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,

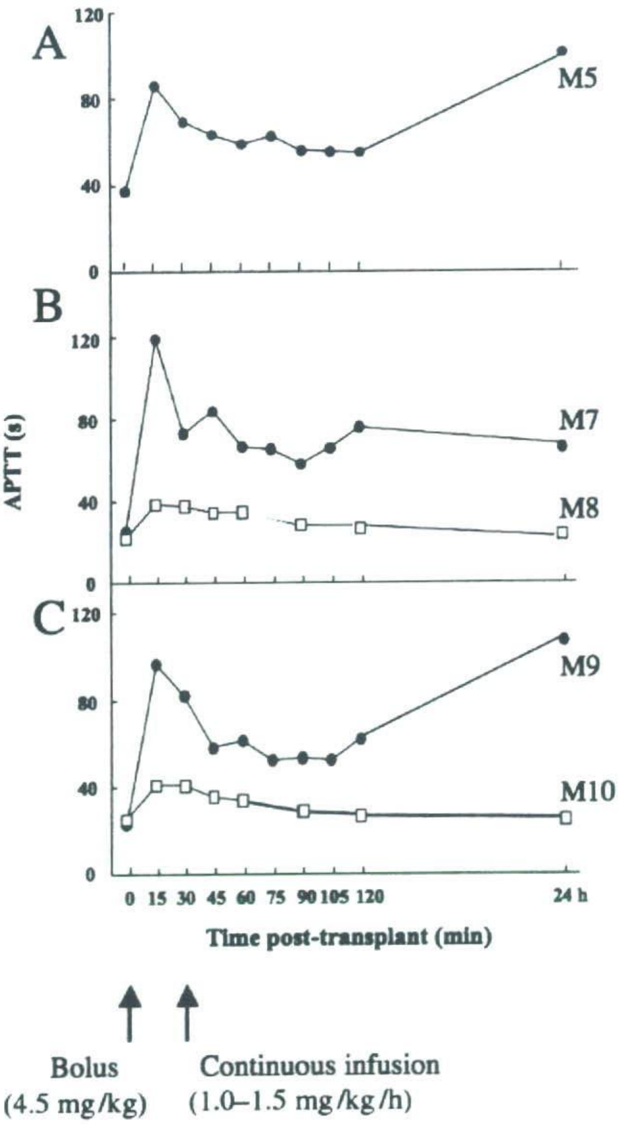

Fig. 1. Plasma APTT values in transplanted diabetic monkeys (M5 and M7–M10) treated with heparin (squares) or LMW-DS (circles).

LMW-DS did not trigger an increase of plasma IL-1β, TNFα, or CRP (not shown).

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.

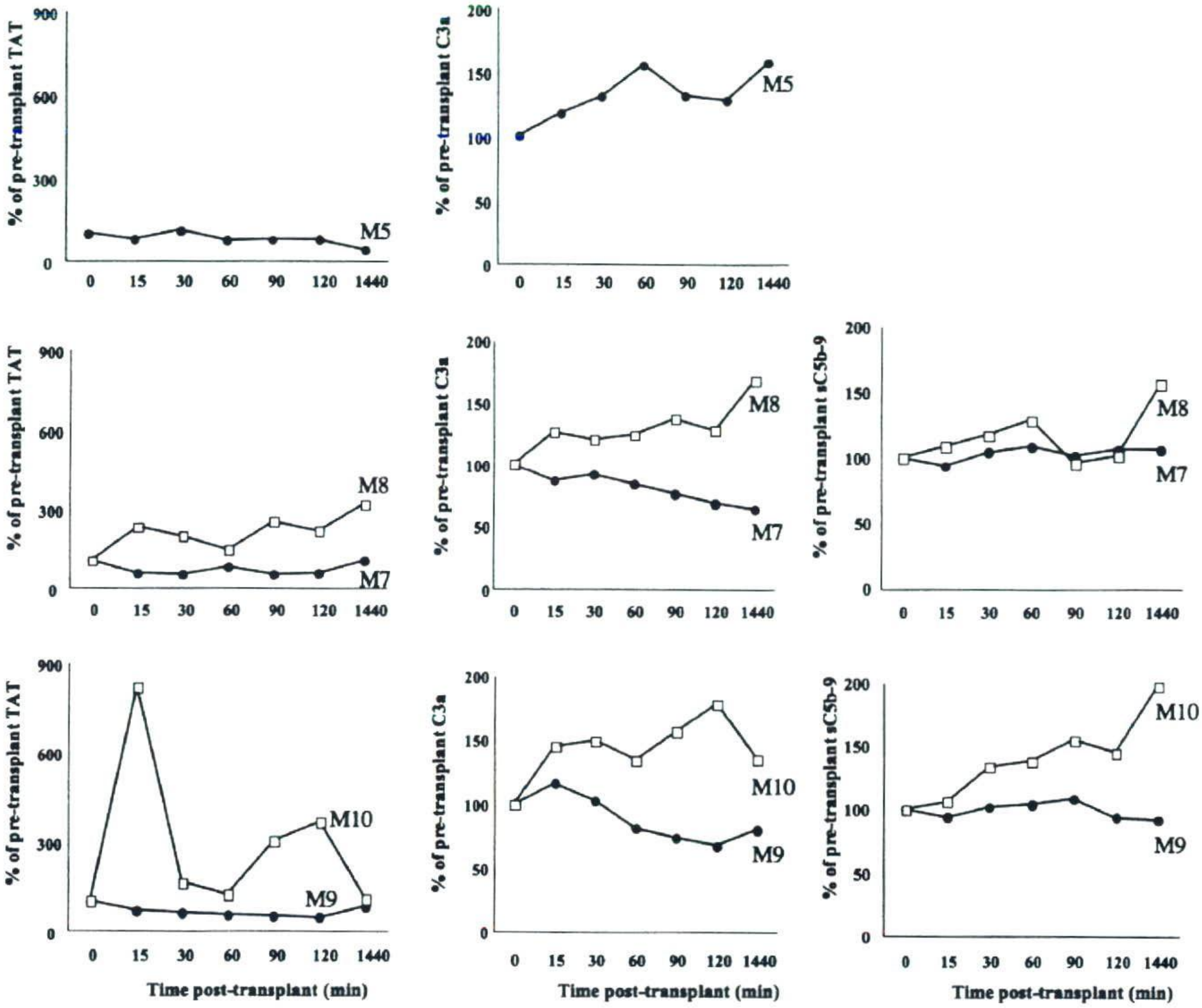

Fig. 2. EDTA blood was drawn from a femoral vein catheter of the transplanted monkeys treated with heparin (squares) or LMW-DS (circles) at varying time points after porcine islet xenotransplantation. TAT, C3a, and sC5b–9 levels were assessed and expressed as percentage of the pre-transplant values.

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

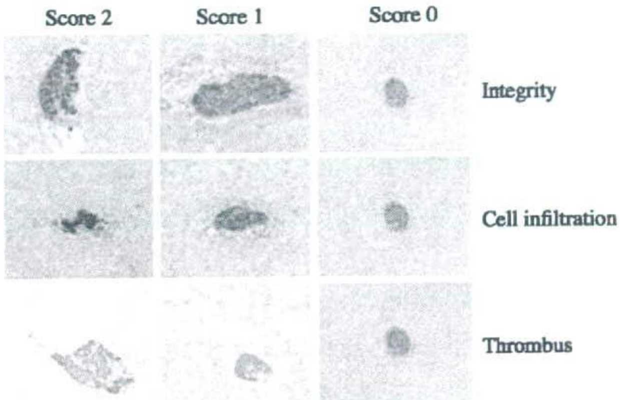

Fig. 3. Visual examples of the morphological scoring system used to quantify different aspects of the IBMIR. Hematoxylin eosin-stained porcine islet grafts retrieved 24 h after intraportal xenotransplantation from diabetic monkeys treated with LMW-DS or heparin were used. A summary of all transplanted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

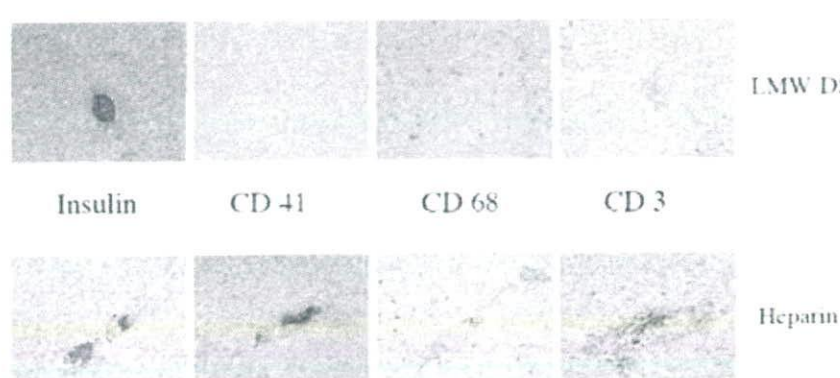

Fig. 4. Immunohistochemical staining of porcine islet grafts retrieved 24 h after intraportal xenotransplantation from diabetic monkeys treated with LMW-DS or heparin. The figure shows representative expression of insulin and of CD41 (platelets), CD68 (macrophages), and CD3 (T cells) in the grafts. A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

