio (heat shock stress: 0 seconds, 0.73 ± 0.05, 40 seconds, 0.99 ± 0.13, 50 seconds, 0.86 ± 0.10, 60 seconds, 0.91 ± 0.06, 80 seconds, 1.12 ± 0.06; cured group: 0.78 ± 0.04; noncured group: 1.03 ± 0.05, respectively), and Trypan blue exclusion test (heat shock stress: 0 seconds, 100.0 ± 0.0, 40 seconds, 98.8 ± 0.6, 50 seconds, 99.3 ± 0.5, 60 seconds, 99.9 ± 0.1, 80 seconds, 94.9 ± 1.8; cured group: 99.5 ± 0.3; noncured group: 97.8 ± 0.8, respectively). The glucose-stimulated respiratory activity in islet preparations that could cure diabetic rats was significantly higher than in those unable to cure diabetes ($P < .0001$). Rat islets with glucose-stimulated respiratory activity more than 1.5 consistently cured diabetic rats, whereas rat islets with a value <1.5 hardly cured any rats ($P < .0001$) (Fig 1). Notably, the predictive rate for curing diabetic rats was 91% when glucose-stimulated respiratory activity was used.

DISCUSSION

It is well known that unexpectedly poor effects of grafts are still seen in the field of islet transplantation even using the current refined procedures. Most likely, this is attributed to suboptimal quality of the isolated islets.

It has been reported that the current methods of islet quality assessment have only a limited ability to predict

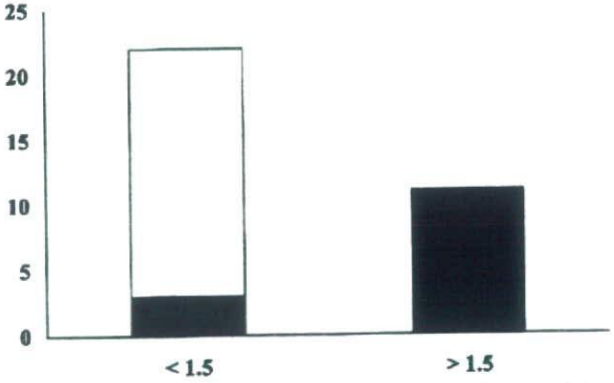

Fig 1. Streptozotocin-induced diabetic rats underwent intraportal transplantation with syngeneic islets that were exposed to various degrees of heat shock stress (0, 40, 50, 60, and 80 seconds). The X-axis indicates the glucose-stimulated respiratory activity, and the Y-axis indicates the number of animals. The black bar shows cured animals; the white bar shows noncured diabetic animals. Rat islets with glucose-stimulated respiratory activity >1.5 consistently cured diabetic rats, whereas rat islets with a value <1.5 hardly cured any rats ($P < .0001$).

outcomes after clinical transplantation.[1,9,10] In vivo bioassay has thus far been regarded as the most reliable assessment.[11,12] However, it is not clinically useful because several days are needed for evaluation. Therefore, we need establishment of reliable, rapid methods enabling accurate prediction of islet potency. This issue is crucial for Japan because only marginal organs from non–heart-beating donors are currently available for islet isolation.

In 2006, we reported that the ADP/ATP ratio was a useful predictive assay for isolated islets.[2] Although the ADP/ATP assay has many advantages as islet quality assessment, its limitation is the absence of a correlation with insulin release from the isolated islets, suggesting that it reflects islet viability rather than function.

As shown in the present study, glucose-stimulated respiratory activity strongly correlated with islet quality. This highly sensitive, noninvasive method made it possible to distinguish respiratory activity even in one islet by visualizing the reduction current in a simple form. Notably, the glucose-stimulated respiratory activity is expected to reflect not only islet viability but also function.

Taken together, measurement of the glucose-stimulated respiratory activity using SECM technique is a novel rapid, potent predictor of the outcome of clinical islet transplantation.

