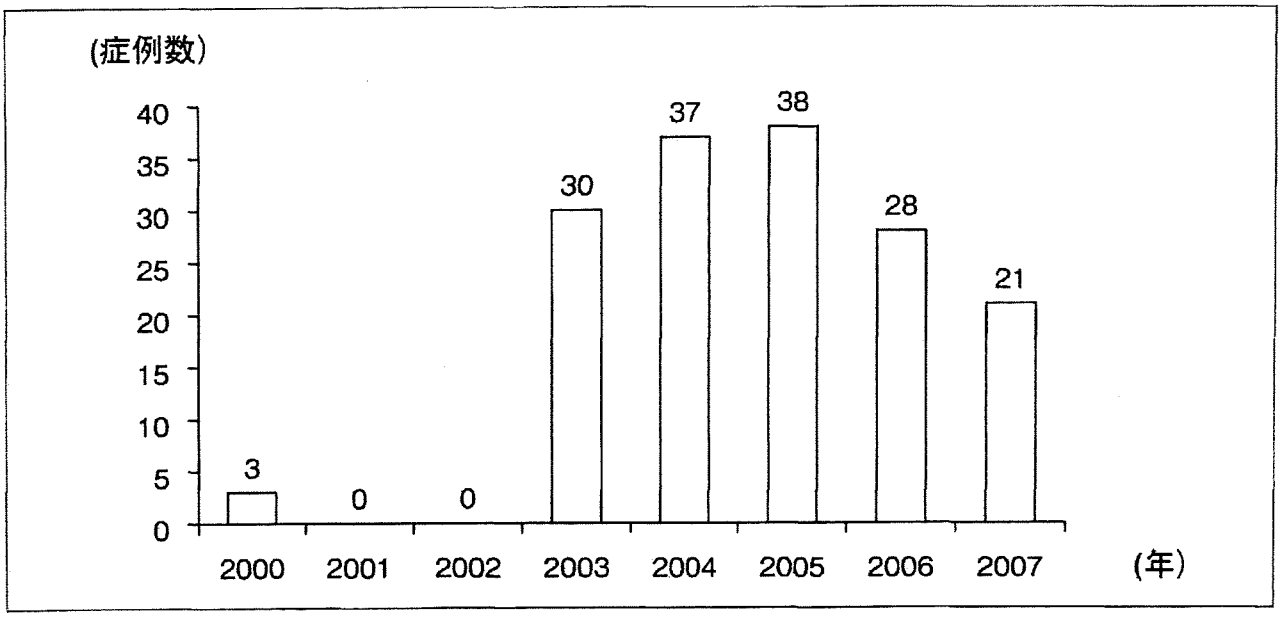

図1 新規登録者数の推移

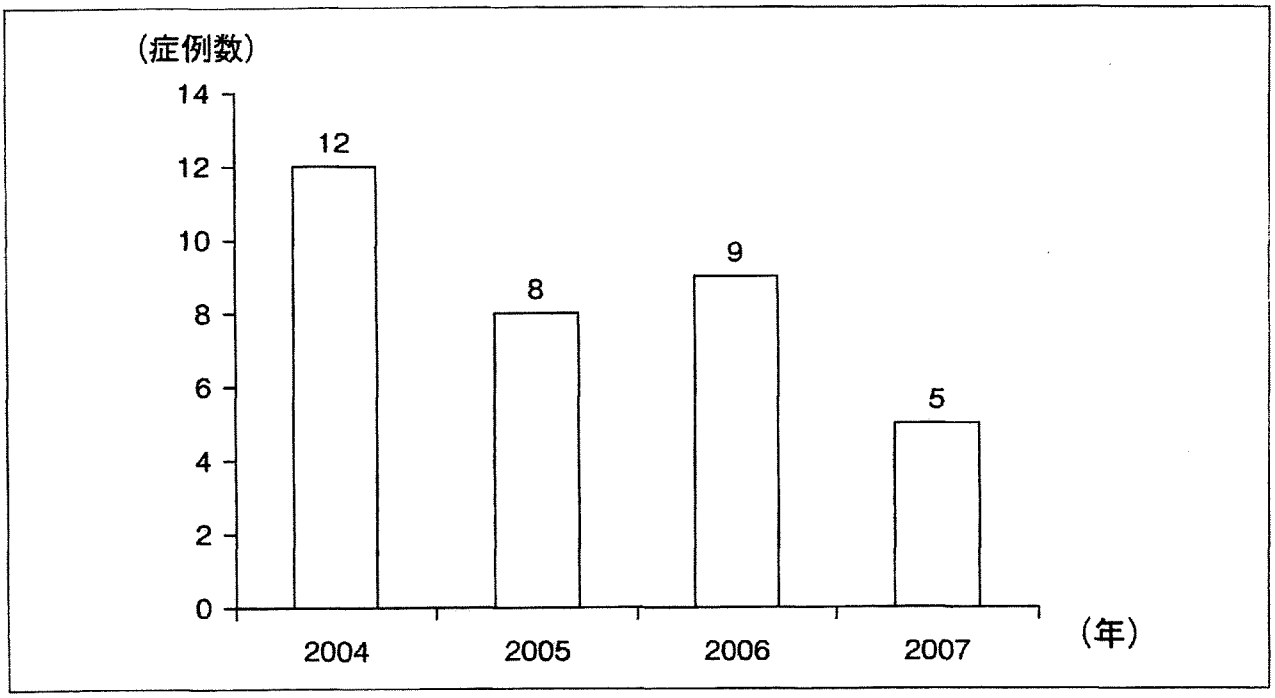

図2 膵島移植症例数の推移

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。

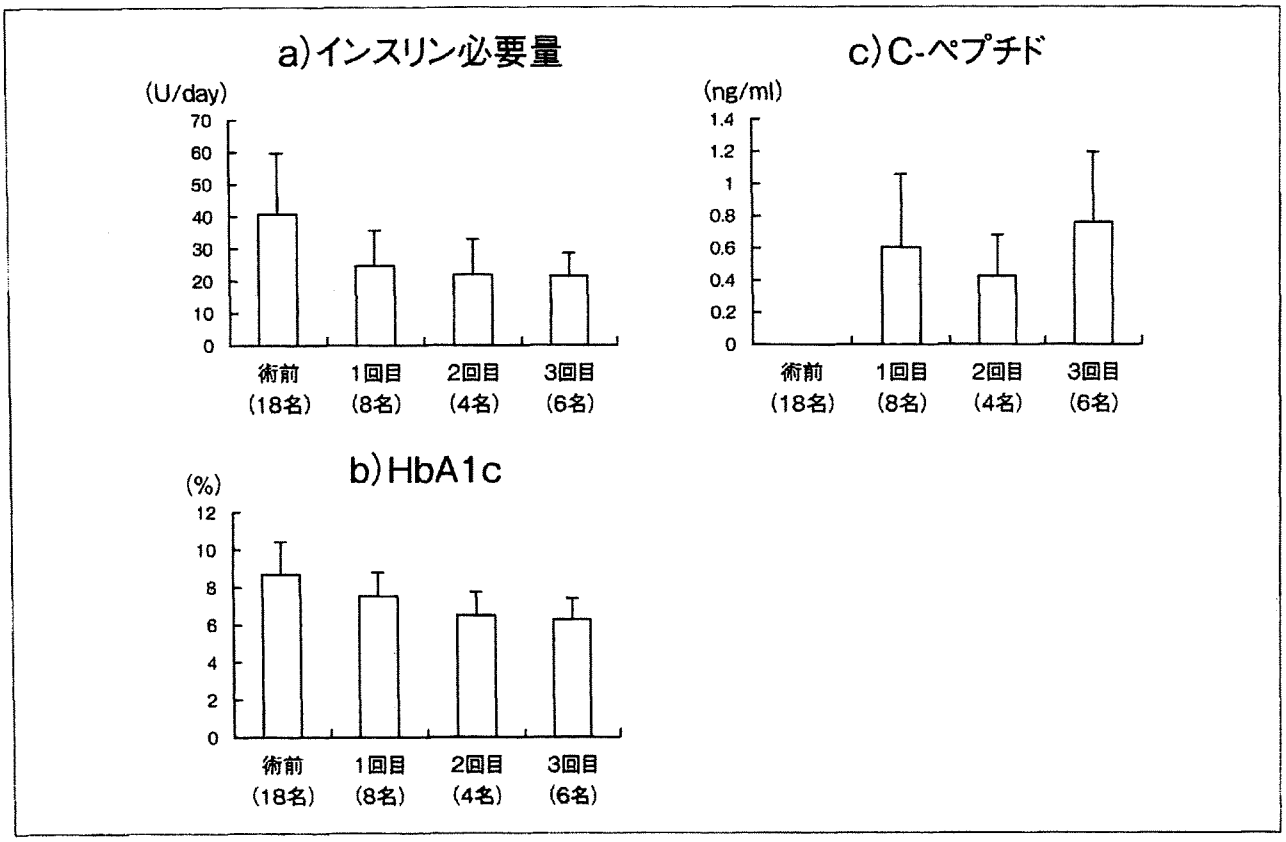

図3　膵島移植前後のインスリン必要量，C-ペプチド，HbA1c の推移

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

