Transplantation 69:427-430; 2000.

25. Rong, Y.; Post, D. E.; Pieper, R. O.; Durden, D. L.; Van Meir, E. G.; Brat, D. J. PTEN and hypoxia regulate tissue factor expression and plasma coagulation by glioblastoma. Cancer Res. 65:1406-1413; 2005.

26. Ryan, E. A.; Lakey, J. R.; Rajotte, R. V.; Korbutt, G. S.; Kin, T.; Imes, S.; Rabinovitch, A.; Elliott, J. F.; Bigam, D.; Kneteman, N. M.; Warnock, G. L.; Larsen, I.; Shapiro, A. M. Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. Diabetes 50:710-719; 2001.

27. Shapiro, A. M.; Lakey, J. R.; Ryan, E. A.; Korbutt, G. S.; Toth, E.; Warnock, G. L.; Kneteman, N. M.; Rajotte, R. V. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N. Engl. J. Med. 343:230-238; 2000.

28. Swift, S.; Kin, T.; Mirbolooki, M.; Wilson, R.; Lakey, J. R. Comparison of cooling systems during islet purification. Cell Transplant. 15:175-180; 2006.

29. Takahashi, H.; Goto, M.; Ogawa, N.; Saito, Y.; Fujimori, K.; Kurokawa, Y.; Doi, H.; Satomi, S. Superiority of fresh islets compared with cultured islets. Transplant. Proc. 41:350-351; 2009.

30. Takahashi, H.; Goto, M.; Ogawa, N.; Saito, Y.; Fujimori, K.; Kurokawa, Y.; Doi,

H.; Satomi, S. Influence of a current style of culture on the quality of isolated pancreatic islets. Transplant. Proc. 40:358-359; 2008.

31. Terasaki, P. I.; Cecka, J. M.; Gjertson, D. W.; Takemoto, S. High survival rates of kidney transplants from spousal and living unrelated donors. N. Engl. J. Med. 333:333-336; 1995.

32. The CITR Group, 2007 Update on Allogeneic Islet Transplantation from the Collaborative Islet Transplant Registry (CITR). Cell Transplant. 18(7):753-767; 2009.

33. Toyama, H.; Takada, M.; Suzuki, Y.; Kuroda, Y. Activation of macrophage-associated molecules after brain death in islets. Cell Transplant. 12:27-32; 2003.

34. Wennberg, L.; Song, Z.; Bennet, W.; Zhang, J.; Nava, S.; Sundberg, B.; Bari, S.; Groth, C. G.; Korsgren, O. Diabetic rats transplanted with adult porcine islets and immunosuppressed with cyclosporine A, mycophenolate mofetil, and leflunomide remain normoglycemic for up to 100 days. Transplantation 71:1024-1033; 2001.

Figure Legends

Figure. 1: Islet recovery and purity after isolation. The appearance of the isolated islets without brain death (A) and with brain death (B) at a magnification of 40×. (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.

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.

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-hour and 48-hour cultures ($P<0.05$).

ELSEVIER

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.

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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: ρ = .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

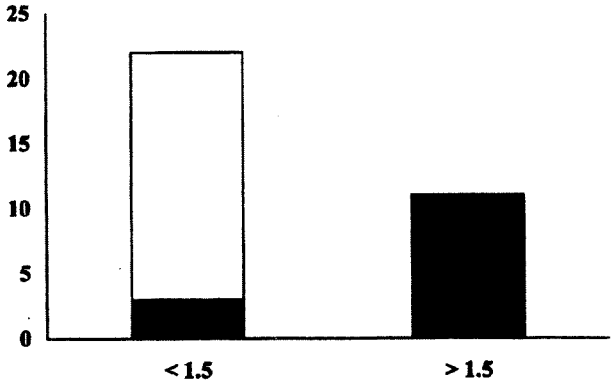

Fig 1. Streptozotocin-induced diabetic rats underwent intraportal transplantation with syngeneic islets that were exposed to various degrees of heat shock stress (0, 40, 50, 60, and 80 seconds). The X-axis indicates the glucose-stimulated respiratory activity, and the Y-axis indicates the number of animals. 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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REFERENCES