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ELSEVIER

C5a Inhibitory Peptide Combined With Gabexate Mesilate Is a Clinically Available Candidate for Preventing the Instant Blood-Mediated Inflammatory Reaction

K. Tokodai, M. Goto, T. Imura, N. Ogawa, K. Fujimori, Y. Kurokawa, H. Okada, and S. Satomi

ABSTRACT

Background. The instant blood-mediated inflammatory reaction, characterized by activation of both the coagulation and complement cascades, is a serious obstacle to successful islet engraftment. No attractive protocol is clinically available as yet. The objective of the present study was to examine whether complementary peptide against an active region of C5a in combination with a clinically available anticoagulant could provide an effective protocol for suppression of the instant blood-mediated inflammatory reaction.

Methods. Three islet equivalents per gram of syngeneic rat grafts were transplanted intraportally into 6 pairs of rats with streptozotocin-induced diabetes. Islets from the same donor were transplanted into each pair. In each pair, one rat was treated with C5a inhibitory peptide in addition to continuous intravenous infusion of gabexate mesilate and the other rat, injected with equivalent amount of saline solution, served as the control. In addition, 6 rats that received transplants from irrelevant donors were treated with the same dose of gabexate mesilate. We evaluated the cure rate, time to normoglycemia, liver insulin concentration in recipients, and results of in vivo glucose tolerance tests.

Results. The cure rate was remarkably improved and the time to normoglycemia in cured animals was significantly shortened with C5a inhibitor plus gabexate treatment. In six rats that received only gabexate mesilate, normoglycemia was not restored during the study.

Conclusions. These data suggest that C5a inhibitory peptide combined with gabexate mesilate could be an attractive drug candidate without adverse effects to control the detrimental innate immune responses induced in clinical islet transplantation.

A SERIOUS OBSTACLE to successful islet engraftment is the instant blood-mediated inflammatory reaction (IBMIR), characterized by activation of both the coagulation and complement cascades.[1,2] We have previously shown that a low-molecular-weight dextran sulfate attenuates xenogeneic IBMIR triggered by porcine islets.[3] No attractive protocol, however, is clinically available as yet. A complementary peptide to an active region of C5a regulates C5a anaphylatoxin and is effective for rapid lethal shock in rat models.[4] Furthermore, this reagent is expected to be safe owing to its extremely low molecular mass. The objective of the present study was to examine whether C5a inhibitory peptide in combination with a clinically available anticoagulant provides an effective protocol for suppression of IBMIR.

MATERIALS AND METHODS

Three islet equivalents per gram of syngeneic rat islet grafts were transplanted intraportally into 6 pairs of rats with streptozotocin-induced diabetes Islets from the same donor were transplanted into

From the Division of Advanced Surgical Science and Technology (K.T., M.G., N.O., S.S.), Tohoku University, Miyaga, Japan; Tohoku University International Advanced Research and Education Organization (M.G., T.I., Y.K.), Tohoku University, Sendai, Japan; Division of Surgical Oncology (K.F.), Tohoku University, Sendai, Japan; Choju Medical Institute (H.O.), Fukushimura Hospital, Toyohashi, Japan.

Address reprint requests to Kazuaki Tokodai, MD, Division of advanced Surgical Science and Technology, Tohoku University, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi, 980-8575 Japan. E-mail: tsu7ka5so8mi@yahoo.co.jp

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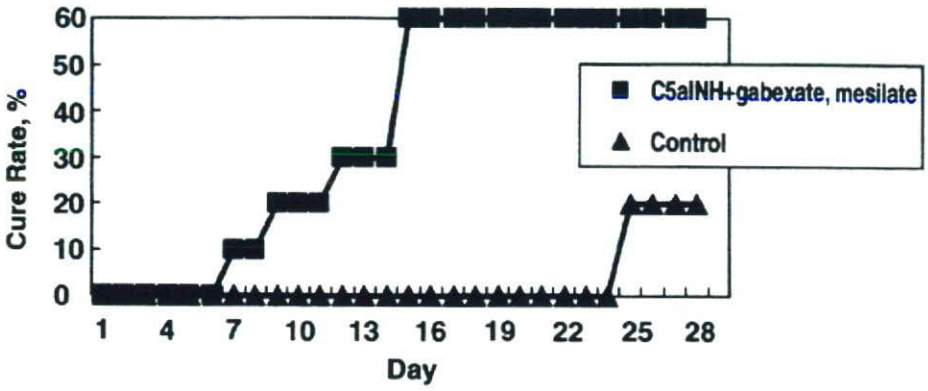