Table 2. Summary of the immunohistochemical staining (as depicted in Fig. 4) of the islet grafts in recipient monkeys receiving LMW-DS or heparin

Treatment	CD41	C3c	C9	Neutrophil elastase	CD68	MAC 387	CD56	CD3	CD20	IgG	IgM
LMW-DS	(−)-(++)	(−)-(++)	(−)-(+++)	(−)-(++)	(−)-(++)	(−)-(++)	(−)-(+)	(−)-(++)	(−)-(+)	(−)	(−)-(+)
(n = 21)	0.59 ± 0.19[a]	0.80 ± 0.37	1.50 ± 0.31	0.42 ± 0.23	1.31 ± 0.21	0.90 ± 0.28	0.10 ± 0.10	0.63 ± 0.20	0.20 ± 0.13	0	0.25 ± 0.25
Heparin (n = 18)	(−)-(+++)	(−)-(++)	(−)-(+++)	(−)-(++)	(++)-(+++)	(+)-(+++)	(−)-(+)	(−)-(+++)	(−)-(++)	(−)	(−)-(++)
	1.60 ± 0.51	0.63 ± 0.26	1.67 ± 0.33	1.08 ± 0.23	2.17 ± 0.11	2.11 ± 0.26	0.22 ± 0.15	1.90 ± 0.35	0.40 ± 0.27	0	0.60 ± 0.24
P value	0.056	0.69	0.71	0.04	0.002	0.01	0.48	0.006	0.83	–	0.36

[a]Values are expressed as mean ± SEM. LMW-DS, Low molecular weight dextran sulfate.

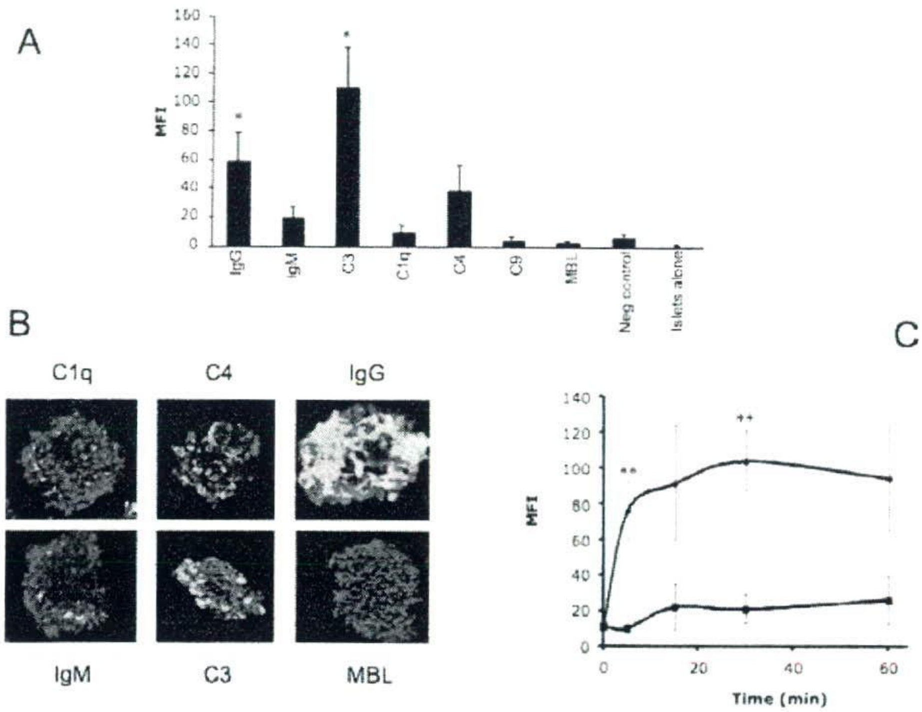

Fig. 5. Porcine islets incubated in hirudin-treated plasma for 30 min. The islets were stained for IgG (n = 5), IgM (n = 5), C3b/iC3b (n = 5), C1q (n = 3), C4 (n = 5), C9 (n = 3), and MBL (n = 3). As negative control, an antibody recognizing mouse IgG was used (n = 5). The islets were analyzed by (A) large particle flow cytometry and (B) confocal microscopy. In (C), the deposition of C3b/iC3b on the islet in the absence and presence of Compstatin is presented after analysis by large particle flow cytometry (n = 5; statistical evaluation was performed at 5 and 30 min where n = 7; *P < 0.05 and **P < 0.01).

far more efficient in inhibiting the IBMIR than heparin. These data confirm those of Rood et al. [19] who recently demonstrated longer porcine islet survival in non-human primates treated with LMW-DS.

In this study, both the morphological findings and the measurements in the plasma were similar to those in our previous studies, in which APIs were surrounded by clots and infiltrated by numerous leukocytes immediately after contact with fresh blood seen in the tubing loop model and our small animal model [9]. Most parameters reflecting the IBMIR, i.e. both coagulation and complement cascades, platelet deposition, and infiltration of macrophages and neutrophils, were attenuated in the monkeys treated with LMW-DS compared to the controls. There was also a tendency that increases in granulocyte count and liver enzymes were attenuated. One control monkey (M6) died of hypoglycemia, suggesting a strong IBMIR. Notably, T-cell infiltration observed in some of the transplanted islet grafts was also effectively suppressed, demonstrating that also the adaptive immune responses are attenuated by LMW-DS.

The effects of LMW-DS on the adaptive immune system may be explained by the effects on complement activation as complement is also of great importance in bridging innate immunity and specific immune responses. In allogeneic whole organ transplantation, C3 is one of the essential factors that trigger rejection in mice [20–22] and humans [23]. It is therefore reasonable to expect that

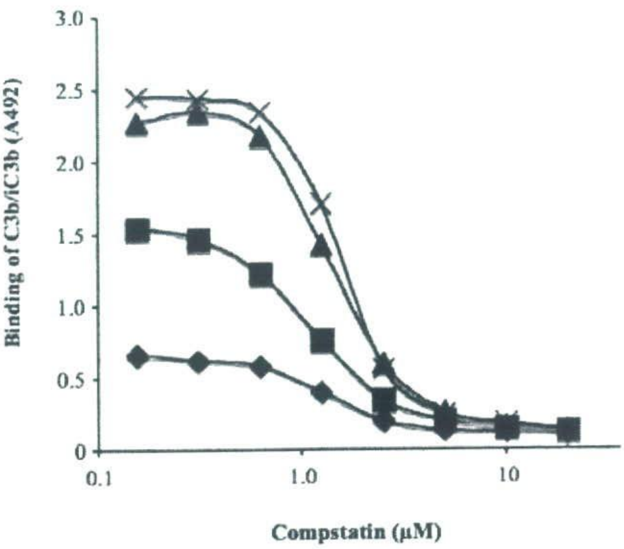

Fig. 6. Binding of C3b/iC3b to the surface of microtiter wells after incubation with 10% serum in the presence of increasing doses of Compstatin for 30 min at 37 °C. 0 (cross), 10 (triangle), 100 (squares), and 1000 (diamond) mg/l of LMW-DS was present in the wells.

complement activation will trigger a profound adaptive immune response raised against the graft, necessitating an unwarrantedly heavy immunosuppressive regimen. Previous studies support such a hypothesis [4,5].

As shown in Table 1, the islet grafts in M9 that reached an APTT of 107 s 24 h after transplantation, demonstrated well-preserved morphology suggesting that this dose of LMW-DS would be preferable. In a recently performed phase I study in normal individuals, we have shown that this concentration can be reached without an increased risk of bleeding or side effects (manuscript under preparation). This makes treatment with LMW-DS during xenogeneic islet transplantation an attractive alternative. It should be noted that a specific concentration of LMW-DS gives different APTT in blood from different individuals both in vitro and in vivo, probably due to that different allotypes of certain coagulation factors interact with LMW-DS differently.

In our previous studies, we showed that complement activation induced in xenogeneic IBMIR occurs secondarily to coagulation activation; a reaction which is also seen in allogeneic IBMIR [9,24] and which is elicited by chondroitin sulfate released by activated platelets [25]. This explains why complement activation in this study was suppressed in parallel with the reduction of coagulation activation at substantially lower concentrations (15–35 mg/l) of LMW-DS than used in other studies aiming for a inhibition of hyper acute rejection [26,27]. However, unlike complement activation in the fluid phase, immunohistochemical analyses showed that complement deposition was still seen on the islet grafts in the monkeys treated with LMW-DS. These reactions were analyzed in detail in vitro using large particle flow cytometry and confocal microscopy to be able to clarify the mechanism of activation. The experiments were performed using human plasma to directly translate the findings to clinical islet xenotransplantation. Pig islets incubated in human plasma revealed an almost instantaneous binding of IgM and IgG antibodies and complement components already after 5 min. This rapid activation was completely inhibited by Compstatin. It is possible that the instantaneous insulin dumping in a non-human primate model previously reported by Bennet et al. [3] and also observed in monkey M6 is explained by this antibody-mediated reaction. The severity of this reaction, which was totally abrogated by the recombinant complement inhibitor CR1, is reflected in the fact that the release of insulin corresponded to about 40% of the insulin in the transplanted islets.