1. Barnett MJ, McGhee-Wilson D, Shapiro AM, et al: Variation in human islet viability based on different membrane integrity stains. Cell Transplant 13:481, 2004

2. Goto M, Holgersson J, Kumagai-Braesch M, et al: The ADP/ATP ratio: a novel predictive assay for quality assessment of isolated pancreatic islets. Am J Transplant 6:2483, 2006

3. Yamashita K, Takagi M, Uchida K, et al: Visualization of DNA microarrays by scanning electrochemical microscopy (SECM). Analyst 126:1210, 2001

4. Oyamatsu D, Hirano Y, Kanaya N, et al: Imaging of enzyme activity by scanning electrochemical microscope equipped with a feedback control for substrate-probe distance. Bioelectrochemistry 60:115, 2003

5. Kasai S, Yokota A, Zhou H, et al: Immunoassay of the MRSA-related toxic protein, leukocidin, with scanning electrochemical microscopy. Anal Chem 72:5761, 2000

6. Yasukawa T, Uchida I, Matsue T: Microamperometric measurements of photosynthetic activity in a single algal protoplast. Biophys J 76:1129, 1999

7. Wennberg L, Song Z, Bennet W, et al: Diabetic rats transplanted with adult porcine islets and immunosuppressed with cyclosporine A, mycophenolate mofetil, and leflunomide remain normoglycemic for up to 100 days. Transplantation 71:1024, 2001

8. Brandhorst D, Brandhorst H, Zwolinski A, et al: Prevention of early islet graft failure by selective inducible nitric oxide synthase inhibitors after pig to nude rat intraportal islet transplantation. Transplantation 71:179, 2001

9. Eckhard M, Brandhorst D, Winter D, et al: The role of current product release criteria for identification of human islet preparations suitable for clinical transplantation. Transplant Proc 36:1528, 2004

10. Gerling IC, Kotb M, Fraga D, et al: No correlation between in vitro and in vivo function of human islets. Transplant Proc 30:587, 1998

11. Hering BJ, Kandaswamy R, Harmon JV, et al: Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. Am J Transplant 4:390, 2004

12. Ricordi C, Scharp DW, Lacy PE: Reversal of diabetes in nude mice after transplantation of fresh and 7-day-cultured (24 degrees C) human pancreatic islets. Transplantation 45:994, 1988

ELSEVIER

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

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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

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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).

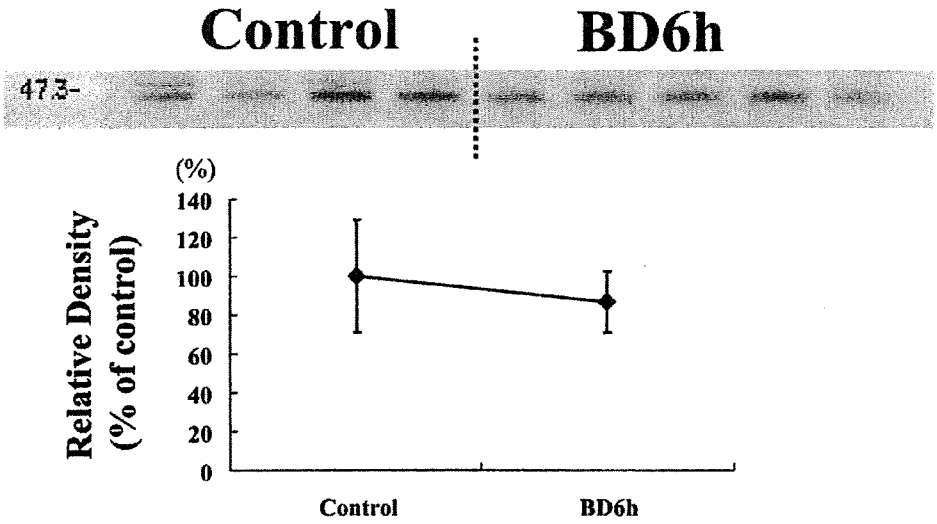

Fig 1. Protein expression of TF in the pancreatic tissues from the donors with/without BD was analyzed using Western blotting assay.