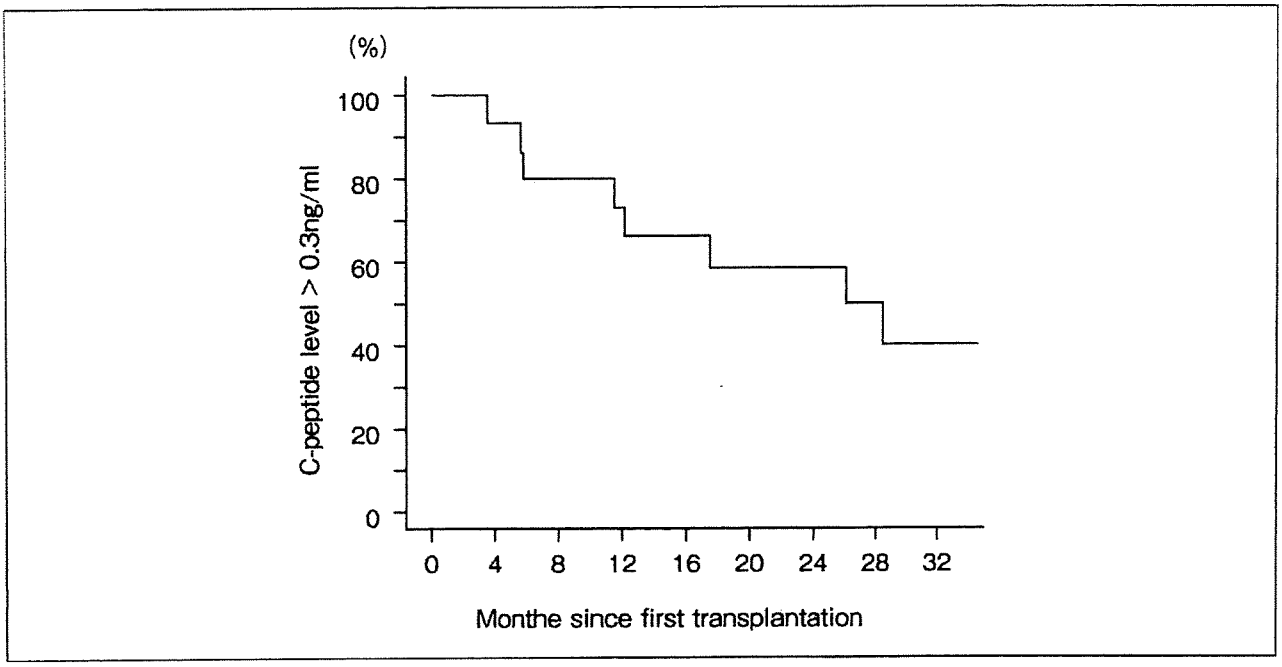

図4 膵島生着率

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
後藤満一, 斎藤拓朗

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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).

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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