One way to fully inhibit complement activation is to increase the dose of LMW-DS, but as shown in our in vitro experiments, doses between 10 and 100 mg/l have only minor effects on complement activation alone. Moreover, higher doses of LMW-DS are likely to give side effects. It is therefore obvious that LMW-DS must be combined with a specific complement inhibitor such as Compstatin to block the immediate destructive immunoglobulin-triggered complement activation found both in vitro and in vivo [10,28]. The in vitro studies show that LMW-DS and Compstatin do not interact in human serum.

Taken together, it possible to propose a model of how the different components of IBMIR interact in xenogeneic combinations: (1) Immediately when porcine islets come in contact with human blood there is an instantaneous binding of IgG and IgM antibodies to the islet surface which triggers a deleterious complement activation; (2) This is followed by a clotting reaction with accompanying complement activation. Based upon the experimental data presented, LMW-DS combined with a specific complement inhibitor is an attractive alternative to control the detrimental innate immune responses that are postulated to occur in forthcoming intraportal pre-clinical and clinical islet xenotransplantation trials. We are at the moment in progress to produce sufficient amounts of Compstatin to perform studies in the NHP model with Compstatin combined with LMW-DS.

Acknowledgments

This work was supported by National Institute of Health Grants 5U01-A1065192, GM-62134, GM069736, and AI068730, Juvenile Diabetes Research Foundation, 1-2004-18, Swedish Research Council Grants 5647, and 15244.

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ELSEVIER

Optimization of a Prominent Oxygen-Permeable Device for Pancreatic Islets

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ABSTRACT

Background. We have demonstrated a culture bag system that is useful for pancreatic islet transplantation. To improve and simplify islet transplantation procedures from culture to transplantation, we developed a novel device specific for both islet culture and transplantation (TUBERO Device [TD]) using an oxygen-permeable material.

Materials and Methods. Porcine islets with 30 minutes warm ischemia time were cultured for 24 hours at 37°C in 5% CO_2 and humidified air under three different procedures: (1) ordinary culture flask, (2) culture bag suitable for platelets, and (3) TD. Loss of islets during culture, glucose-stimulated insulin release as an islet functional test, and ADP/ATP ratio as an index of islet viability tests were evaluated to compare the devices. TD was further applied in two clinical islet transplantations using non–heart-beating donors in Japan.

Results. The loss of islets during culture was considerably lower in the TD group. The stimulation index upon glucose challenge tests was significantly higher in the TD group than the others. The ADP/ATP ratio in TD group was significantly lower than that in the ordinary flask group, suggesting that the apoptotic islets were relatively lower among TD. Most importantly, TD was successfully applied both in the clinical islet cultures and in transplantation, resulting in excellent graft function.

Conclusions. We propose that the TD, a novel product, not only simplifies islet transplantation procedures, but also maintain the quality of isolated islets.

WE HAVE demonstrated a culture bag system that is useful for pancreatic islet transplantation.[1] To improve and simplify the islet transplantation procedures, we have described a novel device for both islet culture and transplantation (*T*UBERO *D*evice [TD]) using a prominent oxygen-permeable material.

MATERIALS AND METHODS

The TD was made from polyethylene film. The oxygen permeation coefficient of the polyethylene film is 3,000 cm^3/m^2 atm. Porcine islets with 30 minutes warm ischemia time were cultured for 24 hours at 37°C in 5% CO_2 and humidified air under three different procedures: (1) ordinary culture flasks, (2) culture bags suitable for platelets, and (3) TD. The loss of islets during culture, the glucose-stimulated insulin release as a islet functional test, and the ADP/ATP assay[2] as an islet viability test were evaluated to compare the devices. TD was further applied in two clinical islet transplantations using non–heart-beating donors in Japan.

RESULTS

The loss of islets during culture tender to be lower among the TD group: (1) 22.9 ± 4.4, (2) 23.9 ± 10.0, and (3) 38.9 ± 6.0: (P = .09, Fig 1). The stimulation index on glucose challenge tests was significantly higher in TD group than

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Supported by grants from the Japanese Special Coordination Funds for Promoting Science and Technology, the Japanese Grant-in-Aid for Scientific Research (B).

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0041-1345/08/$–see front matter
doi:10.1016/j.transproceed.2008.01.056

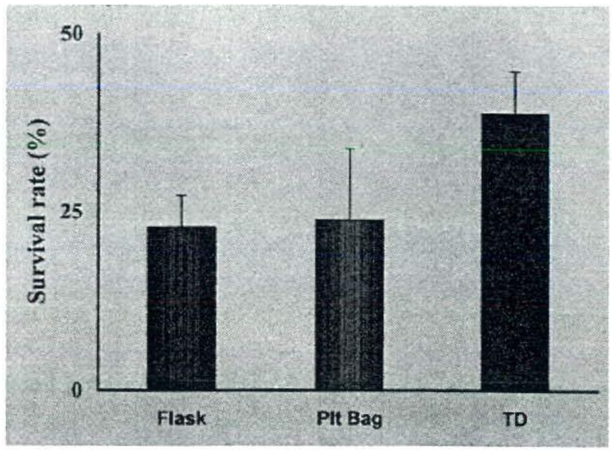

Fig 1. The loss of porcine islets during 24-hour culture was evaluated as the survival rate.

the other methods: (1) 1.46 ± 0.15, (2) 1.67 ± 0.17, and (3) 2.09 ± 0.18 ($P = .03$; Fig 2). The ADP/ATP ratio in the TD group was significantly lower than that in the ordinary flask

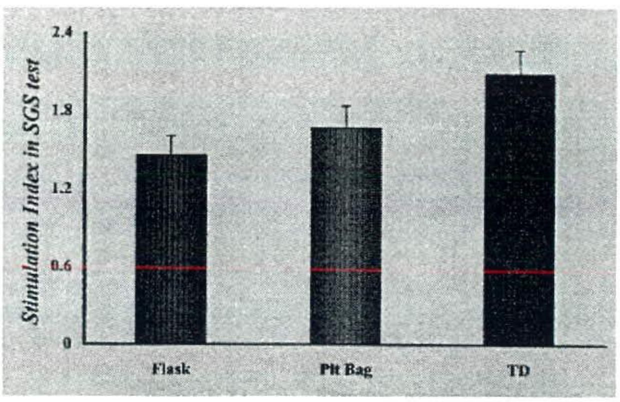

Fig 2. Glucose-stimulated insulin release was evaluated as a functional test.

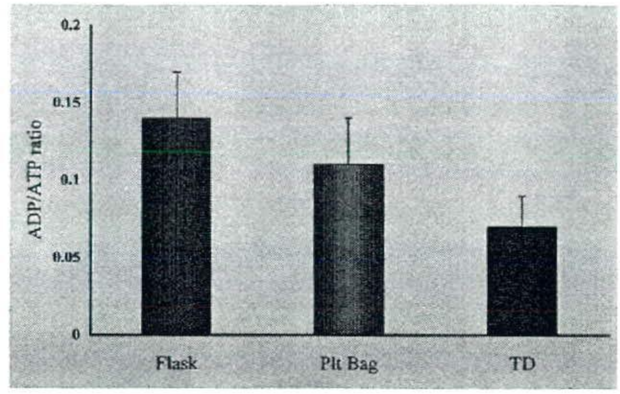

Fig 3. The ADP/ATP assay was evaluated as a viability test.

group (TD 0.07 ± 0.02 vs. flask 0.14 ± 0.03; $P = .07$; Fig 3), suggesting fever apoptotic islets with TD. Most importantly, TD was successfully applied in transplantation, resulting in excellent graft function: fasting C-peptide levels during 6 months after transplantation: 1.2 to 2.7 ng/mL and secretory units of islets in transplantation (SUIT) index[3]: 15 to 45.

In conclusion, the TD simplified islet transplantation procedures and maintained the quality of isolated islets.

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ELSEVIER

Influence of a Current Style of Culture on the Quality of Isolated Pancreatic Islets

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

ABSTRACT

Objective. Comparable outcomes of islet transplantation with short periods of culture may be achieved with various culture media. To clarify the influence of a style of culture on isolated pancreatic islets, islet quality of fresh islets was compared with those cultured in several different fashions including not only for viability but also for inflammatory mediators.

Materials and Methods. Wistar rat islets were cultured for 48 hours with CMRL including 10% allogeneic serum; CMRL including 0.5% human serum albumin (HSA); and Miami medium including 0.5% HSA. The influence of culture conditions on islet integrity was evaluated by survival rate of islets during culture and visual scoring. The influence of culture conditions on islet function and viability was examined by ADP/ATP tests, insulin/DNA content, and glucose stimulation tests.

Results. Although the survival rates were similar for all groups, the visual scoring was lower in Miami medium. The stimulation index in glucose challenge tests was higher for fresh islets than the media ($P = .02$). Insulin/DNA ratios revealed the same tendency as glucose challenge tests ($P = .0005$). ADP/ATP ratio was lower in both the fresh and serum groups than in the others ($P = .38$), suggesting that apoptotic islets are relatively fewer in both fresh and serum groups. Most importantly, the expression of tissue factor (TF) on the islets was considerably lower in the fresh group, suggesting that a current style of culture could enhance TF-dependent instant blood-mediated inflammatory reactions after transplantation.

Conclusion. In conclusion, Isolated islets without prior culture shows characteristics beneficial for transplantation using current modes of culture.