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.

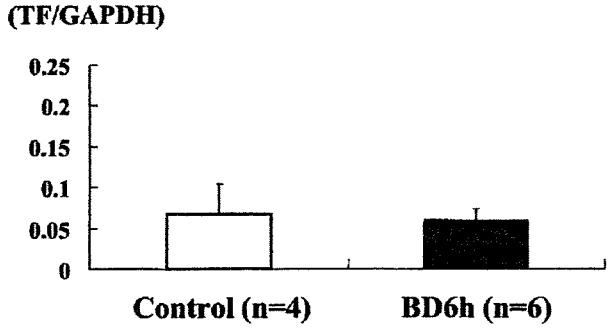

Fig 2. mRNA expression of TF in the pancreatic tissues from the donors with/without BD was analyzed using real-time PCR assay.

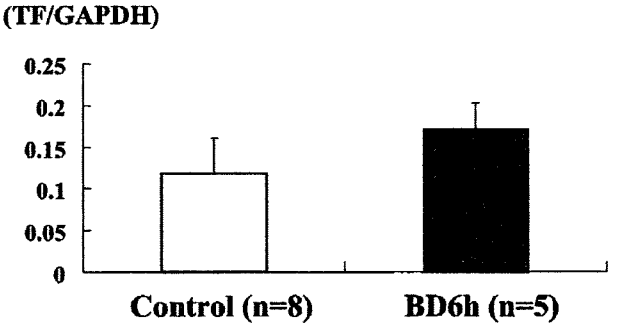

Fig 3. mRNA expression of TF in the isolated islets from the donors with/without BD was analyzed using real-time PCR assay.

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We thank Takehiro Imura and Megumi Goto for their excellent technical assistance.

REFERENCES

1. Moberg L, Johansson H, Lukinius A, et al: Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. Lancet 360: 2039, 2002

2. Berman DM, Caberera O, Kenyon NM, et al: Interference with tissue factor prolongs intrahepatic islet allograft survival in a nonhuman primate marginal mass model. Transplantation 84:308, 2007

3. Goto M, Johansson U, Eich TM, et al: Key factors for human islet isolation and clinical transplantation. Transplant Proc 37:1315, 2005

4. Terasaki PI, Cecka JM, Gjertson DW, et al: High survival rates of kidney transplants from spousal and living unrelated donors. N Engl J Med 333:333, 1995

5. Contreras JL, Eckstein C, Smyth CA, et al: Brain death significantly reduces isolated pancreatic islet yields and functionality in vitro and in vivo after transplantation in rats. Diabetes 52:2935, 2003

6. Pratschke J, Wilhelm MJ, Kusaka M, et al: A model of gradual onset brain death for transplant-associated studies in rats. Transplantation 69:427, 2000

7. Goto M, Holgersson J, Kumagai-Braesch M, et al: The ADP/ATP ratio: a novel predictive assay for quality assessment of isolated pancreatic islets. Am J Transplant 6:2483, 2006

8. Wennberg L, Song Z, Bennet W, et al: Diabetic rats transplanted with adult porcine islets and immunosuppressed with cyclosporineA, mycophenolate mofetil, and leflunomide remain normoglycemic for up to 100 days. Transplantation 71:1024, 2001

9. Goto T, Tanioka Y, Sakai T, et al: Application of the two-layer method on pancreas digestion results in improved islet yield and maintained viability of isolated islets. Transplantation 83:754, 2007

10. Avila JG, Wang Y, Barbaro B, et al: Improved outcomes in islet isolation and transplantation by the use of a novel hemoglobin-based O_2 carrier. Am J Transplant 6:2861, 2006

11. Swift S, Kin T, Mirbolooki M, et al: Comparison of cooling systems during islet purification. Cell Transplant 15:175, 2006

ELSEVIER

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 ±

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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).