Fig 1. Cure rate and time to normoglycemia.

each pair. In each pair, one rat was treated with C5a inhibitory peptide (bolus, 4 mg/kg; continuous infusion, 0.42 mg/kg/h for 1 day followed by 0.08 mg/kg/h for 2 days) plus continuous intravenous infusion of gabexate mesilate (2 mg/kg/h) 30 minutes before islet infusion and up to 1 hour after the infusion. The other rat was injected with an equivalent amount of saline solution and served as the control. In addition, 6 rats who received transplants from irrelevant donors were treated with the same dosage of gabexate mesilate only. We evaluated the cure rate, time to normoglycemia, liver insulin concentration, and results of in vivo glucose tolerance tests.

RESULTS

The cure rate was remarkably improved (100% vs 33.3%; $P < .005$) and the mean (SD) time to normoglycemia in cured animals (12.2 [1.4] vs 25.0 [0.0]; $P < .05$) was significantly shortened in the group treated with C5a inhibitor plus gabexate (Fig 1). The six rats that received only gabexate mesilate did not achieve normoglycemia during the study. No difference was observed between the groups in terms of glucose tolerance (area under the curve, $P = .85$; glucose disappearance rate; $P = .35$). The mean (SD) liver insulin concentration was considerably higher in the treated group compared with the control group (18.0 [3.2] vs 12.0 [1.0] ng/IEQs, $P = .14$). The increased body weight, 128.6% [1.2%] vs 127.7% [3.3%]; $P = .80$) in the recipient rats was not affected by the treatment, which suggests that C5a inhibitory peptide combined with gabexate mesilate does not produce this adverse effect.

DISCUSSION

Our findings suggest that C5a inhibitory peptide combined with gabexate mesilate could be an attractive drug candidate to control the detrimental innate immune responses induced by clinical islet transplantation.

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ELSEVIER

The Influence of Brain Death on Tissue Factor Expression in the Pancreatic Tissues and Isolated Islets in Rats

Y. Saito, M. Goto, K. Maya, N. Ogawa, K. Fujimori, Y. Kurokawa, and S. Satomi

ABSTRACT

Introduction. Tissue factor (TF) in islets has been identified as the main trigger of the instant blood-mediated inflammatory reaction. Because the crucial events that directly induce TF remain to be determined, we focused on the influence of brain death (BD) on TF expression in pancreatic tissues and isolated islets.

Materials and Methods. BD was induced in male Lewis rats weighing 250–300 g by inflation of a Fogarty catheter placed intracranially. The rats were mechanically ventilated for 6 hours until removal of the pancreas. The expression of TF protein in pancreatic tissues was examined using Western blotting assay. Messenger RNA (mRNA) expressions of TF in pancreatic tissue and isolated islets were analyzed using real-time polymerase chain reaction (PCR) assay. The influence of BD on the isolation outcome was evaluated by islet yield, purity, viability, and function.

Results. TF protein and mRNA levels in the pancreatic tissues were similar between the groups. However, TF mRNA in the isolated islets of the BD group was significantly greater than that of the control group ($P = .04$). Islet yield was considerably lower, and purity significantly lower in the BD than the control group ($P = .002$). Unexpectedly, ATP/DNA ratio and respiratory activity were comparable between the groups.

Conclusions. Although BD per se was not sufficient to induce TF expression in pancreatic tissues, BD combined with subsequent warm ischemic damage during isolation procedures remarkably up-regulated TF expression in isolated islets, suggesting that BD is of great importance as an initiator of TF induction in the islet grafts. The present study demonstrated that the expression of inflammatory mediators rather than islet viability is more susceptible to BD.

TISSUE factor (TF), a 47-kd transmembrane glycoprotein, acts as the initiator of the extrinsic coagulation system. It is pivotal for activation of the intrinsic pathway as well. Pancreatic islets have thus far been reported to express TF.[1] It has been revealed that TF in islets has been identified as the main trigger of the instant blood-mediated inflammatory reaction.[1,2] Low expression of TF in the graft has been correlated with high C-peptide values after clinical islet transplantation.[3] However, the crucial procedures to directly induce TF remain to be determined.