ALTHOUGH ONE KEY FACTOR of the Edmonton protocol is transplantation of fresh islets just after isolation, it has recently been reported that the outcomes are comparable with short periods of culture. This observation may be based upon specific culture media used in an institution. To clarify the influence of culture media on isolated pancreatic islets, the present study compared islet quality including not only viability but also expressed inflammatory mediators of fresh islets with those cultured in several different fashions.

MATERIALS AND METHODS

Wistar rat islets cultured for 48 hours with CMRL including 10% allogeneic serum or 0.5% human serum albumin (HSA), and Miami medium including 0.5% HSA are the most established current media. The influence of culture on islet integrity was evaluated by the survival rate of islets during culture and by visual scoring.[1] The influence of culture conditions on islet function and viability was examined by glucose stimulation tests[1] (Fig 1), insulin/DNA ratios (Fig 2), and ADP/ATP assays[2] (Fig 3). The influence of culture on the expression of inflammatory mediators in the islets

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0041-1345/08/$–see front matter
doi:10.1016/j.transproceed.2008.01.022

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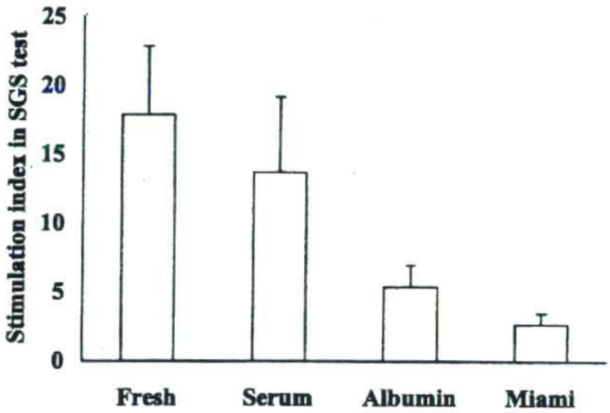

Fig 1. The influence of culture conditions on islet function was examined by static glucose stimulation tests (SGS).

was examined by Western blotting assays for tissue factor (TF), which is the initiator of detrimental instant blood-mediated inflammatory reactions (IBMIR). Statistical analyses were performed by using ANOVA.

RESULTS

Although the survival rates were similar for all groups, the visual scoring was lower in Miami medium. The stimulation index in glucose challenge tests was higher among fresh than the other islets: fresh = 17.89 ± 4.93; serum = 13.69 ± 5.44; HSA = 5.36 ± 1.60; Miami = 2.69 ± 0.82 (P = .02; serum vs Miami; n = 5). Insulin/DNA ratios revealed the same tendency as glucose challenge tests: fresh = 1.02 ± 0.07; serum = 0.83 ± 0.11; HSA = 0.52 ± 0.07; Miami = 0.37 ± 0.08 (P = .0005; fresh vs HSA, fresh vs Miami; n = 5). ADP/ATP ratios were lower among both fresh and serum than the other groups: fresh = 0.047 ± 0.021; serum = 0.054 ± 0.017; HSA = 0.086 ± 0.004; Miami = 0.084 ± 0.026 (P = .38), suggesting a relatively lower content of apoptotic islets in both fresh and serum groups. Most importantly, the expression of TF on the islets was considerably lower in the fresh group, suggesting that the current style of culture enhanced tissue factor-dependent IBMIR after transplantation.

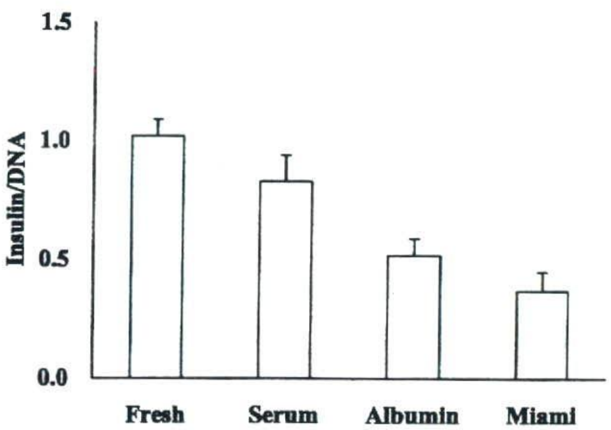

Fig 2. The influence of culture conditions on islet function was examined by insulin/DNA assays.

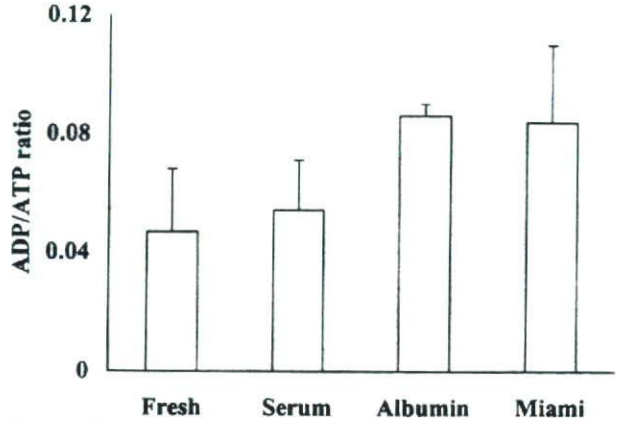

Fig 3. The influence of culture conditions on islet viability was examined by ADP/ATP assays.

DISCUSSION

Isolated islets without prior culture were beneficial for transplantation using current modes of culture. Further improvements are required to optimize a substitute for the serum supplement.

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報告

膵島移植症例登録報告(2008)

膵・膵島移植研究会膵島移植班

Islet transplantation in Japan
—Report from Japanese Islet Transplantation Registry—

The Japanese Society for Pancreas and Islet transplantation

【Summary】

The program of islet transplantation in Japan was started in 2004. One hundred twenty nine recipients are currently registered. Sixty five isolations and 34 transplants were performed in 18 insulin-dependent diabetes mellitus recipients including 2 IAK recipients. All but one donor (64/65) were non-heart-beating donors. All recipients were free from hypoglycemic episodes after transplantation. Three of these recipients became insulin independent transiently and most the other patients are currently being followed with a minimal dose of exogenous insulin.

Keywords: Japanese Society for Pancreas and Islet Transplantation, non heart-beating donor, islet transplantation, type I diabetes mellitus

I. はじめに

わが国の膵島移植は膵・膵島移植研究会の膵島移植班を中心として準備を進め，2004年から開始された。膵・膵島移植研究会膵島移植班では実施症例ごとにワーキンググループで検討を重ねてきたが，2007年より移植学会を中心として開始された全臓器を対象とした移植症例登録と報告に伴い，膵島移植例の成績について，昨年第1回目の報告を行った[1]。しかし，2007年3月，Liberase HIによるCreutzfeldt-Jakob病(以下，CJD)感染の可能性が明らかとなり，わが国における膵島移植は停止している。現在，製造過程で哺乳類由来の抽出物を使用しない新しい酵素製剤の開発が進められており，2009年より膵島移植を再開しうる見込みである。今回は，2007年12月末までの膵島分離・移植症例の集計結果およびLiberase問題に対する膵・膵島移植研究会の対応について報告する。

II. 対象と方法

2007年から開始された日本移植学会への各臓器の症例登録作業に伴い，膵・膵島移植研究会膵島移植班で作成した膵島移植登録に関するフォーマットをもとに集計を行った。

1. 膵島移植施設認定および実施体制

膵・膵島移植研究会では，実際に膵島の分離・凍結・移植が可能であることを確認するために施設基準を設け，新たに膵島移植施設の申請があった場合はこの施設基準をもとに膵・膵島移植研究会内の施設認定委員会で検討し，施設認定を行っている[2]。これまでに新鮮膵島分離・凍結・移植施設として，北から東北大学，福島県立医科大学，国立千葉東病院，京都大学，大阪大学，神戸大学，福岡大学の7施設が認定されている。施設認定を受けた各施設は膵・膵島移植研究会内のシェアリング委員会における協議決定に従い，その施設が存在する地域(県)および隣接する地域を担当する形で地域を分担しブロック体制を形成しているが，2007年は新たな申請施設がなく，膵島移植実施体制に変更はない。

2. レシピエント登録

膵島移植の適応基準は，①内因性インスリン分泌が著しく低下し，インスリン治療を必要とする状態で，②糖尿病専門医の治療努力によっても血糖コントロールが困難な，③75歳以下の患者，としている。禁忌条件としては重度の心・肝疾患，アルコール中毒，感染