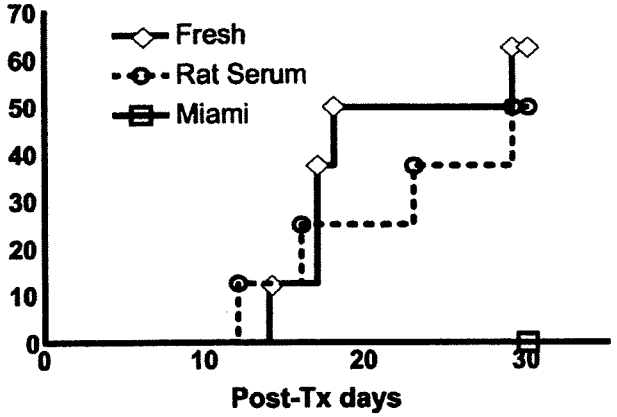

Fig 1. The influence of culture on islet quality was evaluated by intraportal transplantation of syngeneic islets into streptozotocin-induced diabetic rats. In an in vivo transplantation model, the curative rate was higher in the fresh islet group than in the others.

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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REFERENCES

1. Goto M, Eich TM, Felldin M, et al: Refinement of the automated method for human islet isolation and presentation of a closed system for in vitro islet culture. Transplantation 78:1367, 2004

2. Goto M, Holgersson J, Kumagai-Braesch M, et al: A novel predictive assay for quality assessment of isolated pancreatic islets. Am J Transplant 6:2483, 2006

3. Moberg L, Johansson H, Lukinius A, et al: Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. Lancet 360: 2039, 2002

4. Goto M, Groth CG, Nilsson B, et al: Intraportal pig islet xenotransplantation into athymic mice as an in vivo model for the study of the instant blood-mediated inflammatory reaction. Xenotransplantation 11:195, 2004

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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).