It is well known that the outcome of organ transplantation is highly influenced by brain death (BD). The success rate of kidney transplantations derived from cadaveric

From the Division of Advanced Surgical Science and Technology (Y.S., M.G., N.O., S.S.), Tohoku University International Advanced Research and Education Organization (M.G., K.M., Y.K.), and Division of Surgical Oncology (K.F.), Tohoku University, Sendai, Japan.

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Address reprint requests to Yukihiko Saito, MD, Division of Advanced Surgical Science and Technology, Tohoku University, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi, 980-8575, Japan. E-mail: yuki-hiko@poppy.ocn.ne.jp

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donors remains significantly inferior to that from living donors regardless of their genetic relationship to the recipient.[4] Contreras et al presented data that demonstrated BD to reduce isolated pancreatic islet yield and function, as well as up-regulation of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 in the serum and pancreatic tissues from BD donors.[5] In the present study, therefore, we focused on the influence of BD on TF expression in pancreatic tissues and isolated islets.

MATERIALS AND METHODS

Rodent BD Model

BD was induced in male Lewis rats weighing 250–300 g by inflation of a Fogarty catheter placed intracranially. The rats were mechanically ventilated for 6 hours until removal of the pancreas as previously described.[5,6]

Western Blotting Assay of 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 = 5 and n = 4, respectively). Approximately 10 mg of pancreatic biopsy specimens prepared on dry ice were immediately transferred into phosphate-buffered saline containing 5 mmol/L EDTA, 10 mmol/L benzamidine (Merck-Schuchardt, Hohenbrunn, Germany), 0.1 g/L soybean trypsin inhibitor (Sigma-Aldrich, Steinheim, Germany), and 1 mmol/L phenyl methyl sulfonyl fluoride (Sigma). The samples were then homogenized using Polytron PT 1300D (Kinematica AG, Littau-Lucerne, Switzerland) and Vibra-Cell (Sonics & Materials Inc, Newtown, Conn, USA) for 30 seconds each. Thereafter the samples were centrifuged at 4°C at 10,000g for 30 minutes to collect the supernate. The samples, containing 2.5 mg/mL of protein measured by BCA Protein Assay kit (Thermo Prod, Rockford, Ill, USA), were separated using SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Invitrogen, Carlsbad, Calif, USA). Membranes were incubated with rabbit anti-rat TF polyclonal antibody (Hokudo, Sapporo, Japan) at 4°C overnight and subsequently with goat anti-rabbit immunoglobulin (Ig)G-horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, Calif) antibody for 1 hour at room temperature. TF antigen was visualized using enhanced chemiluminescence Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK).

Determination of TF 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 prepared using the RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. RNA concentrations were estimated from absorbance at 260 nm. First-strand complementary DNA (cDNA) was synthesized from 2500 ng total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, Ind, USA). The cDNAs were amplified by PCR using rat TF primer probe set (Nihon Gene Research Laboratories Inc., Sendai, Japan) and rat GAPDH primer probe set (Nihon Gene Research Laboratories Inc.) with a Lightcycler (Roche Diagnostics).

Islet Isolation and Culture

Before removal of the pancreas, the cannulated bile duct was injected with 10 mL of cold Hanks' Balanced Salt Solutions (HBSS) containing 1 mg/mL Coragenase (Sigma type V; Sigma Chemicals, St. Louis, Mo). After addition of 10 mL HBSS the pancreas was digested at 37°C for 14 minutes. Thereafter, density-gradient centrifugation was performed using Histopaque-1119 (Sigma Diagnostics) and Lymphoprep (Nycomed Pharma AS, Oslo, Norway) to isolate pancreatic islets. The islet count was performed as islet equivalents (IEQ) under a scaled microscope using diphenylthiocarbazone (Wako, Osaka, Japan) staining (BD, n = 8; control, n = 7). One IEQ was the islet tissue 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% FBS at 37°C in 5% CO_2 and humidified air before examination.