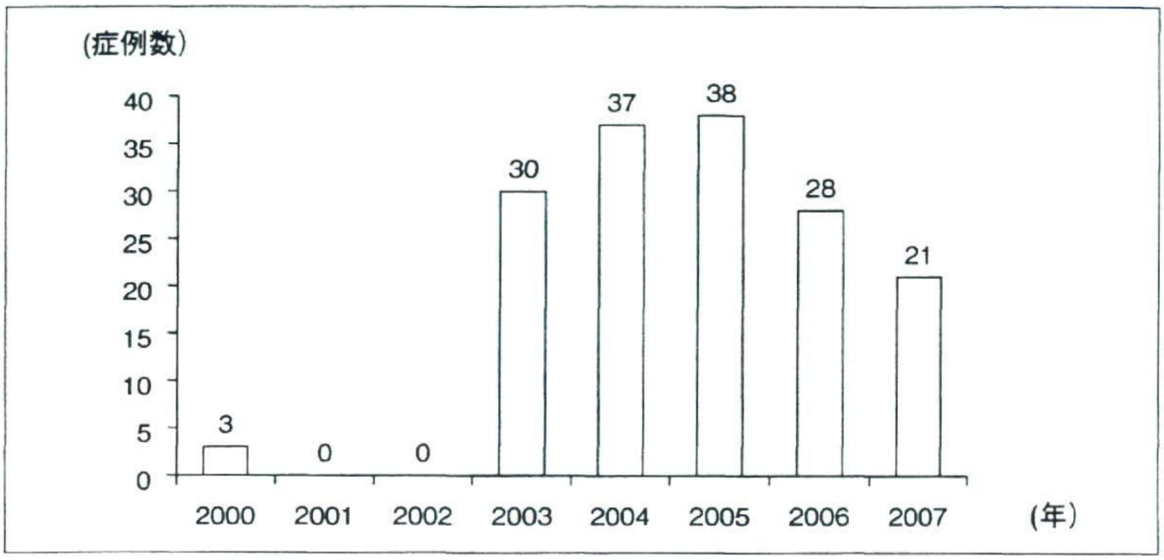

図1　新規登録者数の推移

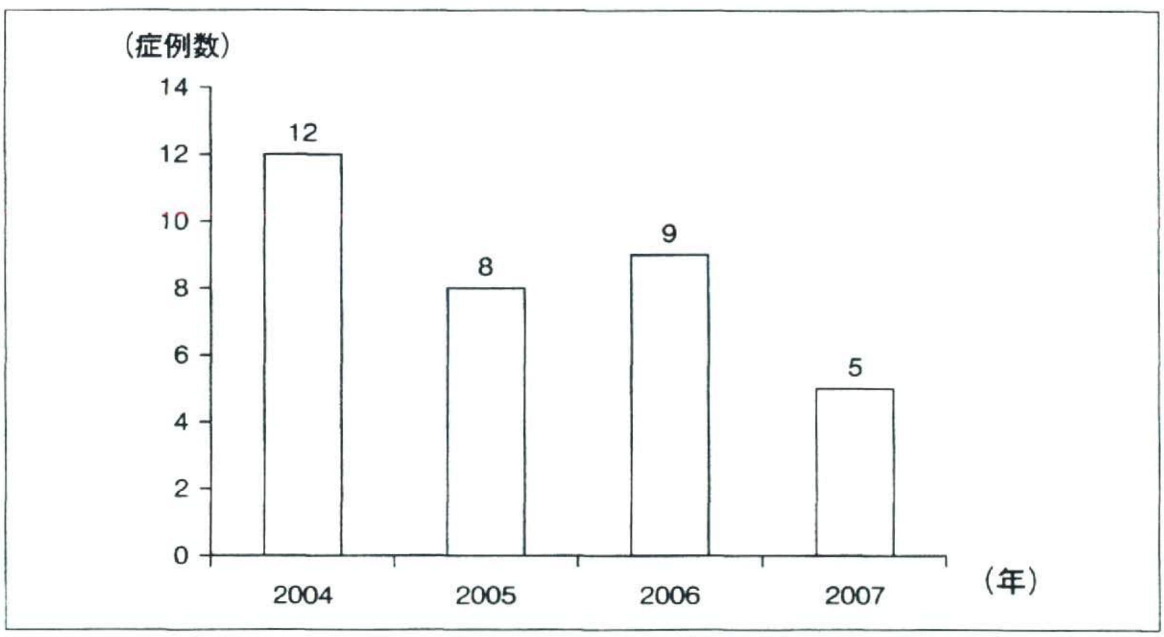

図2　膵島移植症例数の推移

症，悪性腫瘍の既往，重症肥満，未処置の網膜症などが挙げられている[2]。糖尿病性腎症に関しては，膵島単独移植の場合はⅢA期までを適応とし，腎移植後膵島移植症例では，移植後6カ月以上経過し，クレアチニン 1.8 mg/dl 以下で直近6カ月の血清クレアチニンの上昇が 0.2 以下で，ステロイド内服量 10 mg/dl 以下，などの基準を満たす症例を移植の対象としている[2]。2007年12月末の時点で157名が登録され，3回移植あるいはインスリン離脱例が7名，再判定にて適応外となったものが2名，辞退者13名，待機中死亡5名あり，レシピエント候補者として130名が登録されている。2000年以降の新規登録者数の推移を**図1**に示す。

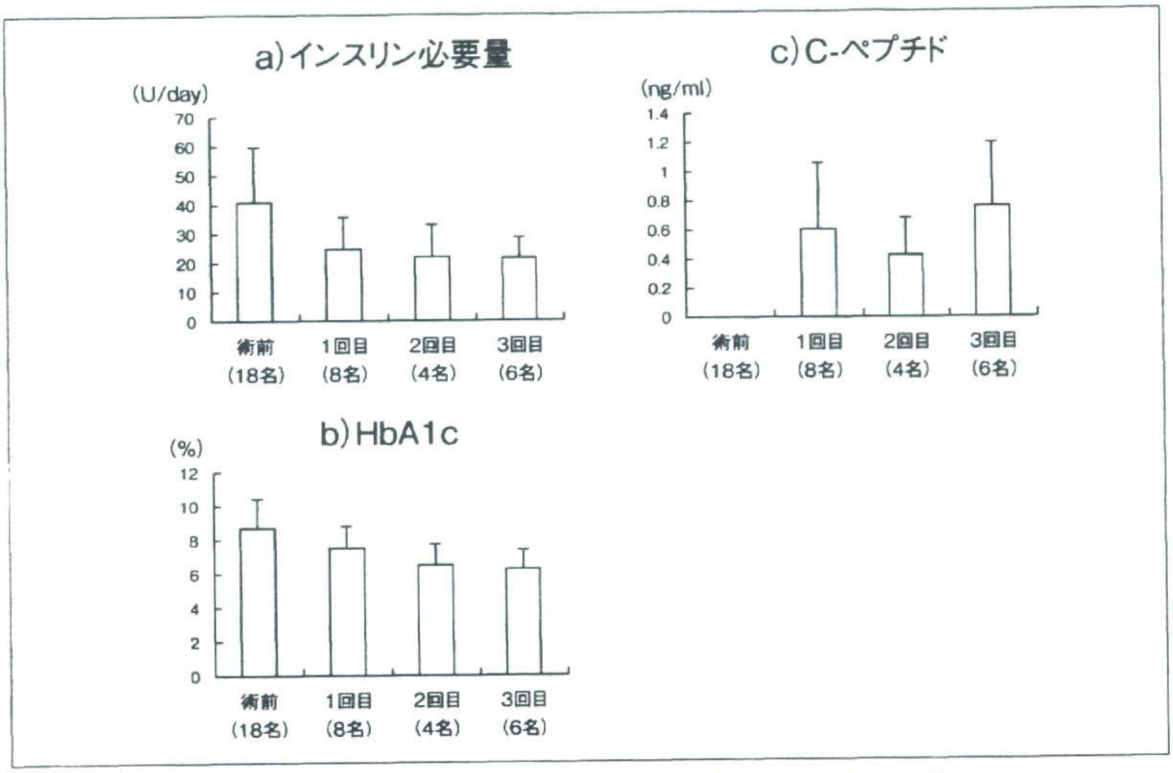

図3 膵島移植前後のインスリン必要量，C-ペプチド，HbA1c の推移

III. 結果と考察

2007年12月までに65回の膵島分離が行われ，1例の脳死ドナーを除く64回は心停止ドナーからの提供であった。このうち34回で移植の条件を満たしていたため18症例（男性5例，女性13例）に対して膵島移植が行われた（移植率：移植回数/分離回数×100＝52%）。移植症例の平均年齢は37.3歳，糖尿病歴は6～37年（平均20.8年）であった。移植症例数の年次推移を**図2**に示す。

膵島移植は3回まで行うことが可能で，これらの18例に対する移植回数は1回8名，2回4名，3回6名であった。術前，1回移植後，2回移植後，3回移植後における，インスリン必要量，HbA1c値，C-ペプチド値はそれぞれインスリン必要量：39.7±18.0 U/day，24.2±11.0 U/day，21.4±11.5 U/day，21.0±7.7 U/day（**図3a**），HbA1c値：8.8±1.8%，7.5±1.4%，6.5±1.4%，6.2±1.2%（**図3b**），C-ペプチド：感度以下，0.5±0.4 ng/ml，0.4±0.2 ng/ml，0.8±0.4 ng/ml（**図3c**）と，インスリン必要量およびHbA1c値は術前に比して減少し，術前陰性であったC-ペプチドは移植後に陽性となっている。これらの症例のうち，2回移植の1例と3回移植の2例の計3症例で一時的にインスリン離脱を認めた。本邦における膵島移植症例にエドモントンプロトコールによる膵島移植の多施設共同研究における膵島生着の基準である，basal C-peptide level が0.3 ng/ml以上を当てはめると，初回移植後6カ月，1年，2年時における膵島生着率はそれぞれ80.0%，73.3%，58.7%であった（**図4**）。

ところで，2007年3月，Liberase HIによるCJD感染の可能性が明らかとなり，わが国における膵島移植は停止しているが，これに関する膵・膵島移植研究会の対応について述べる。本研究会では，2007年3月27日，Liberase HIの製造過程におけるClostridium histolyticum培養の強化培地に含まれるウシ脳抽出物がTSE危険地域由来であるため，NIHが全米のLiberase HIを用いた膵島移植を停止したとの情報を得たため，ただちにわが国における膵島移植を停止した。また，本邦で実施された心停止ドナーによる膵島移植18例と生体膵島移植1例に対するCJDをはじめとする