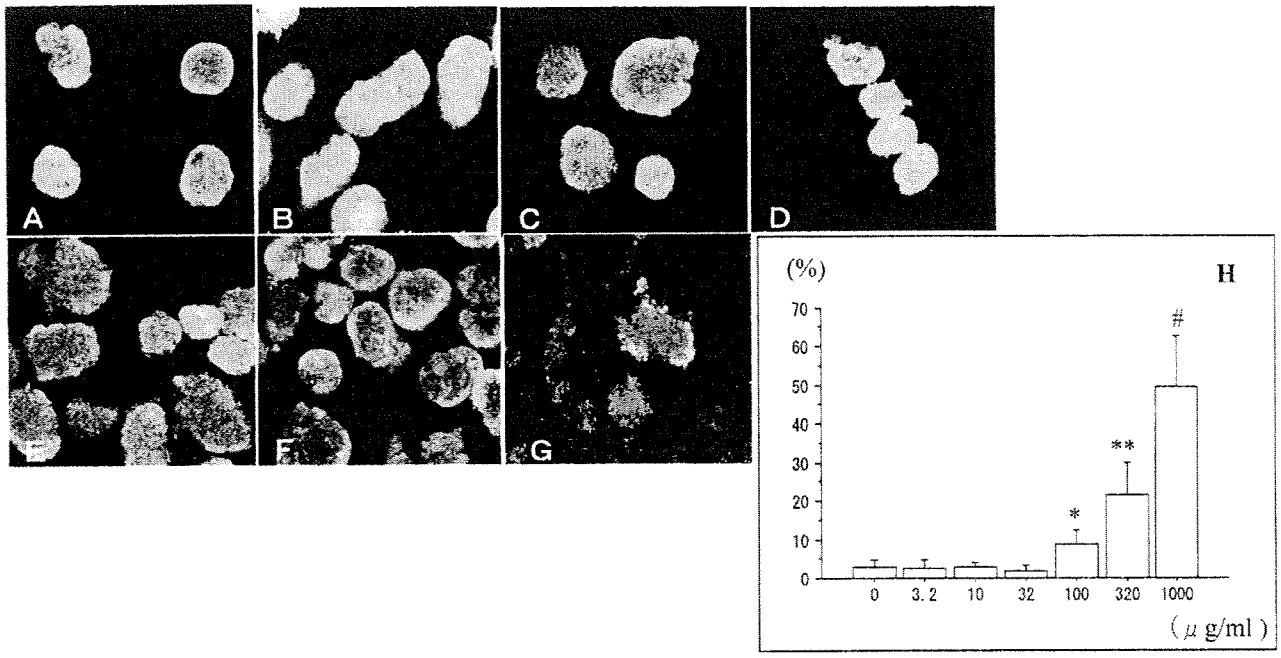

Figure 1. Vital staining of MMC-treated islets using PI and AO (original magnification ×100). Islets treated with MMC at various concentrations followed by culture for 20 h were tested for membrane integrity by vital staining. Islets shown in (A), (B), (C), (D), (E), (F), and (G) were obtained after 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.

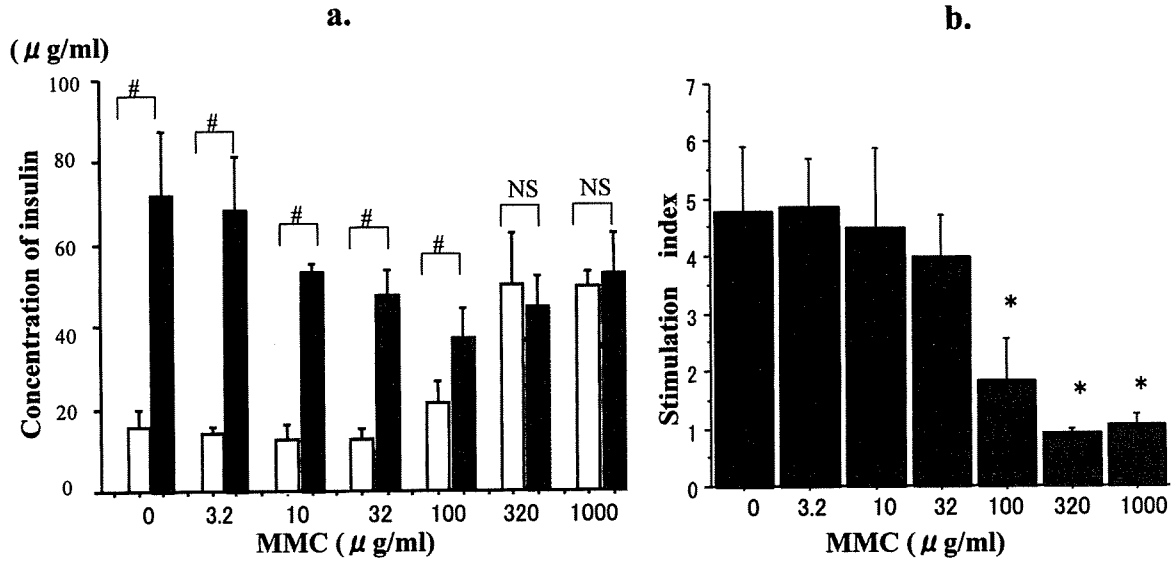

Figure 2. Insulin secretory responses of MMC-treated islets. MMC-treated islets at various concentrations (0, 3.2, 10, 32, 100, 320, and 1000 μg/ml) followed by 20-h culture were tested for insulin secretory capacities to low (3.3 mM; open bars) and high (20 mM; filled bars) glucose. Significant increase 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

