福岡大医紀(*Med. Bull. Fukuoka Univ.*): 36(2), 103-112, 2009

Beneficial Effects of Activated Protein C on Amelioration of Hyperglycemia in Streptozotocin-induced Diabetic Mice Receiving Intrahepatic Syngenic Islets From a Single Donor

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Abstract: The inability to achieve successful islet transplantation from one donor to one recipient has been a major obstacle facing clinical islet transplantation. The present study focused on the effects of activated protein C(APC) which plays a key role in crosstalk between coagulation and inflammation and determined whether APC has any beneficial effect on engraftments of islets transplanted into the liver of mice. Streptozotocin(STZ)-induced diabetic mice(n=8) receiving intrahepatic 200 syngeneic islets, the number of islets isolated from a single donor, remained hyperglycemic after transplantation. In marked contrast, all of diabetic mice(n=7) receiving 200 islets and treated with APC(40 μg, i.v. at 0, 2 and 4 hr) became normoglycemic. A histological examination revealed that APC prevented islet graft loss during the early post-transplant period and more of the islets were detected in the liver of the APC treated mice than in the controls. Sixty days after transplantation, the APC treated mice showed better glucose tolerance than the control mice. A flow cytometry analysis disclosed that Gr-1^{+}CD11b^{+} cells (neutrophils) with a high production of proinflammatory cytokines had accumulated in the liver of control mice at a peak of 6 hr after transplantation. In mice receiving islets and treated with APC, the production of proinflammatory cytokines in these cells was down-regulated without affecting their number. These findings show that APC prevents early loss of transplanted islets by inhibiting the production of proinflammatory cytokines deleterious to islet grafts, enabling successful transplantation from one donor to one recipient in mice. The present study indicates that APC may improve the efficiency of clinical islet transplantation when the effect of APC is also the case in human.

Key words: Islet transplantation, Engraftment, Activated protein C(APC), Proinflammatory cytokine

Introduction

Although, the remarkable success of islet transplantation for type 1 diabetes patients was first reported by the Edmonton group in 2000[1] and subsequently confirmed by other groups,[2)-6)] sequential transplantations with the use of islets from 2 or 3 donors are still required to achieve insulin independence after transplantation. Thus, the inability of producing successful islet transplantation from one donor to one recipient has been a ma-

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This paper was presented at American Transplant Congress 2005 in Chicago, USA.

jor obstacle facing clinical islet transplantation. Moreover, patients who underwent islet transplantation had a β-cell function of only 20-30% of those in healthy individuals even though they had received islets from more than one donor.[7] Therefore, new strategies to prevent islet graft loss during the posttransplant period are needed for successful islet transplantation.

Since Kemp *et al.*[8] reported that syngeneic islet transplantation into the liver reversed drug-induced diabetes in rats in 1973, the liver has been regarded as an appropriate site for islet transplantation. In clinical settings, isolated islets are transplanted into the liver of recipients via the portal vein[1)-6)] and form an embolism in the small portal veins stimulating blood coagulation. Thus, coagulatory events may participate in inflammatory response deleterious to transplanted islet grafts.

Under physiologic conditions, the endothelial cells inhibit blood coagulation by expressing thrombomodulin(TM). TM binds to thrombin and makes a complex on the surface of the endothelial cells. TM-thrombin complex activates protein C to generate the anticoagulant enzyme activated protein C(APC), a vitamin K-dependent serine protease, and this activation is enhanced by the endothelial protein C receptor(EPCR).[9] Therefore, APC makes a complex with protein S and inhibits coagulation by inactivating two critical regulatory proteins, factor Va and VIIIa.[10] In animal models of thrombus formation, APC was revealed to play a very important role in preventing blood coagulation.[11][12] Recently, many investigators have reported that there is a crosstalk between coagulation and inflammation, and APC plays a central role in regulating this interaction.[13)-16)] Bernard *et al.* demonstrated that recombinant human APC significantly reduces the mortality of patients with severe sepsis, which was defined as sepsis associated with acute organ dysfunction resulting from a generalized inflammatory and procoagulant response to an infection, in their randomized multi-center trial.[17] Moreover, APC has been reported to have cytoprotective effects on renal cells and neurons, and reduced organ damages in animal models of ischemic-reperfusion injury and stroke.[18)-20)]

This study investigated whether APC prevents islet graft loss during the early posttransplant period to improve the engraftment of intrahepatic islet grafts, facilitating to produce successful islet transplantation from one donor to one recipient in mice.