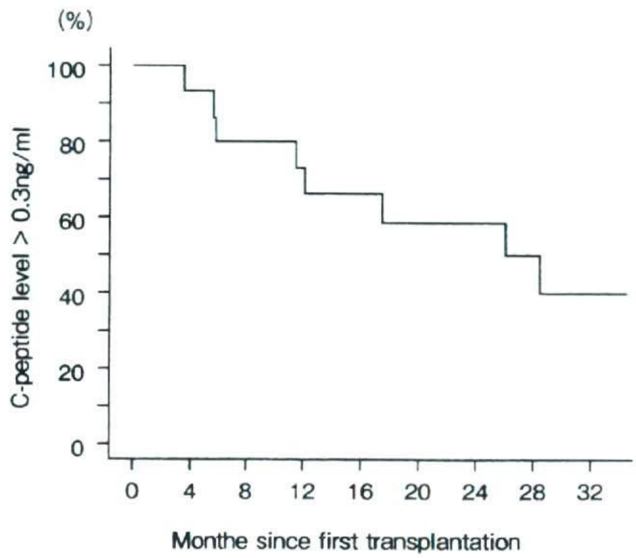

図4　膵島生着率

transmissible spongiform encephalopathy（以下，TSE）感染の可能性について，基礎と臨床のCJD専門家にコンサルトし，感染の可能性はきわめて低いものの完全に否定することはできないとの回答を得た。このため，本研究会は本邦における膵島移植を停止することを各ブロック事務局，日本臓器移植ネットワーク，日本組織移植学会，厚生労働省へ通達した。また，移植症例および移植待機症例に対してこれらの情報を文書で伝え，さらに各移植施設を通じて直接患者説明を行った。その中で，献血および臓器・組織移植のドナーとならないこと，外科手術を受ける際は必ず連絡していただくことをお願いした。さらにすべての移植症例でdiffusion MRIと脳波を測定しCJD専門医による診断と経過観察を受ける体制を整えた。また，コラゲナーゼ中のプリオンによるCJD感染の危険性評価のため，移植症例と同ロットのLiberase HIを用い，ヒト化プリオンのノックインマウスを用いた高感度バイオアッセイ系を用い，感染性の可能性を調査する体制を整えた。これらの対応は厚生労働省および遅発性ウイルス感染調査研究班のご協力のもとに1カ月以内で終了した。

今後は，前述のCJD対策を継続するとともに，移植再開に向けて安全性の高い酵素製剤を検討しており，また膵島移植の費用負担の問題を解決するために高度医療への申請に向けて，使用薬剤のプロトコールを検討中である。

IV. おわりに

膵・膵島移植研究会が長年にわたって準備を進め，2004年から開始された膵島移植症例の第2回の集計結果を誌上で公にすることができた。膵・膵島移植研究会会員をはじめとする関係各位のご協力の賜であり，稿を終えるにあたり改めて感謝の意を表したい。

文責：膵・膵島移植研究会膵島移植班事務局
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Cell Transplantation, Vol. 17, pp. 619–629, 2008
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Mitomycin-C Treatment Followed by Culture Produces Long-Term Survival of Islet Xenografts in a Rat-to Mouse Model

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One of the goals of islet transplantation is to transplant viable islets without host immunosuppression. The present study was designed to determine whether pretreatment of islets with mitomycin-C (MMC) followed by culture enhances islet survival in a rat-to-mouse xenogeneic combination. WS(RT1k) rat islets pretreated with various concentrations of MMC (0, 3.2, 10, 32, 100, 320, and 1000 μg/ml) were tested for viability by in vitro insulin secretory capacity and vital staining of islets. The MMC-treated islets (10 μg/ml) cultured for various periods (4, 20, or 40 h, 3 or 7 days) were transplanted into the renal subcapsular space of STZ-induced diabetic C57BL/6 (B6: H-2b) mice. MMC-treated or nontreated islets were subjected to microarray gene analysis and immunohistological study. Evaluation of in vitro insulin secretory capacity and vital staining of islets indicated that MMC at a dose ≤32 μg/ml is nontoxic and preserves islet function. Marked prolongation of graft survival was noted with half of islet grafts surviving indefinitely (>100 days) when 10 μg/ml of MMC-treated islets was transplanted after 40 h or 3 days in culture, but not when they were transplanted within 4 h following treatment or at 7 days following treatment, indicating that there is a critical culture period necessary for successful islet graft survival. Microarray analysis suggested possible genes for this prolongation with TGF-β highly expressed in MMC-treated islets subjected to culture for 3 days. Our results indicate that MMC treatment followed by a critical culture period induces marked prolongation of rat islet xenograft survival in nonimmunosuppressed recipient mice, offering a strategy for islet transplantation without immunosuppression.

Key words: Mitomycin-C; Culture; Islet xenograft; Insulin secretion

INTRODUCTION

Since the Edmonton group's successful report on human islet transplantation, in which seven consecutive patients quickly attained sustained insulin independence after transplantation (22), more than 471 patients have received islets at 43 institutions worldwide in the past 5 years (21). High rates of insulin independence have been observed at a 1 year follow-up at the leading centers (21). However, insulin independence was lost in the majority of recipients by 5 years (21), and the side effects of immunosuppressants necessitate stringent inclusion criteria for islet-alone candidates. To improve outcome, antirejection drugs have been used based on their reduced toxicity to islets when compared to steroids, but they still harm the cells (2). Another possible result following human islet transplantation includes rejection or recurrence of autoimmune diabetes. These possibilities are reasonably assumed because the rate of late graft failure is small when islet autografts were carried out in patients who had undergone a total pancreatectomy for pain caused by chronic pancreatitis (19).

An important step to encourage the use of this treatment modality widely used for the cure of diabetes is to transplant islets without immunosuppression. We have shown that the treatment of islets with mitomycin-C (MMC) and overnight culture before transplantation induces significant prolongation of graft survival in an islet allotransplantation (BALB/c to B6) (17) and xenotransplantation (rat to mouse) (8) model, and that the effect of MMC treatment is concentration dependent. Thus, 3.2–32 μg/ml of MMC can potentially prolong graft survival, although higher concentrations were toxic with regard to glucose regulation in vivo (8,17). In the allograft model, half of grafts survived indefinitely with low-grade antigen-specific unresponsiveness (17). In the xenograft model, prolongation was significant, but was limited with no indefinite survival (8).

Received September 8, 2006; final acceptance January 7, 2008.
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For clinical application, some modification of the above protocol is necessary. Here, we show that MMC treatment followed by 3-day culture of islets at 37°C results in indefinite survival of islet xenografts in 50% of recipients, whereas culture alone was less effective. Together with these results, we identified potential genes responsible for prolongation of graft survival based on a microarray gene expression profile study. Among these genes, TGF-β expression was demonstrated to be upregulated histologically in these islets.

MATERIALS AND METHODS

Animals

Seven- to 8-week-old male WS (RT1k) rats (WS rats, Shionogi Pharmaceutical Co., Aburabi, Shiga, Japan) were used as islet donors. Diabetes was induced in 6- to 9-week-old male C57BL/6 (B6: H-2b) mice (Nihon Clea Inc., Shizuoka, Japan) by IP injection of streptozotocin (250 mg/kg Sigma, Japan) under anesthesia with ether inhalation. Recipients were mice with nonfasting blood glucose levels over 400 mg/dl for 2 consecutive days before transplantation. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Fukushima Medical University, and all procedures in this experiment were performed according to the guidelines of the National Research Council's *Guide for the Care and Use of Laboratory Animals*.

Islet Isolation, MMC Treatment, and Transplantation

The islets were isolated using a protocol similar to that reported previously (6). Briefly, we injected 3.0 ml of Hank's balanced salt solution (Nissui, Tokyo, Japan) containing 2 mg/ml collagenase (collagenase S-1, Nitta Gelatin, Japan) into the common bile duct. The distended pancreas was removed and incubated at 37°C for 35 min. Collagenase-digested islets were purified by centrifugation on gradients composed of three different Ficoll (type 400, Sigma Chemical Co., St. Louis, MO) densities (1.120, 1.090, and 1.050). After centrifugation, the distinct layer of islets was collected and washed. These islets containing immunogenic contaminants (crude islets) were incubated for 30 min with MMC (Kyowa Hakko Kogyo, Tokyo, Japan) at different doses (0, 3.2, 10, 32, 100, 320, and 1000 μg/ml). The islets were then washed three times with RPMI-1640 containing 2% fetal bovine serum (FBS) and cultured in complete medium [RPMI-1640 with HEPES (10 mM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), and 10% fetal calf serum] for 20 h at 37°C under 5% CO_2/95% air in a humidified atmosphere. The islets were handpicked and their viability was assessed by the following in vitro assay.