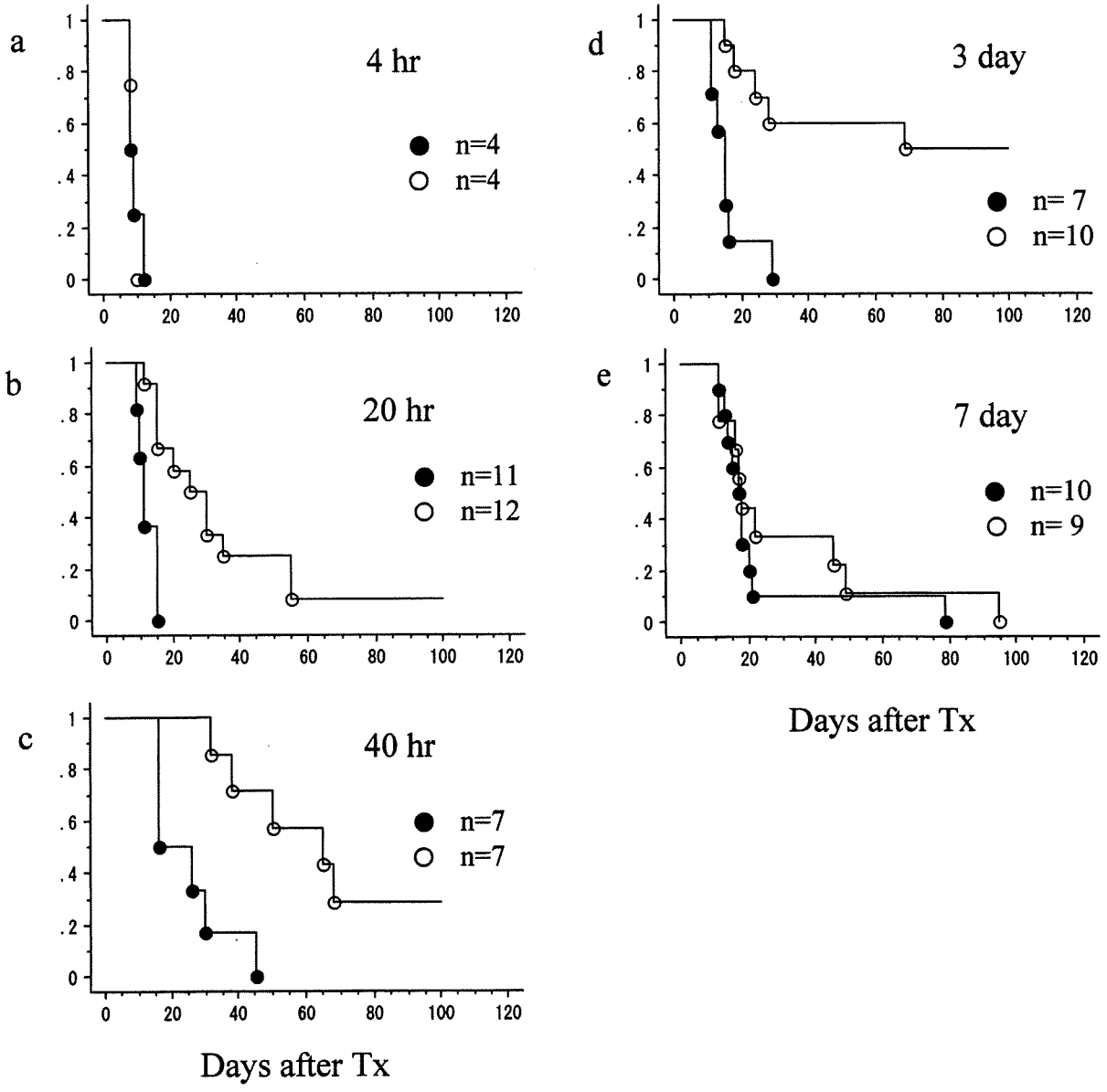

Figure 3. Graft survival time of MMC-treated and untreated islet xenografts after various culture periods. Significant prolongation of MMC-treated islets (open circles) compared with untreated islets (solid circles) was observed when islets were cultured for 20 h ($p < 0.01$), 40 h ($p < 0.002$), or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

insulin, glucagons, and somatostatin with minimal inflammatory cell infiltration (Fig. 4). The secondary grafts from the original donor strain were rejected on days 15, 37, and 42, respectively, which was somewhat delayed when compared to controls (11.9 ± 0.8 days).

Microarray Gene Expression Profile After 3-Day Culture of MMC-Treated Islets

We compared the gene expression profile of MMC-treated and untreated islets cultured for 20 h and 3 days following MMC treatment. The genes expressed in MMC-treated islets followed by 20-h culture were compared to those of islets in culture alone. The fold change (FC) in gene expression of MMC-treated islets was quite similar to that of islets in culture alone when the values were plotted in relation to the expression value of the cultured alone islets (Fig. 5a). Upregulation (FC >2) and downregulation (FC <0.5) of gene expression was detected in 9 and 16 genes, while most of the remaining 1067 genes among 1091 were within the values of $0.5 < FC < 2.0$. On the other hand, prolongation of the culture period to 3 days induced various changes in gene expression, which included the upregulated expression of 442 genes and the downregulated expression of 158 genes with 490 genes within the value of $0.5 < FC < 2.0$ (Fig. 5b). Similarly, MMC treatment followed by 3-day culture resulted in upregulated expression of 236 genes and downregulated expression of 155 genes with 699

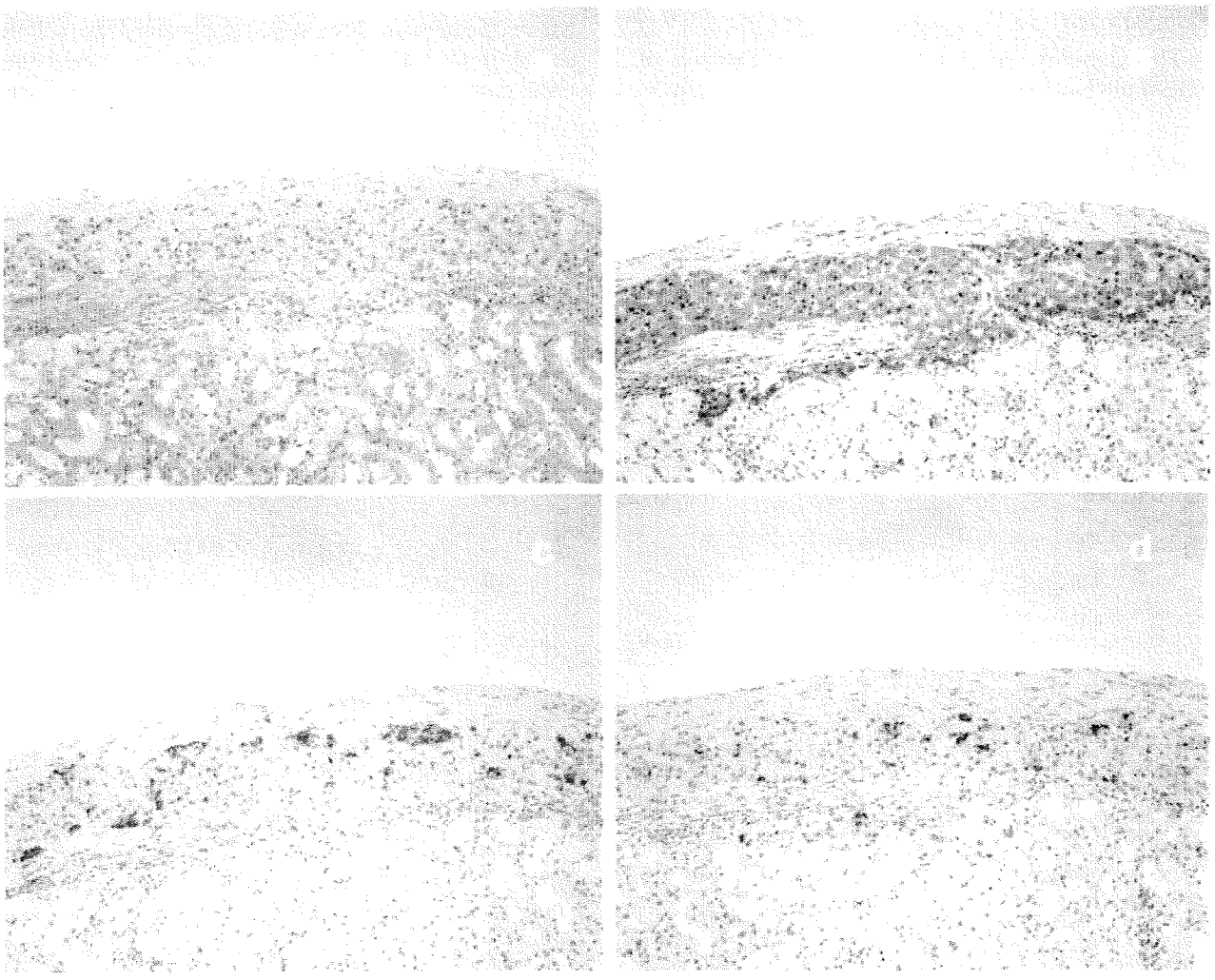

Figure 4. Immunohistological study of long-term functioning graft. One (No. 7557) of the long-term functioning xenografts was sacrificed on day 135 postgrafting and stained with H&E (a), anti-insulin (b), anti-glucagon (c), and anti-somatostatin (d) antibodies (original magnification ×40). The islet xenografts showed intact hormone-containing cells with minimal infiltration of inflammatory cells.

genes being within the value of 0.5 < FC < 2.0 (Fig. 5c), suggesting that MMC treatment tended to downregulate the expression of many genes.