Islet Viability and Function

ATP/DNA ratio was measured to evaluate the energy status of isolated islets. Eighty islet equivalents of islets with overnight culture were used in both BD and control groups (BD, n = 6; control, n = 5). The ApoGlow kit (Lonza Rockland Inc, Rockland, ME, USA) was used for ATP measurement as described previously.[7] Using the same sample, the DNA content was measured using DNA Quantify kit (Primary Cell, Sapporo, Japan) as described previously.[8] We evaluated the respiratory activity of isolated islets with overnight culture using scanning electrochemical microscopy (BD, n = 6; control, n = 5). 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 was applied as a rapid, potent predictor for the outcome of clinical islet transplantation.

Determination of TF mRNA in the Isolated Islets

Total RNA extracted from the 40 islets after 3-hour culture was prepared using RNeasy Micro Kit (Qiagen) according to the manufacturer's protocol (BD, n = 5; control, n = 8). RNA concentration was estimated from absorbance at 260 nm. First-strand cDNA was synthesized from 100 ng total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). The cDNAs were amplified by PCR, using rat TF primer probe set (Nihon Gene Research Laboratories Inc.) and rat GAPDH primer probe set (Nihon Gene Research Laboratories Inc.) with a Lightcycler (Roche Diagnostics).

Statistical Analysis

All data are expressed as mean values ± SD. Comparisons between groups were performed by student t test using Statcel 2nd Edition (Oms Publishing, Osaka, Japan). Statistical significance was established at $P < .05$.

RESULTS

Tissue factor protein and mRNA levels in the pancreatic tissues were similar between the groups (Fig 1 and 2). However, TF mRNA in the isolated islets of the BD group was significantly greater than that of the control group (TF/GAPDH BD, 0.169 ± 0.033; control, 0.119 ± 0.041; P = .04) (Fig 3). Islet yield was considerably lower (BD, 2110 ± 231 IEQs; control, 2390 ± 528 IEQs; P = .19), and purity was significantly lower in the BD than the control group (BD, 87.7 ± 7.5%; control, 97.0 ± 2.6%; P = .002). Unexpectedly, the ATP/DNA ratio and respiratory activity were comparable between the groups (ATP/DNA BD, 51.6 ± 12.8; control, 59.1 ± 3.47; P = .20; and BD, 2.39 ± 0.55; control, 2.58 ± 0.19; P = .45).

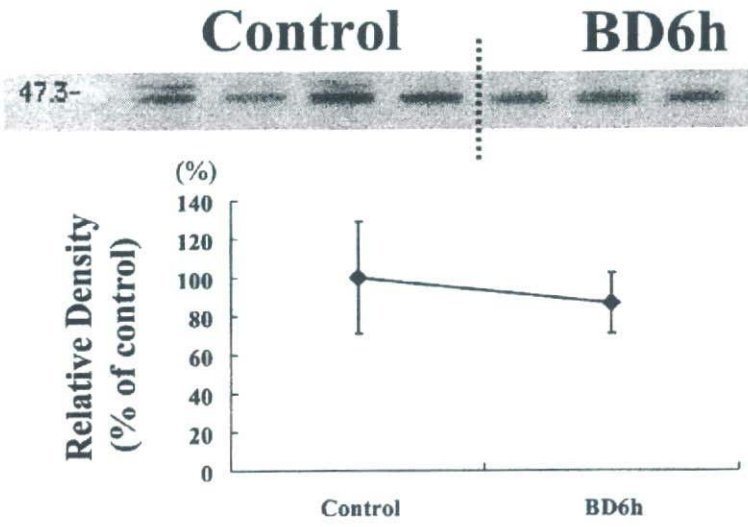

Fig 1. Protein expression of TF in the pancreatic tissues from the donors with/without BD was analyzed using Western blotting assay.

DISCUSSION

In the present study, our data showed that BD influenced TF expression in isolated islets but not in pancreatic tissues prior to the digestion procedure. It may be speculated that the difference was attributable to warm ischemic damage during the digestion procedure. In islet transplantation, unlike other organ transplantations, islet grafts are placed at 37°C during whole digestion procedure. This period could theoretically be considered as one kind of "warm ischemia," a concept that is supported by many investigators in the field of islet transplantation.[9–11] As shown in the present study, TF was not up-regulated in the isolated islets from the donors without BD, suggesting that warm ischemic damage during digestion procedure per se was not sufficient to induce TF in isolated islets. We therefore believe that the induction of TF from BD was accelerated by warm ischemic damage during the digestion procedure.