Research Design and Methods

Mice

Male C57BL/6 mice were purchased from Charles River Inc., Japan(Kanagawa, Japan). Diabetes was induced in the recipients by an intravenous injection of 180 mg/kg streptozotocin(STZ; Sigma-Aldrich Japan, Tokyo, Japan). Mice whose plasma glucose levels exceeded 400 mg/dl at 2 days after STZ injection were used as recipients, and they remained hyperglycemic until the time of islet transplantation.

Reagents

APC, purified from fresh frozen plasma by immunoaffinity chromatography using monoclonal antibody to protein C, was supplied from The Chemo-Sero- Therapeutic Research Institute(Kumamoto, Japan). The dosage of 40 μg of APC was dissolved in 0.2 ml of sterile normal saline and injected 3 times(just before islet transplantation, 2 and 4 hr after transplantation) intravenously via the tail vein. The mice of the control group were injected equivalent volume of saline in the same manner. Anti-EPCR antibody was a monoclonal antibody to recombinant mouse EPCR, and was a kind gift from Dr. Masaru Taniguchi(RIKEN Research Center for Allergy and Immunology, Yokohama, Japan). Anti-EPCR antibody 100 μg was administered intraperitoneally on the day before transplantation. All other reagents were purchased from Sigma-Aldrich Japan(Tokyo, Japan).

Islet isolation and transplantation

Islets were isolated by the static digestion method using collagenase[21] and then they were separated by centrifugation on Ficoll-Conray gradients.[22] The islets were collected manually using a Pasteur pipette with the aid of a dissecting microscope. Only the islets measuring 150 to 250 μm in diameter were hand-picked and used for the

experiments. The size of individual islets on each islet isolation procedure was confirmed using a phase-contrast microscope equipped with a scale in the eye piece. Hand-picked islets were transplanted into the liver via the portal vein of the recipients at 3 days after the induction of diabetes by STZ injection.

Monitoring plasma glucose and body weight

The nonfasting plasma glucose level and body weight were monitored three times a week in all the recipients after islet transplantation. The plasma glucose levels were measured using a Beckman glucose analyzer(Beckman Japan, Tokyo, Japan). Normoglycemia was defined to have occurred when the two consecutive plasma glucose levels after transplantation were less than 200 mg/dl.

Morphological study

The livers bearing the islet grafts were examined morphologically at the appropriate time after transplantation. The recipient mice were sacrificed, and the liver bearing the grafts were removed. To compare the numbers of islet grafts in the group of mice treated with APC and those of the control group, the whole liver was cut into 2 mm thin slices, fixed with Bouin's solution and embedded in paraffin. The embedded specimen was sliced from the bottom of the case into fifty continuous 5 μm thick sections. The sections were stained with hematoxylin and eosin(HE) and for insulin(DAKO Co., Carpinteria, CA) immunohistochemically. The number of islets in the first and last sections were counted to avoid counting the same islet graft twice because the transplanted islets ranged from 150 to 250 μm in diameter.

Intraperitoneal glucose tolerance test(IPGTT)

IPGTT was performed on day 60 after islet transplantation. The mice were fasted for 15 hr prior to the examination. Blood samples were obtained at 0, 30, and 120 min after the intraperitoneal administration of glucose(1.0 g/kg body weight). The plasma glucose was measured as previously described.

Preparation of hepatic mononuclear cells

Hepatic mononuclear cells(HMNCs) were prepared as described previously.[23] In brief, an excised liver was pressed through a stainless steel mesh, then the resulting dissociated liver was suspended in Dulbecco's modified Eagle medium(D-MEM/F-12; Life Technologies, Tokyo, Japan) and washed in PBS. Next the mixture was resuspended in an isotonic 33% Percoll solution containing heparin(67 U/ml), and centrifuge 2,000×g at 4℃ for 15 min. The resulting pellet was resuspended in 0.83% ammonium chloride solution to lyse the erythrocytes. After counting, these HMNCs were then washed twice in PBS and used for further analyses.