To determine the MMC-treatment associated gene expression profile rather than culture related effect, we compared the gene expression of MMC-treated and nontreated islets in culture for 3 days (Fig. 5d). Twenty-five upregulated genes (FC >5) were found in MMC-treated islets, with an expression value of >100 (Table 1). Furthermore, three downregulated (FC <0.2) genes were identified in cultured islets with an expression value of >100 (Table 1). Among upregulated genes, TGF-β, as well as type II activin receptor, which binds TGF-β superfamily, were both highly upregulated following MMC treatment compared to culture alone.

TGF-β Protein Expression of MMC-Treated and 3-Day Cultured Islets

To determine whether the strong upregulation of TGF-β mRNA expression detected in MMC-treated islets was associated with production of TGF-β protein within the islets, we analyzed TGF-β protein expression in MMC-treated islets (Fig. 6). Although vital staining of islets in both groups showed relatively compact shapes with some islets having PI-positive areas in the center, the process of fixing and embedding in paraffin affected the vulnerability of nontreated islets, compared to MMC-treated islets. TGF-β was expressed strongly in peripheral areas of MMC-treated islets (grade 4) compared with those of nontreated islets (grade 3).

DISCUSSION

We previously reported that crude digested islets pretreated with MMC prolonged graft survival time in a xenogeneic rat-to-mouse model when they were cultured at 37°C for 20 h (8). While the difference was significant, all xenografts eventually showed signs of rejection within 35 days. In this study, we extended the culture period up to 7 days. Marked prolongation of graft survival time was noted when MMC-treated islets were

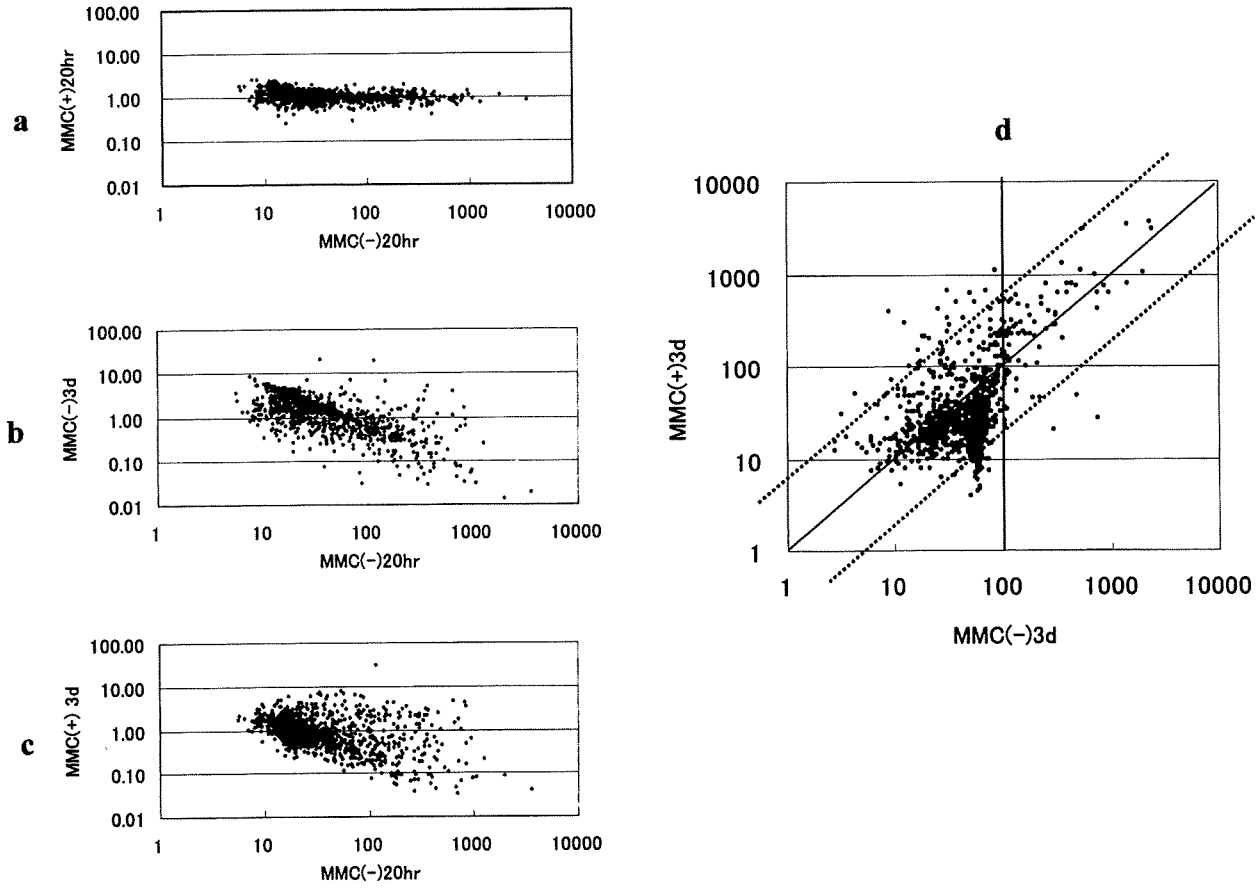

Figure 5. Gene expression of MMC-treated islets followed by 20-h or 3-day culture. Expression levels of various genes in MMC-treated islets cultured for 20 h (a) or 3 days (b) and those of untreated islets cultured for 3 days (c) were compared to those in untreated islets cultured for 20 h. Gene expression of MMC-treated and nontreated islets in culture for 3 days were compared between the two groups (d). Data are fold changes at 5, 1, or 0.2.