In the present study, islet yield and purity were certainly affected by BD. However, the difference was extremely small compared with a previous report,[5] moreover, almost no influence was observed in terms of islet viability. One possible explanation for this discrepancy is a difference in isolation procedures. In our isolation procedures, pancreatic tissues were kept on ice except during the digestion phase. Furthermore, at the density-gradient centrifugation phase, we applied Histopaque-1119 and Lymphoprep, in contrast, a 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 conclusion, although BD per se was not sufficient to induce TF expression in pancreatic tissues, BD combined with warm ischemic damage during isolation procedures remarkably up-regulated TF expression in isolated islets, suggesting that BD is of great importance as an initiator of TF induction in islet grafts.

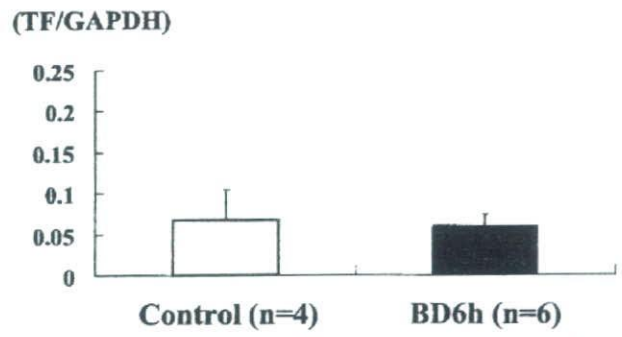

Fig 2. mRNA expression of TF in the pancreatic tissues from the donors with/without BD was analyzed using real-time PCR assay.

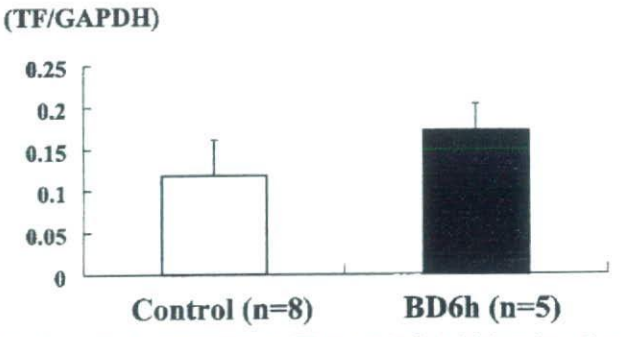

Fig 3. mRNA expression of TF in the isolated islets from the donors with/without BD was analyzed using real-time PCR assay.

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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.

Masafumi Goto,[1] Jenny Tjernberg,[2] Denis Dufrane,[3] Graciela Elgue,[2] Daniel Brandhorst,[2] Kristina Nilsson Ekdahl,[2] Heidi Brandhorst,[2] Lars Wennberg,[4] Yoshimochi Kurokawa,[5] Susumu Satomi,[6] John D. Lambris,[7] Pierre Gianello,[3] Olle Korsgren[2] and Bo Nilsson[2]

[1]Tohoku University International Advanced Research and Education Organization, Tohoku University, Sendai, Japan, [2]Department of Radiology, Oncology and Clinical Immunology, Division of Clinical Immunology, The Rudbeck Laboratory, University Hospital, Uppsala, Sweden, [3]Universiteé Catholique de Louvain, Faculty of Medicine, Experimental Surgery Unit, Brussels, Belgium, [4]Department of Transplantation Surgery, Karolinska Institute, Huddinge University Hospital, Stockholm, Sweden, [5]Tohoku University Innovation of New Biomedical Engineering Center, Tohoku University, Sendai, Japan, [6]Division of Advanced Surgical Science and Technology, Tohoku University, Sendai, Japan, [7]Department of Pathology and Laboratory Medicine, University of Pennsylvania, Medical School, Philadelphia, PA, USA

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Address reprints request to Bo Nilsson, Department of Radiology, Oncology and Clinical Immunology, Division of Clinical Immunology, The Rudbeck Laboratory, University Hospital, Uppsala, Sweden (E-mail: bo.nilsson@klinimm.uu.se)

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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