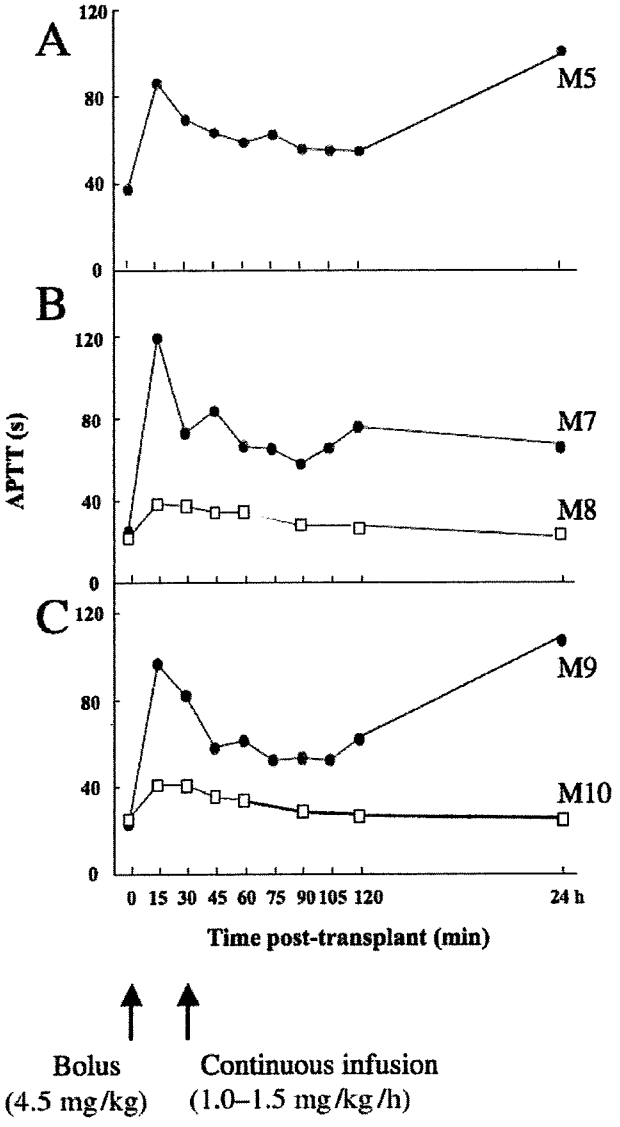

Fig. 1. Plasma APTT values in transplanted diabetic monkeys (M5 and M7–M10) treated with heparin (squares) or LMW-DS (circles).

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.