Table 1. Marked Up- or Downregulated Genes Following MMC Treatment and Culture for 3 Days Over Culture Alone

Affy No.	Genbank No.	Gene Title	Functions	Fold Change
Upregulated				
705	U03491	transforming growth factor-β3 (TGF-β3)	growth factors, cytokines, and chemokines	5.01
741	M32167	glioma-derived vascular endothelial cell growth factor	growth factors, cytokines, and chemokines	7.30
152	M35105	ros1 proto-oncogene	growth factor & chemokine receptors	5.09
534	X61479	macrophage colony-stimulating factor I receptor (CSF1R)	growth factor & chemokine receptors	6.57
536	U54791	LCR-1; G protein-coupled receptor	growth factor & chemokine receptors	5.23
559	S48190	type II activin receptor	growth factor & chemokine receptors	8.27
562	M84009	dopamine receptor D4 (D4 receptor; DRD4)	neurotransmitter receptors	24.01
1067	S47609	adenosine A2A receptor (ADORA2A)	other receptors (by ligands)	20.28
158	M86389	heat shock 27-kDa protein (HSP27)	heat shock proteins	6.15
162	X96394	multidrug resistance protein (MRP)	drug resistance proteins	8.83
750	U10156	growth hormone-releasing hormone (GHRH)	neuropeptides	11.36
896	L29090	guanine nucleotide-binding protein G(i)/G(s)/G(t) beta subunit 3	G-proteins	13.21
892	L19699	Ral B; GTP-binding protein	GTP/GDP exchangers and G-protein; GTPase activity modulators	7.17
1098	U57715	fibroblast growth factor receptor-activating protein 1 (FRAG1)	adaptors and receptor-associated proteins	10.65
453	M17086	cAMP-dependent protein kinase type I alpha regulatory subunit (PRKAR1A)	kinase activators and inhibitors	11.74
353	L20822	syntaxin 5 (STX5)	targeting	6.44
355	M95735	syntaxin 1B (STX1B)	targeting	7.19
350	D28512	synaptotagmin III (SYT3)	general trafficking	15.55
178	J02627	cytochrome P450 2E1 (CYP2E1)	simple lipid metabolism	9.06
373	M64797	testis fructose-6-phosphate 2-kinase/fructose 2,6-biphosphate (testis 6PF-2-K/fru-2,6-P2ase)	simple carbohydrate metabolism	10.51
376	AF019973	neuron-specific enolase (NSE)	energy metabolism	43.58
186	D83044	organic cation transporter 2 (OCT2)	xenobiotic transporters	5.28
184	AF008221 + AB004559	renal organic anion transporter (ROAT1) + multispecific organic anion transporter (OAT1)	xenobiotic transporters	11.51
253	M88751	voltage-gated dihydropyridine-sensitive L-type calcium channel beta 3 subunit (CCHB3)	voltage-gated ion channels	8.81
977	M16736	growth-accentuating protein 43 (GAP43)	functionally unclassified	8.85
Downregulated				
56	D26307	junD proto-oncogene	basic transcription factors	0.10
809	D31873	LIM domain kinase 1 (LIMK1)	nonreceptor protein kinases	0.07
983	L12382	ADP-ribosylation factor 3 (ARF3)	trafficking/targeting proteins	0.03

transplanted after 40 h or 3 days in culture following MMC treatment. Half of rat islet grafts survived indefinitely in B6 recipient mice with chemically induced diabetes. Although culture alone induced some prolongation of graft survival, the grafts were all eventually rejected.

Various modalities have been used to immunomodulate islet graft survival. These include islet culture (11,25), ultraviolet irradiation (UV) (15), specific monoclonal antibodies such as anti-Ia (4), anti-dendritic cells (5), and anti-ICAM-1 molecules (7). However, it has been difficult to produce indefinite survival of xenoislets without host immunosuppression except in one report by Hardy et al. (9), who showed that UV-irradiated Lew

islet xenografts could survive indefinitely in B10.BR recipient mice. However, this was not the case when the same islets were grafted into Balb/C mice, which eventually rejected them before 90 days postgrafting. In most of these experiments as well as those reported by Hardy et al., handpicked islets were used, which are less immunogenic compared to crude-digested islets that contain highly immunogenic contaminants. In the present study, we used crude-digested islets to test the feasibility of a treatment modality for preclinical islet transplantation.

We investigated the appropriate dose of MMC necessary to induce graft prolongation but not islet toxicity. We reported that MMC at doses of 10, 32, 50 and 100 μg/ml are effective for prolongation of graft survival, while glucose metabolism posttransplantation deteriorated when the dose exceeded 32 μg/ml in a rat-to-mouse combination (8). In a mouse model, a significant adverse effect was detected in both isografts and allografts when the dose of MMC was >32 μg/ml at 3 to 5 days postgrafting, whereas no adverse effect was found in mice bearing long-term functioning isografts regardless of the dose of MMC (3.2, 10, 32, 100 μg/ml) (17).

To consider the application of this treatment for human patients, it is necessary to evaluate the effect of MMC treatment on islets prior to transplantation. In this study, we examined islet viability by vital staining of islets and insulin secretory capacity in response to glucose and compared those data with previous in vivo findings. Vital staining using AO and PI demonstrated that MMC at doses <32 μg/ml appeared to be nontoxic without increasing the number of AO-positive cells. Insulin secretory capacity in response to glucose, which is a gold standard test for islet function in vitro, showed that the stimulation index was maintained when the MMC dose was <32 μg/ml. Both in vitro studies indicated that MMC treatment at a dose <32 μg/ml is nontoxic and preserves islet function at 20 h following MMC treatment. This finding correlates well with the