Antibodies and a flow cytometry analysis

The antibodies(Abs) used for the flow cytometry analysis were: Fc block(anti-mouse FcRγ II/III mAb, 2.4G2), Phycoerythrin(PE)-conjugated anti-mouse-CD11b mAb(clone M1/70, Integrin-α M chain, Mac-1α chain, Rat IgG2b, κ), Peridinin chlorophyll protein(PerCP)-Cy5.5-conjugated Rat anti-mouse Ly-6G and Ly-6C(Gr-1) mAb(clone RB6-8C5, Rat IgG2b, κ), Allophycocyanin(APC) -conjugated anti-mouse-IFN-γ mAb(clone XMG 1.2, Rat IgG1), anti-mouse-TNF-α mAb(clone MP6-XT22, RatvIgG1), and their isotype control (clone R3-34, Rat IgG1) were purchased from PharMingen(San Diego, CA). For intracellular staining, the cells were incubated with blocking FcRγ II/III mAb, fixed and permeabilized by Cytofix/Cytoperm solution(PharMingen), and stained with anti-IFN-γ, anti-TNF-α, and their isotype control according to the manufacturer's instruction. The stained cells were analyzed on a FACSCalibur(Becton Dickinson, Mountain View, CA) and the data were processed by the CELLQuest software program(Becton Dickinson). Ten thousands viable HMNCs were collected for Dot-Plot to analyze function of each cell population.

Statistical analysis

Data are presented as the mean±SE. Differences between the groups were analyzed by Mann-Whitney's U-test and Fisher's exact probability test. The differences were considered significant when the p values were less than 0.05.

Results

The beneficial effects of APC on amelioration of hyperglycemia in STZ-diabetic mice receiving 200 syngenic islets into the liver from a single donor

As shown previously,[24] hyperglycemica of STZ-diabetic mice was ameliorated by transplantation of 400 but not 200 syngenic islets isolated from a single mouse pancreas into the liver. Thus, all mice(n=8) receiving intrahepatic 200 islets and treated with saline remained hyperglycemic at 60 days after transplantation(Figure 1A). In marked contrast, all of the recipient mice treated with three 40 μg APC injections(0, 2, and 4 hr after transplantation) became normoglycemic at 5.4 ±1.4 days(n=7) after transplantation(Figure 1B). To determine whether this effect of APC was mediated by EPCR, the mice receiving islet grafts were injected 100 μg of anti-EPCR antibody intraperitoneally on the day before transplantation and treated with 3 APC injections. Anti-EPCR antibody completely abolished the effect of APC, and all mice(n=3) remained hyperglycemic on day 60 after transplantation(Figure 1C). The administration of control antibody(rat IgG2a) did not affect the effect of APC treatment, and all mice(n =4) treated with APC combined with control antibody became normoglycemic at 7.0±2.4 days after transplantation(Figure 1D).

Morphology at 6 and 24 hr after transplantation

The livers bearing the islet grafts were examined at 6 or 24 hr after transplantation to analyze the effect of APC. In the liver of the mice in the control group, the hepatocytes surrounding the islet grafts degenerated in a wide area because of an inflammatory reaction caused by the islet grafts.

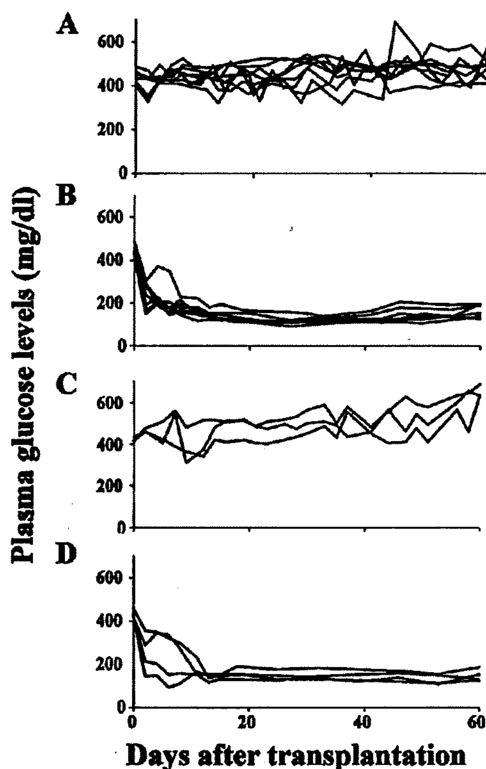

Figure 1. The plasma glucose levels after the transplantation of 200 syngeneic islets
A: Saline, B: APC, C: APC+Anti-EPCR antibody, D: APC+Control antibody
The individual line represents the plasma glucose levels of each animal.