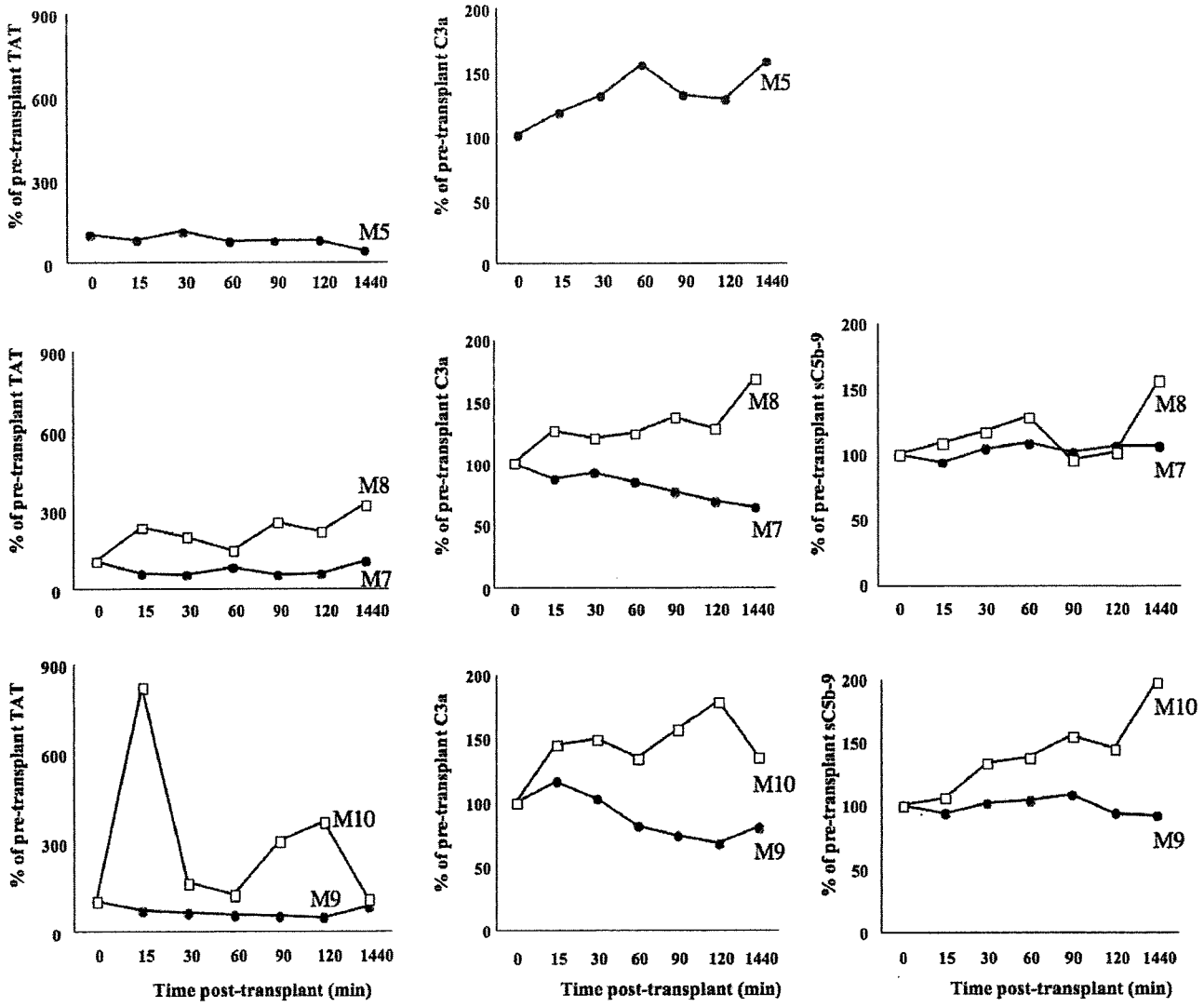

Fig. 2. EDTA blood was drawn from a femoral vein catheter of the transplanted monkeys treated with heparin (squares) or LMW-DS (circles) at varying time points after porcine islet xenotransplantation. TAT, C3a, and sC5b--9 levels were assessed and expressed as percentage of the pre-transplant values.

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

The immunohistochemical findings from the grafts were summarized in Fig. 4 and Table 2. As expected, most parameters involved in innate immune responses were active after 24 h post-islet transplantation in the controls M8 and M10. In particular, CD68+ macrophages, and neutrophil elastase positive PMNs were abrogated in the monkeys treated with LMW-DS compared with the controls given heparin. Also, CD41+ platelets tended to be lower in the LMW-DS treated animals. CD56+ natural killer cells were found only occasionally. Unlike the soluble complement markers there was no clear inhibition of complement activation as reflected in deposition of C3 fragments and C9 on the surface of the islets. Furthermore, IgM antibodies were found on islet both in LMW-DS and heparin-treated animals. Most of parameters reflecting specific immune responses were yet silent. However, CD3+ T-cell infiltration was already seen in the islet grafts of the controls M8 and M10. Notably, this infiltration was effectively suppressed by LMW-DS.

Binding of complement components to porcine islets after incubation in human plasma

After incubation in hirudin-treated plasma, the porcine islets were stained with FITC-conjugated antibodies recognizing IgG, IgM, C1q, C3b/iC3b, C4 fragments, C9, and MBL. Large particle flow cytometry and confocal microscopy demonstrated that antibodies against IgG, IgM, C1q, C4, and C3

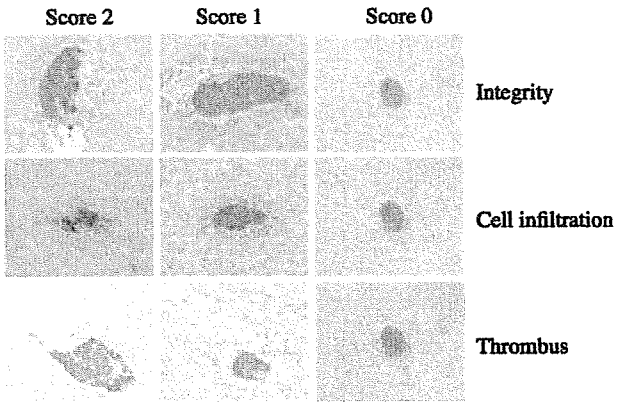

Fig. 3. Visual examples of the morphological scoring system used to quantify different aspects of the IBMIR. Hematoxylin eosin-stained porcine islet grafts retrieved 24 h after intraportal xenotransplantation from diabetic monkeys treated with LMW-DS or heparin were used. A summary of all transplanted monkeys is presented in Table 1.

bound strongly to the islets, but the binding of MBL and C9 was less prominent (Fig. 5A and B). C3b/iC3b fragments were detected on the islets after only 5 min, and the binding of C3b/iC3b continued to increase over time. Addition of Compstatin significantly reduced the binding of C3b/iC3b to the islets (Fig. 5C). Confocal microscopy analyses confirmed these results (not shown).