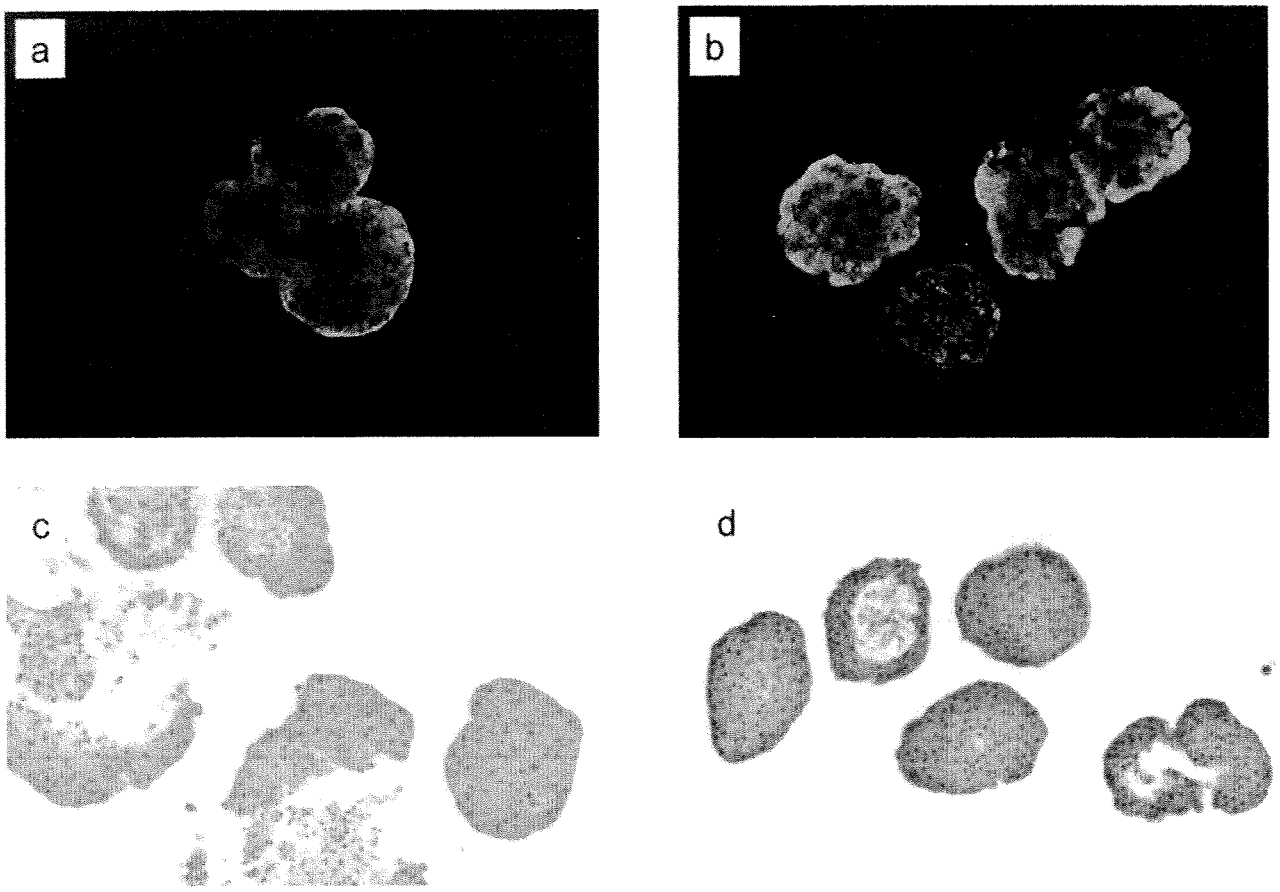

Figure 6. Vital staining and immunohistological study of MMC-treated and nontreated islets cultured for 3 days. MMC-treated (b, d) and nontreated islets (a, c) that were cultured for 3 days were stained by PI and AO (a, b), and were immunohisotologically studied using anti-TGF-β antibody (c, d). Vital staining of islets in both groups showed relatively compact shape with some islets having PI-positive areas in the center. TGF-β was expressed strongly in peripheral areas of MMC-treated islets as compared with those of nontreated islets.

previous results of an in vivo study (17), suggesting that these modalities could be applicable for testing viability after MMC treatment in human islet preparations.

Previous studies showed that culture alone did not induce indefinite survival of rat islets in mouse recipients even with a variety of modifications, including temperature (11), high oxygen concentration (14), and culture duration (25). This study also showed that prolongation of the culture period alone induced a significant, but only marginal, effect on protection of grafted islets from immune destruction. MMC treatment had a significant impact on graft survival time over culture for 20 and 40 h and 3 days, but not for 4 h or 7 days. Thus, there is a critical window in the post-MMC culture period that is necessary for significant graft survival. We previously showed that transient upregulation of TGF-β was responsible for prolongation of graft survival in a study using MMC-treated and 20-h cultured islets (10). Extending culture periods up to 40 h or 3 days induced further prolongation of graft survival time. Furthermore, some kind of unresponsiveness was induced in animals bearing long-term functioning grafts.

To determine the effect of MMC treatment on islets during the culture period, we examined the gene expression profiles of MMC-treated islets and found 25 relatively high-grade upregulated genes and 3 relatively high-grade downregulated genes. The highly upregulated genes (FC >20) included neuron-specific enolase, dopamine receptor D4, and adenosine A2A receptor, which were reported to be involved in the glycolytic pathway (18), neural–immune interactions (20), and signaling reactions (13), respectively. Interestingly, TGF-β, as well as type II activin receptor, which binds TGF-β superfamily (24), were both highly upregulated following MMC treatment compared to culture alone. TGF-β superfamily of ligands and receptors are known to stimulate cellular events in diverse processes ranging from cell fate specification during development to immune suppression (16). Data of microarray analysis were consistent with the immunohistological study of MMC-treated islets in which TGF-β upregulation was demonstrated after culture for 3 days. There have been some reports on various gene expressions of pancreatic islets during culture (3) or tolerated islets after transplantation (12); however, it is not yet determined which gene expression would be responsible for tolerance induction. In this study we were able to show that MMC treatment induced a variety of up- or downregulated genes. One of the responsible genes is upregulated TGF-β, and others hopefully will be identified in the future.

In conclusion, MMC pretreatment of rat islets and culture for 3 days at 37°C induced marked prolongation of graft survival in nonimmunosuppressed recipient mice, with half of the grafts surviving indefinitely. This effect was obtained within a specific culture period and was supported by microarray gene profile analysis. The results of these two manipulations were reproducible and may offer a strategy for the preclinical application of this protocol in human islet transplantation.

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膵島移植の成績

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はじめに

膵島移植は，糖尿病専門医の指導によっても血糖コントロールが不良な症例で，生活の質の低下のみならず重症低血糖発作あるいはケトアシドーシスなどを来すⅠ型糖尿病あるいは難治性糖尿病に対して行われている[1]。このような糖尿病に対する移植医療としては，現在，膵臓移植と膵島移植が行われているが，膵島移植は膵臓を構成する細胞の中から膵島のみを取り出して移植する治療法で，経皮経肝的に門脈を穿刺してカテーテルを留置し局所麻酔下に移植するため，膵臓移植に比し低侵襲の治療法である。

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[2]。膵島移植班は，1996年に千葉大学，東京女子医科大学，国立佐倉病院，筑波大学により発足したワーキンググループを母体とし，1997年からは膵・膵島移植研究会ワーキンググループ内の膵島移植班として活動を開始した。開始当初の事務局は国立病院機構千葉東病院にあり，『膵島移植の指針（1998年）』，『膵島移植実施マニュアル第1版（2002年）・第2版（2004年）』などの編集・刊行をはじめとする臨床膵島移植開始へ向けて重要な部分が形作られた。2004年7月に事務局が国立病院機構千葉東病院から福島県立医科大学外科学第一講座へ移転し，臨床膵島移植の実施に伴う問題点を修正した『膵島移植実施マニュアル第3版（2006年）』を発刊した。