Most of the nuclei of the islet cells also degenerated and showed picnosis. In the APC treated group, the degeneration of hepatocytes was prevented, although more islets were observed in the livers than in the control group. In addition, most of the islet cells nuclei were intact in the APC treated group(Figure 2A). At both 6 and 24 hr after transplantation, more islet grafts were detected in the livers of the APC group than the control group(13.3±4.7 islets/preparation vs. 7.7±2.4 islets/preparation at 6 hr, 10.0±1.5 vs. 3.8±0.4 at 24 hr; n=6). The difference was statistically significant at 24 hr after transplantation(Figure 2B).

IPGTT on day 60 after transplantation

To evaluate the function of islet grafts in the liver of recipient mice, IPGTT was performed on day 60 after transplantation. The results are summarized in Figure 3. The plasma glucose levels of naive untreated C57BL/6 mice(n=4) were 62.0±1.1, 464.0±15.3 and 178.0±7.5 mg/dl at 0, 30 and 120 minutes, respectively, after the intraperitoneally injection of 1.0 g/kg glucose(white circle), and those of the STZ-induced diabetic mice without

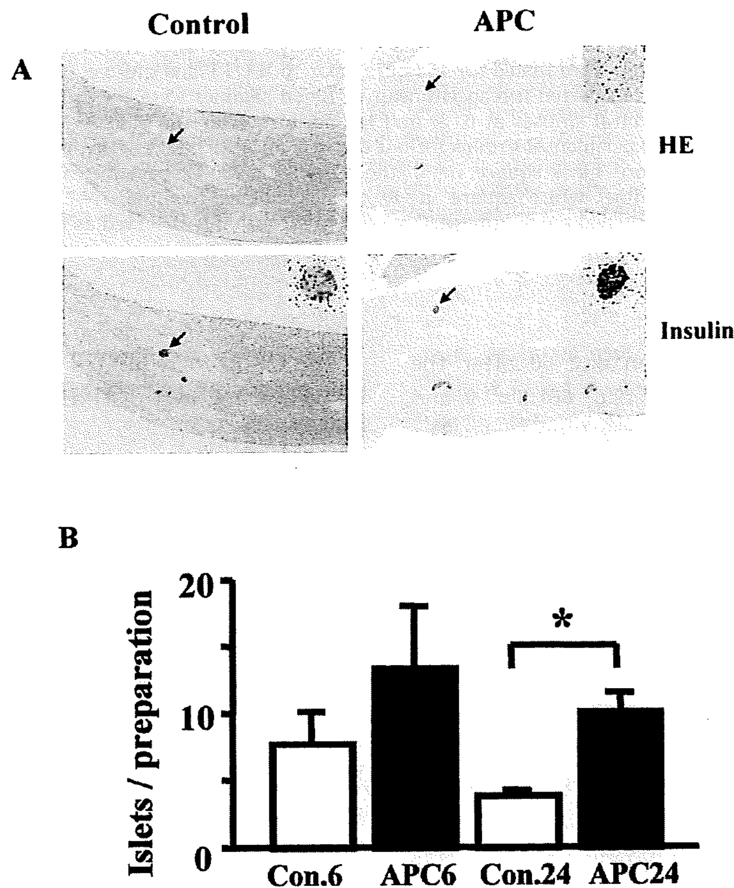

Figure 2. Beneficial effects of APC on engraftment of islets in the liver
A: Photomicrography of the mouse liver receiving islets and treated with saline(left column) or APC(right column).
Islet grafts indicated by arrows are magnified in right upper part. The sections were stained with hematoxylin and eosin(upper panel) and immunohitochemically for insulin (lower panel). Original magnification= ×40
B: The number of islet grafts detected in the liver of mice after transplantation.
At both 6 and 24 hr after transplantation, more islet grafts were detected in the liver of mice treated with APC in comparison to control mice. The difference is statistically significant at 24 hr after transplantation(n=6, $p<0.01$, Mann-Whitney's U-test)