Assessment of Cell Viability of MMC-Treated Islets

The viability of MMC-treated islet cells was assessed by simultaneous use of the inclusion and exclusion dyes acridine orange (AO) and propidium iodide (PI), as described previously (1). Briefly, 10 to 50 islets were stained for 10 min with 0.67 μM AO and 75 μM PI in phosphate-buffered saline (PBS). Then islets were examined under confocal microscopy (model FV300, Olympus, Japan). Fluorescent images were obtained under a fluorescent microscope (Eclipse E800, Nikon, Japan) and a confocal laser microscope (FV300, Olympus, Japan). We used a fluorescent illuminator consisting of a 100-W mercurial light source with a 490-nm excitation filter and a 510-nm barrier filter. This filter combination permits simultaneous visualization of the green emission of AO, and the red emission of PI. Digital images of the islets treated with various concentrations of MMC (0, 3.2, 10, 32, 100, 320, and 1000 μg/ml) were analyzed for AO- or PI-positive areas using Adobe Photoshop™ 3.0J and NIH-Image software (Version 1.61/fat).

Glucose-Stimulated Insulin Secretion

Insulin secretory capacities to low (3.3 mM) and high (20 mM) glucose were evaluated for the islets treated with various concentrations of MMC (0, 3.2, 10, 32, 100, 320, and 1000 μg/ml, five measurements for each concentration) and cultured for 20 h or for 3 days according to the method described in a manual of clinical islet transplantation in Japan (23). Briefly, 10 islets, each measuring 150 μm in diameter, handpicked from the petri dish, were placed in a 12-well transwell microplate (Corning Transwell 3403, pore size 12 μm) with 3.3 mM glucose RPMI-1640 and 0.1% FCS at 37°C for 60 min under 5% CO_2 and 95% air for stabilization. After preincubation, the transwell was placed in a second well cluster containing 3.3 mM glucose RPMI-1640 and 0.1% FCS and incubated at 37°C for 60 min. Then, the transwell was placed in a third well cluster containing 20 mM glucose. Insulin content in the media of the second and third well clusters was measured for insulin secretion in response to low and high glucose loads. Stimulation index was calculated by dividing insulin secretion at high glucose by one at low glucose.

Extension of Culture Period of MMC-Treated Islets and Transplantation

In our previous reports, the effect of MMC treatment was tested after overnight (20 h) culture following MMC treatment (8). To determine the optimal culture period for maximum graft survival of MMC-treated (10 μg/ml) islets, the culture period following MMC treatment was either 4, 20, 40 h, 3 or 7 days. Three to 400 MMC-treated or untreated (only cultured for 20 h) crude-digested islets were transplanted into the renal subcapsular space of STZ-induced diabetic B6 mice as described previously (6). Rejection was defined as a blood glucose level higher than 300 mg/dl in two consecutive measurements.

Histology and Immunohistochemistry of Islet Graft

The long-term functioning (>100 days) islet grafts were removed to examine whether normoglycemia is maintained by grafted islets and also for immunohistological study. In some of these animals untreated islets, which were only cultured for 20 h, were transplanted into the right renal subcapsular space. Removed tissues were fixed in 10% formalin, and then stained with hematoxylin-eosin (H&E). For detection of insulin and glucagon in the graft, the paraffin sections were stained using rabbit antibodies specific for insulin, glucagons, and a peroxidase-labeled biotin-avidin detection system Histofine SAB-PO kit (Nichirei, Japan). For detection of somatostatin, goat polyclonal antibody specific for somatostatin (D-20:sc-7819) and peroxidase-conjugated rabbit anti-goat immunoglobulin (Wako:P0160) were used.

To examine TGF-β expression in MMC-treated and cultured islets, islets cultured for 3 days were fixed in 4% paraformaldehyde at 4°C for 4 h, and embedded in paraffin. A set of four serial sections (thickness 3 μm) was cut from each paraffin block, stained with mouse anti-human TGF-β1, -β2, and -β3 monoclonal antibodies (Genzyme Techne, CA), and biotin-conjugated goat affinity-purified antibody to mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were used. To compare the level of active TGF-β staining in the graft between two groups, quantification was blindly performed using the following semiquantitative scoring system: grade 0—no positive cells; grade 1—few positive cells; grade 2—more than few but <10% of cells show intense staining; grade 3—10% to 50% of cells show intense staining; grade 4—>50% of cells show intense staining for TGF-β.

Microarray Gene Expression Study

Islets isolated from 10 rats were pooled together and divided into two groups. One islet group was treated with MMC at 10 μg/ml for 30 min while the other group was treated in a similar fashion without MMC and cultured for 20 h or 72 h.

Total RNA was purified with the Micro RNA Isolation Kit (Stratagene, San Diego, CA) according to the instructions supplied by the manufacturer. Total RNA (20 μg) was labeled with Cy3 fluorescent dye using the Atras Fluorescent Labeling Kit (Clontech, Palo Alto, CA). Cy3-labeled probes were hybridized to rat Atras Grass microarray 1.0 (Clontech), which includes 1091 genes with various functional categories, such as the gene related to oncogenes and tumor suppressors, cellular signaling, apoptosis, and transcription regulators, at 50°C for 16 h. The microarrays were washed according to the instructions provided by the manufacturer and the slides were air-blown dried, prepared for scanning, and scanned for fluorescence with GenePrix (Axon Instruments, Union City, CA). The intensity ratios were normalized by the median to compare ratios between arrays.

Statistical Analysis

Data were expressed as mean ± SD. Graft survival in different experimental groups was compared using the Kaplan-Meier test. Ratios of PI-positive to AO-positive area, and insulin secretion in different experimental groups were compared using unpaired Student's *t*-test. Values of $p < 0.05$ were considered statistically significant. All statistical calculations were performed using Statview-J5.0 system software (SAS Institute Inc., Cary, NC).

RESULTS

Assessment of Cell Viability of MMC-Treated Islets by Vital Staining Using PI and AO

PI-positive cells showing loss of membrane integrity were found in the central area of islets significantly more often when the MMC concentration was greater than 100 μg/ml (Fig. 1). These cells also increased in number with greater concentrations of MMC, and most cells within the islets were PI positive at a MMC concentration of 1 mg/ml. These results suggest that MMC concentrations of ≤32 μg/ml are not toxic to the islets under membrane integrity examination.

Assessment of Cell Viability of MMC-Treated Islets Based on In Vitro Insulin Secretion

Significant increase of insulin secretory response to high glucose as compared to low glucose was observed when the MMC concentration was ≤100 μg/ml (Fig. 2a). The stimulation index of MMC-treated islets was maintained when the MMC concentration was ≤32 μg/ml (Fig. 2b), but the index decreased significantly when the MMC concentration was ≥100 μg/ml. These results indicate that MMC concentrations ≤32 μg/ml are not toxic to the islets based on insulin secretory capacity in response to glucose.

Effect of Duration of Culture of MMC-Treated Islets on Survival Time

We compared the graft survival time of MMC-treated and untreated islets cultured for 4, 20, and 40 h, and 3 and 7 days. Although the mean graft survival time of MMC-treated and untreated islets that were cultured for 4 h was not different (9.0 ± 0.4 vs. 9.3 ± 0.9 days), significant prolongation of graft survival was demonstrated when the culture period was extended up to 3 days (20

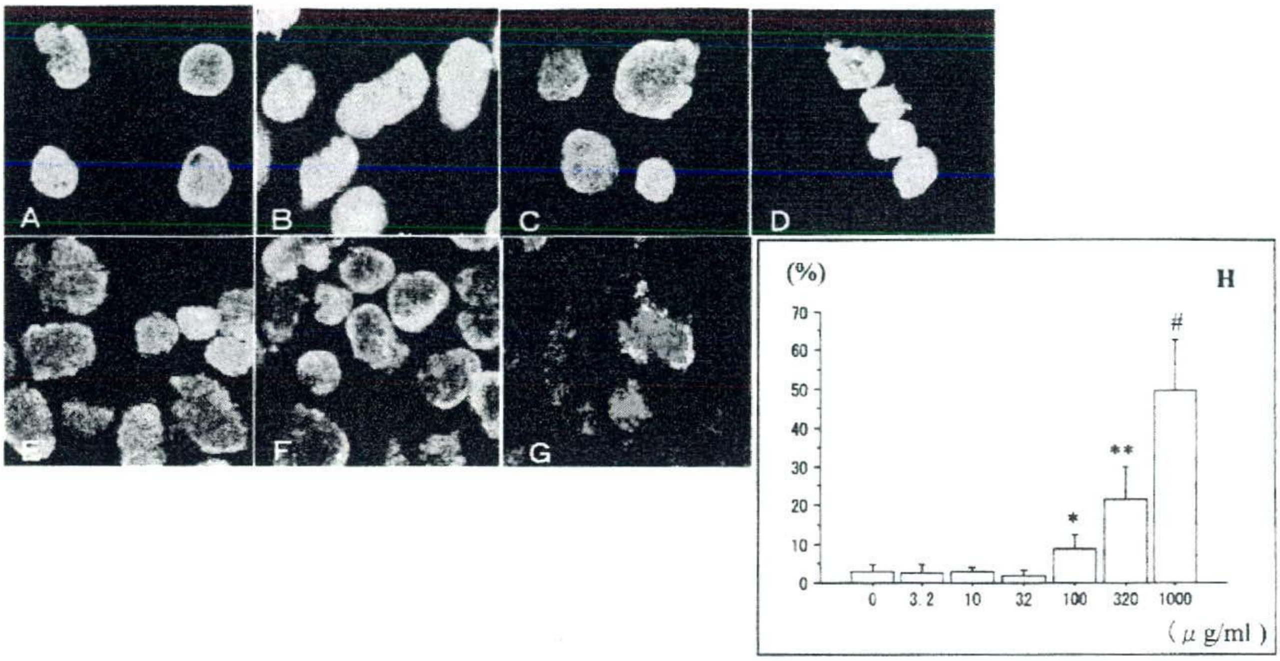

Figure 1. Vital staining of MMC-treated islets using PI and AO (original magnification ×100). Islets treated with MMC at various concentrations followed by culture for 20 h were tested for membrane integrity by vital staining. Islets shown in (A), (B), (C), (D), (E), (F), and (G) were obtained after treatment with 0, 3.2, 10, 32, 100, 320, and 1000 μg/ml MMC, respectively. Mean ratios of PI-positive area to PI- and AO-positive areas were calculated for each group (H). PI-positive areas were significantly increased when MMC concentration was >100 μg/ml. Data are mean ± SD. *$p < 0.05$, **$p < 0.01$, #$p < 0.001$ versus the mean value of untreated group.