Inhibition of complement activation by LMW-DS and Compstatin

Ten percentage (v/v) human serum was incubated in wells of microtiter plates in the presence of LMW-DS and/or Compstatin for 30 min at 37 °C (Fig. 6). In the presence of Compstatin there was no effect below 0.5 μM of the compound, but at higher concentrations Compstatin gradually inhibited complement activation. At 5 μM total inhibition was achieved. LMW-DS inhibited complement activation only marginally between 10 and 100 mg/l, but the effect was more pronounced at concentrations above this level. There was no indication of interaction between the drugs regarding this effect on complement activation in serum.

Discussion

We have previously shown that LMW-DS efficiently prevents clotting that occurs in both allogeneic and xenogeneic IBMIR triggered by APIs both in vitro and in vivo in a small animal model [9,18]. Here, we confirm that LMW-DS is efficient also in a primate model mimicking the clinical setting. The effect of LMW-DS was compared with that of heparin, which is routinely used in clinical islet transplantation. LMW-DS was proved to be

Table 1. Summary of the morphological score (as depicted in Fig. 3) of the islets grafts in recipient monkeys M5 and M7–M10

Monkey number	Treatment	Integrity	Thrombus	Cell infiltration	Percentage of score 0[a] (%)	APTT at 24 h after transplantation (s)
M5	LMW-DS, n = 113	0.66 ± 0.04[b]	0.26 ± 0.03	0.90 ± 0.07	37.2	101
M7	LMW-DS, n = 134	0.93 ± 0.06	0.52 ± 0.06	1.08 ± 0.06	26.1	66
M8	Heparin, n = 149	1.05 ± 0.05	0.62 ± 0.06	1.17 ± 0.06	20.1	24
P-value[c]		0.13	0.28	0.32	0.23	
M9	LMW-DS, n = 134	0.63 ± 0.05	0.37 ± 0.05	0.85 ± 0.06	44.0	107
M10	Heparin, n = 125	0.95 ± 0.06	0.54 ± 0.06	1.25 ± 0.07	22.4	25
P-value[d]		<0.0001	<0.05	<0.0001	<0.001	

[a]Percentage islets with no signs of IBMIR (score 0); [b]Values are expressed as mean ± SEM; [c]P-values for M7 and M8; [d]P-values for M9 and M10. APTT, activated partial thromboplastin time; IBMIR, instant blood-mediated inflammatory reaction; LMW-DS, Low molecular weight dextran sulfate.

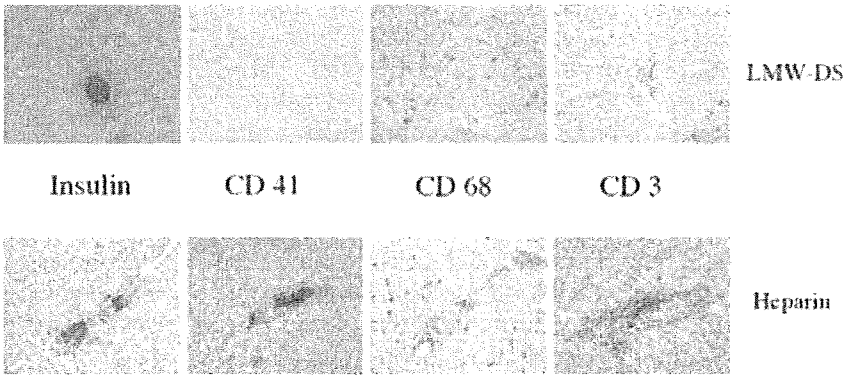

Fig. 4. Immunohistochemical staining of porcine islet grafts retrieved 24 h after intraportal xenotransplantation from diabetic monkeys treated with LMW-DS or heparin. The figure shows representative expression of insulin and of CD41 (platelets), CD68 (macrophages), and CD3 (T cells) in the grafts. A summary of all transplanted monkeys is presented in Table 2. Magnification 200×.