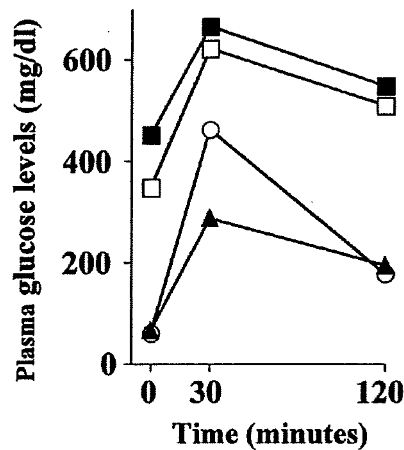

Figure 3. Intraperitoneal glucose tolerance test(IPGTT)
Intraperitoneal glucose tolerance test(IPGTT) in STZ-induced diabetic mice was performed at 60 days after islet transplantation. Mice were fasted for 15 hours prior to IPGTT and glucose(1.0 g/kg) was injected intraperitoneally. Blood samples were taken from the orbital sinuses at 0, 30 and 120 minutes after the glucose injection. Experimental groups include diabetic mice without islet transplantation(black square, n=5), those receiving 200 islets and treated with saline(white square, n=8) or APC(black triangle, n=7). Age-matched naive untreated mice(white circle, n=4) served as controls.

islet transplantation(n=5) on day 60 after the injection of STZ were 454.4±19.6, 667.6±7.9 and 549.0±7.0 mg/dl, respectively(black square). The plasma glucose levels of diabetic mice(n=8) that received 200 islets and were treated with saline were 349.1±23.3, 622.8±31.5 and 509.6±32.7 mg/dl, respectively(white square). The difference in the plasma glucose levels at 0 minute between the diabetic mice without islet transplantation and the mice the received 200 islets and were treated with saline was statistically significant, but the differences at 30 and 120 minutes were not statistically significant. On the other hand, the plasma glucose levels of the normoglycemic mice(n=7) that received 200 islets and were treated with 40 μg APC (0, 2, and 4 hr after transplantation) were 65.1±10.6, 289.0±13.1 and 196.3±13.2 mg/dl(black triangle). The differences in the values at each time point between the mice receiving 200 syngeneic islets and treated with saline(white square) and the mice treated with APC after transplantation (black triangle) were statistically significant.

APC down-regulates proinflammatory cytokine productions of HMNCs in mice receiving 200 islet grafts

A flow cytometry analysis was performed to examine the effects of APC. This analysis disclosed that increased numbers of Gr-1$^+$CD11b$^+$ cells accumulated in the liver of both the control and APC treated mice with a peak at 6 hr after islet transplantation in comparison to naive mice(21.3% in naive mice vs. 81.6% in control mice and 80.5% in APC treated mice). The production of IFN-γ and TNF-α in Gr-1$^+$ or CD11b$^+$ cells in the mice of the APC treated group was found to be remarkably down-regulated without affecting the accumulation of these cells in the liver. Representative data of 2 to 3 experiments are shown(Figure 4).

Discussion

Franklin *et al.*[25] described the sequential morphological changes of intrahepatic islet grafts in rodent models over both brief and longer periods. Islets and non-islet tissues were found lodged in the peripheries of the portal system and associated