膵島移植班では膵グラフトのドナーとして脳死・心停止を想定しているが，脳死ドナーは主として膵臓移植に用いられるため，わが国の膵島移植ドナーのほとんどは心停止ドナーで，これはわが国の膵島移植の特徴の1つである。今回，わが国の膵島移植の現況と移植成績について報告する。

対象と方法

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[1]。その内容は，分離・凍結施設としてGMP基準を満たす膵島分離施設を有することに加え，倫理委員会の承認，機器・薬剤の整備，ヒトまたは大動物の膵島移植・凍結に習熟した医師が常勤していること，ドナー発生に対する24時間体制の対応，また膵島移植施設としては門脈穿刺の経験，糖尿病学会専門医などの協力，免疫抑制剤の使用経験，膵島移植実施マニュアルに従って移植を実施できること，などの諸項目である。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，2009年3月からは神戸大学を除く6施設となった。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。

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。しかし，重度の心・肝疾患，アルコール中毒，感染症，悪性腫瘍の既往，重症肥満，未処置の網膜症などを認める場合は禁忌としている[1]。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[1]。2008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。

結果と考察

2008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（平均20.8）年であった。

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，HbA1c値：8.8±1.8%，7.5±1.4%，6.5±1.4%，6.2±1.2%，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1**）[3]。

2000年にShapiroらは，膵島移植を行った7症例すべてにおいて1年後にインスリン離脱が達成されたと報告した[4]。これはいわゆるエドモントンプロトコールと呼ばれ，①対象を腎症発症前のⅠ型糖尿病で無自覚低血糖が頻発あるいは血糖が非常に不安定な症例に限定し，②免疫抑制剤としてステロイドを使用せず，抗CD 25モノクローナル抗体であるdaclizumabを導入療法とし，維持療法としてsirolimusとカルシニューリン阻害薬であるtacrolimusを低容量で使用，③膵島はウシ血清などの異種タンパクを含まないヒトのアルブミンを用いて処理し，④レシピエントの体重あたり10,000 IEs（islet equivalents）/kg以上の膵島を移植するため，1症例あたり2回から3回の移植を行う，などの特徴を有していた[4]。その後の多施設共同研究でもエドモントンプロトコールの移植早期におけるインスリン離脱率の再現性は確認されている[5,6]。

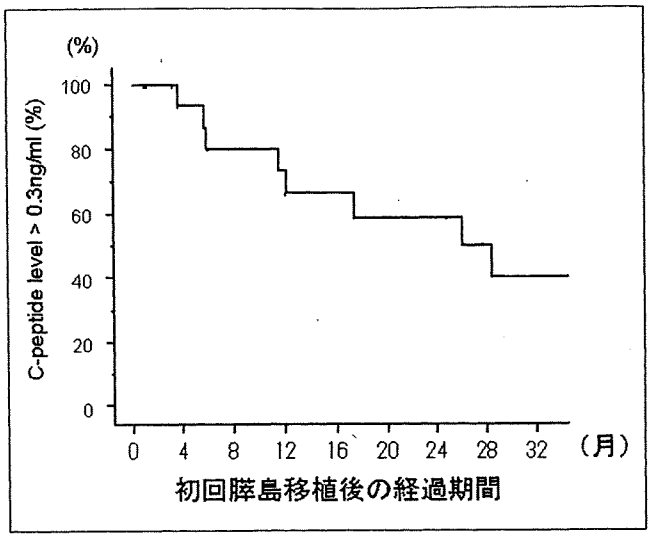

図1　膵島生着率移

その後，海外の施設では種々の免疫抑制療法が試みられ，エドモントンプロトコールを凌駕する成績が報告されている。特に，導入時に抗ヒト胸腺細胞ウサギ免疫グロブリン（ATG, Thymoglobulin）およびヒト型可溶性TNFαレセプター製剤を用い，維持免疫抑制としてカルシニューリン阻害剤とeverolimusあるいはmicophenolate mofetilなどを組み合わせる免疫抑制法により，インスリン離脱率の向上と，インスリン離脱達成後のインスリン離脱期間の延長が報告されている[7]。また，現在，欧米においては膵島移植を一般医療として確立するための最終段階であるPhaseⅢの治験を行うべく，Clinical Islet Transplantation Consortium（CITC）が組織され，その準備がなされているが，その中では初回移植時の導入療法としてThymoglobulinが使用されている。このような現状を受けて，わが国の膵・膵島移植研究会では，膵島移植の安全性と有効性を検証するために，初回移植の導入時にThymoglobulinを用いる新しい免疫抑制プロトコールを立案し，膵島移植実施施設による本多施設共同研究として申請している。今後，これらの研究により膵島移植の安全性と有効性が確認されればより一般的な医療として普及する可能性がある。

ところで，2007年3月，Liberase HIによるCreutzfeldt-Jakob病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。しかし，移植再開に向けてすでに安全性の高い酵素製剤を入手し，他施設参加の動物実験によりその有効性は確認した。臨床膵島移植を再開する準備は整っており，2009年内には再開できる見込みである。

おわりに

臨床移植医療の中で，最も新しい膵島移植は，膵・膵島移植研究会が長年にわたって準備を進め，脳死移植10年の節目としての本企画に参加することができた。これは，日本移植学会および膵・膵島移植研究会の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。

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Brain death in combination with warm ischemic stress during isolation procedures induces the expression of crucial inflammatory mediators in the isolated islets

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Abstract

Tissue factor (TF) and monocyte chemoattractant protein (MCP)-1 expressed on the islets have been identified as the main trigger of the instant blood-mediated inflammatory reaction (IBMIR), in islet transplantation. Since the key steps that directly induce TF and MCP-1 remain to be determined, we focused on the influence of brain death (BD) on TF and MCP-1 expression in the pancreatic tissues and isolated islets using a rodent model. Tissue factor and MCP-1 mRNA levels in the pancreatic tissues were similar between the BD and the control group. However, TF and MCP-1 mRNA in the fresh islets of the BD group were significantly higher than that of the control group ($p < 0.01$). BD may thus be suggested to be of great importance as an initiator of TF and MCP-1 induction in the isolated islets. Furthermore, the up-regulation of crucial inflammatory mediators induced by BD could be exacerbated by warm ischemic damage during digestion procedures. In the present study, the islet yield and purity were affected by BD. However, almost no influences were observed with respect to islet viability, indicating that the expression of inflammatory mediators rather than islet viability is more susceptible to BD. According to the change in time course of TF and MCP-1 expression in the isolated islets, the selected time point for islet infusion in