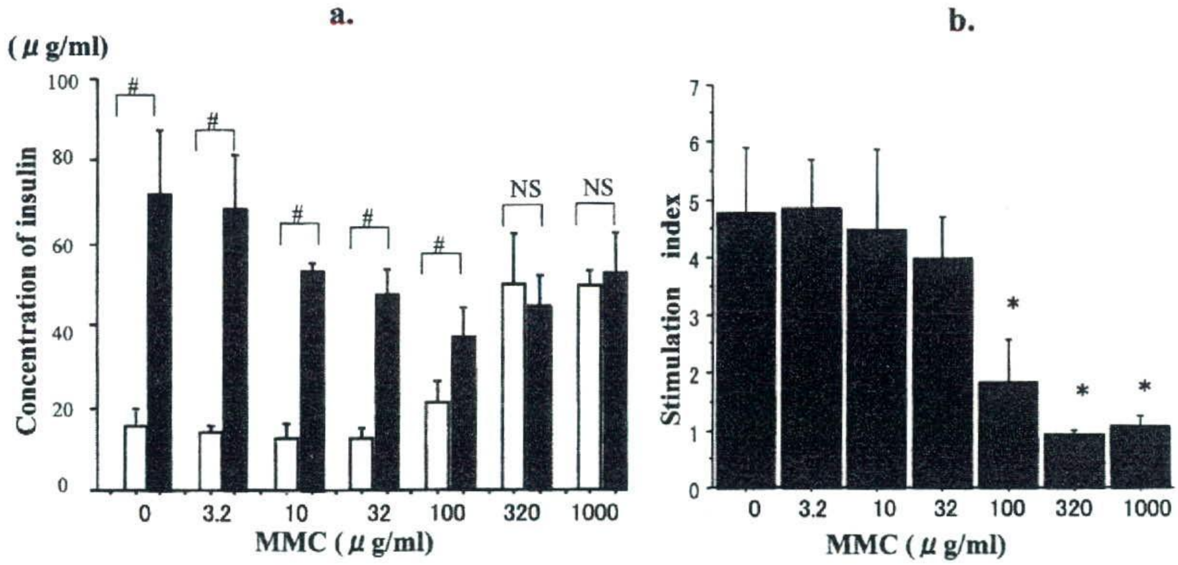

Figure 2. Insulin secretory responses of MMC-treated islets. MMC-treated islets at various concentrations (0, 3.2, 10, 32, 100, 320, and 1000 μg/ml) followed by 20-h culture were tested for insulin secretory capacities to low (3.3 mM; open bars) and high (20 mM; filled bars) glucose. Significant increase in insulin secretory response was noted at high glucose compared with low glucose when MMC concentration was ≤100 μg/ml (a) (#$p < 0.001$). Significant decrease in stimulation index was observed when MMC concentration was ≥100 μg/ml (b) (*$p < 0.05$). Data are mean ± SD.

h: 33.8 ± 7.4 vs. 11.9 ± 0.8, $p < 0.001$; 40 h: 64.7 ± 10.4 vs. 24.8 ± 4.7, $p < 0.002$; 3 days: 65.4 ± 12.4 vs. 15.7 ± 2.3, $p < 0.002$) (Fig. 3). Maximum prolongation was noted in mice transplanted with 3-day cultured MMC-treated islets, with indefinite survival of 50% (>100 days) of islet xenografts. No significant prolongation effect was noted after 7-day culture (31.5 ± 9.2 vs. 22.6 ± 6.3). The stimulation index of MMC-treated islets that were cultured for 3 days decreased to 2.5 ± 0.53, but was better than that of untreated islets (1.7 ± 0.4). Normal nonfasting blood glucose levels were restored within a few days both in animals given MMC-treated and untreated islets, suggesting that MMC treatment had no adverse effect on glucose metabolism.

Histology and Immunohistochemistry of Long-Term Functioning Islet Graft

The animals bearing long-term functioning islet xenografts over 100 days became hyperglycemic following graftectomy, indicating that normoglycemia was maintained by grafted islets. Immunohistological study demonstrated that long-term functioning islet grafts contained

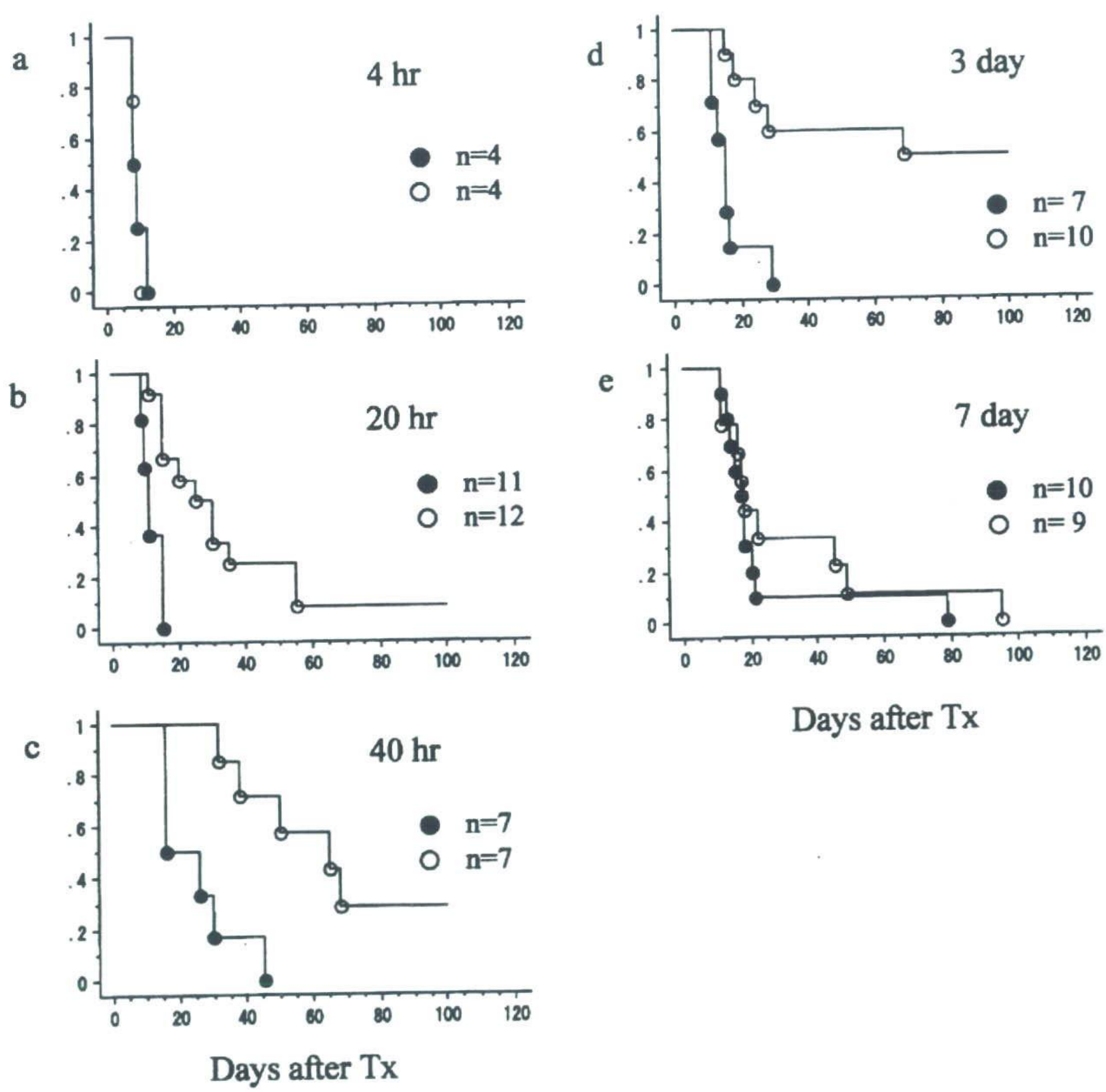

Figure 3. Graft survival time of MMC-treated and untreated islet xenografts after various culture periods. Significant prolongation of MMC-treated islets (open circles) compared with untreated islets (solid circles) was observed when islets were cultured for 20 h ($p < 0.01$), 40 h ($p < 0.002$), or 3 days ($p < 0.002$), but not for 4 h or 7 days.