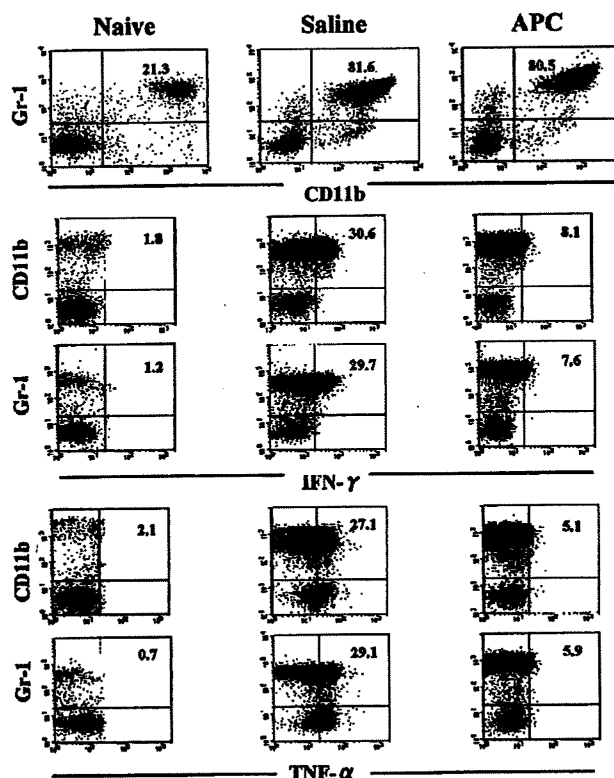

Figure 4. A flow cytometry analysis of the hepatic mononuclear cells(HMNCs) in mice receiving 200 syngenic islets
Mononuclear cells in the liver of naive mice(left panel) were isolated and examined by flow cytometry. Mononuclear cells in the liver of mice receiving islets and treated with saline(middle panel) or APC(right panel) were isolated at 6 hours after transplantation and examined by flow cytometry. The figures show the percentage of the cells in the corresponding area. Representative data of 2 to 3 experiments are shown.

with thrombus formation and necrosis of adjacent hepatocytes one day after transplantation. Korsgren and coworkers noted that a thrombotic reaction occurred when purified human islets were exposed to non-anticoagulated ABO-compatible blood in surface-heparinized polyvinyl chloride tubing loops.[26,27] The same reaction occurred when porcine allogeneic islets were transplanted to the liver of another pig. They termed this thrombus formation after intrahepatic islet transplantation an instant blood-mediated inflammatory reaction (IBMIR). The effects of IBMIR caused a disruption of islet graft morphology entrapped within a thrombus. On the other hand, in the first step of blood coagulation, tissue factor(TF) is expressed on endothelial cells and monocytes triggered by various stimulations such as endotoxin and the inflammatory cytokines.[28,29] In addition, isolated islets produced TF and this production triggered IBMIR and enhanced the destruction of islet grafts.[30,31] In a preliminary study, TF was expressed in islets transplanted in the liver, and APC treatment prevented this TF expression. From their nuclear shape and insulin negative character, most of the TF expressing cells in the transplanted islets were MNCs infiltrating into grafted islets (data not shown). $Gr\text{-}1^+CD11b^+$ cells generated by transplantation and their IFN-γ production triggered by Vα14 NKT cells are an essential component and a major cause of early graft loss following islet transplantation.[32] In this study, APC remarkably down-regulated the inflammatory cytokine production, including IFN-γ and TNF-α, by $Gr\text{-}1^+CD11b^+$ HMNCs after intrahe-

patic islet transplantation. The fact that APC has both anti-inflammatory and anti-coagulation effects is very important in preventing the loss of intrahepatic islet grafts. In clinical settings, heparin is used to inhibit portal vein thrombosis after islet transplantation.[1)-6)] Heparin improves the engraftment of intrahepatic islet grafts.[26)] However, in the current model, the beneficial effect of heparin was unclear and inferior to that of APC (data not shown). APC is now used clinically in the treatment of deep vein thrombosis and acute pulmonary embolism in patients with congenital protein C or S deficiency. APC also shows significant therapeutic effects in the treatment of disseminated intravascular coagulation(DIC), with less risk of bleeding than heparin.[33)] For the application of APC in clinical islet transplantation, it is very important that the use of APC is associated with a low risk of bleeding because bleeding is one of the major complications in clinical islet transplantation.[1)-6)]

Interestingly, APC remarkably inhibited the production of IFN-γ and TNF-α in Gr-1^+CD11b^+ MNCs without affecting the accumulation of these cells in the liver. Migration of MNCs into the liver and inflammatory cytokine production by these cells may be controlled by different mechanisms. Another possibility is that some populations of Gr-1^+CD11b^+ MNCs in the livers of APC treated mice had an anti-inflammatory effect instead of producing inflammatory cytokines. In fact, Gr-1^+CD11b^+ MNCs are recognized as myeloid suppressor cells in tumor metastasis models.[34) 35)] They produce TGF-β and inhibit the cytotoxic T lymphocyte activity. Further studies are required to fully elucidate the function and role of Gr-1^+CD11b^+ MNCs in transplant models.

In this study, the hepatocytes surrounding islet grafts in a wide area and most of the islet cells were degenerated in the livers of the control mice at 24 hr after islet transplantation. In contrast, most of islet cells were intact and the regions of degenerated hepatocytes were relatively small in the livers of the APC treated mice. The cytoprotective effects of APC may contribute this result. Contreras *et al.* also has reported that recombinant murine APC inhibits the apoptosis of syngeneic islet grafts transplanted into the liver in rodent models.[36)] In their paper, APC also reduced IL-1β and TNF-α mRNA expression in the liver of the recipient mice.

In summary, this study demonstrated that APC remarkably down-regulated the production of inflammatory cytokines of accumulating Gr-1^+CD11b^+ cells in the liver of mice receiving islets facilitating to prevent early loss of transplanted islets. APC may play a key role in improvement of the efficiency of islet transplantation, restoring insulin independence after islet transplantation from one donor to one recipient in a clinical setting.

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(Received on December 27, 2008,
Accepted on January 8, 2009)

7.5 膵島移植

Key Point

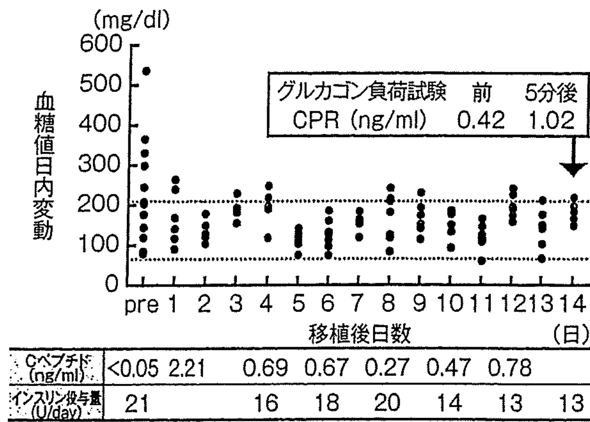
1型糖尿病の移植医療として，近年インスリン産生細胞（膵島）のみを膵外分泌細胞より分離・収集して移植する膵島移植の臨床応用が開始されている。膵島移植は細胞移植であり，臓器移植に比べて侵襲が少なく，より安全な治療法として今後の発展が期待されている。具体的には，門脈にカテーテルを留置し，ドナー膵臓より分離収集した膵島を点滴の要領で肝臓内に注入移植する。移植時の侵襲は通常の血管造影と同等であり，移植翌日より経口摂取が可能で，数日で退院できる。移植された膵島は門脈末端で生着し，血糖値に応じてインスリンを分泌し，血糖を調節する。しかし現状では，1回の膵島移植では血糖値は安定するがインスリン治療からの離脱には成功していない。したがって膵島移植の最も重要な課題は，1人のドナーから1回の膵島移植でインスリン治療より離脱できる方法の開発である。最近の研究で新たな移植膵島細胞障害の機序ならびに制御法が解明され，動物モデルでの膵島移植が成功し，臨床応用が待たれる。また，ブタ膵島をドナーとして用いる異種膵島移植の研究や，再生医療分野からのアプローチも行われており，膵島細胞の移植により1型糖尿病の寛解が得られる日も近いだろう。

❶ 膵島移植の意義，目的と適応

膵島β細胞は血糖を感受するセンサーを有し，高血糖になればインスリンを分泌し，低血糖になればインスリン分泌を停止して血糖を調節している。1型糖尿病ではβ細胞が自己免疫やその他の機序により破壊されて消失するため，注射によりインスリンを補充することが必須である。しかし，遅効型インスリンで基礎分泌を補い，速効型インスリンで追加分泌を補う強化インスリン療法においても血糖管理に難渋する例があり，血糖の不安定性は細小血管障害あるいは動脈硬化を進展させる。また頻回に低血糖を起こせば低血糖に対する自律神経系の応答が鈍麻して，低血糖を認識できない無自覚低血糖発作を生じて，ついには意識障害をきたして生命に重大な影響を及ぼすことになる。移植医療の目的は，インスリン分泌細胞を移植することにより生理的なインスリン分泌を完全な型で補充して血糖値を正常化し，インスリン治療より離脱することである。しかし，現時点では膵島移植によってインスリン離脱ができる率は低く，血糖値の安定化が当面の目標とされ，無自覚低血糖を有する1型糖尿病患者が膵島移植の適応とされている。

膵島移植後のレシピエントの血糖は移植膵島により調節され，移植後はただちに低下して血糖変動幅が少なくなる（図Ⅶ-5-1）。

膵島移植のもうひとつの利点は，糖尿病血管合併症や動脈硬化症に対する効果である。膵島



図Ⅶ-5-1 膵島移植後経過（福岡大学病院でのデータ）
腎移植後膵島移植患者の臨床経過を示す。移植前には50～500mg/dlであった血糖が移植24時間後にはほぼ正常範囲を呈している。膵島移植後血糖管理は良好となった。

移植により血糖が正常化，あるいは安定化すると，これらの障害の進展の阻止や改善が報告されている。

❷ 膵島単離と移植方法

膵島は直径0.1～0.3mmの球状の細胞塊より形成され，膵臓内に島状に散在しており，膵臓容量の約1～2%を占めるにすぎない。このため，ヒト膵臓より全部の膵島を分離収集するのは技術的に困難で，移植に際し充分量の膵島が確保できないことが膵島移植の最大の課題であった。しかし，1990年代に新しいヒト膵島単離法が開発され，膵島移植の臨床応用が開始された（図Ⅶ-5-2）。基本的原理は摘出した膵臓の主膵管[注)]より蛋白分解酵素液を注入して，膵臓の内・外分泌細胞の隔壁を消化し，膵島を膵外分泌細胞から分離することである。膵島は膵外分泌細胞より軽いので，比重勾配で遠心分離して，膵島のみを収集する。

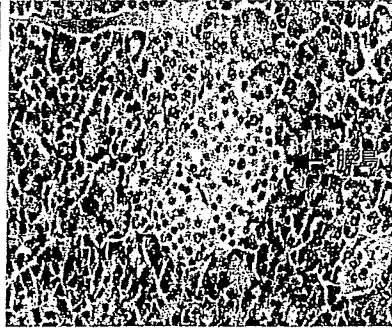
移植は血管造影検査室で放射線科医が行う。超音波で観察しながら肝臓内の血管（門脈）を穿刺してカテーテルを留置し，点滴の要領で膵島を肝臓内に注入（移植）する（図Ⅶ-5-3）。移植された膵島は門脈の末端に留まり，肝臓内で生着し，その部位で血糖を感知して血糖値に応じてインスリンを産生，分泌する。麻酔は穿刺部位の局所麻酔のみで，レシピエントは移植操作中覚醒している。移植に要する時間は20～30分で，造影検査室への搬入から搬出まで約90分で完了する。翌日には経口摂取が可能で，数日で退院できる。

❸ 膵島移植の現況

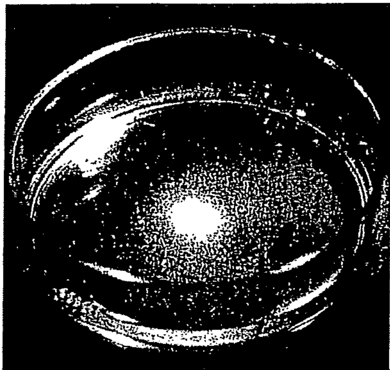
臨床膵島移植は1990年に開始されたが，長らく成績は不良であった。2000年になってカナダのグループより画期的成功例が報告され，以来全世界で症例数が蓄積され，現在600例以上になっている。わが国では2004年に開始され，現在まで18例が実施されている。

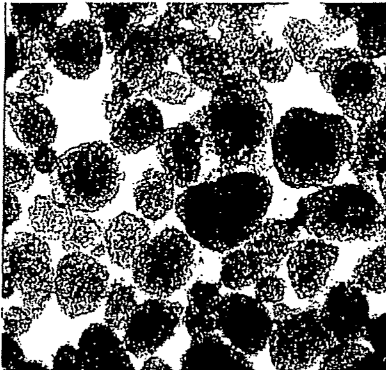
膵島の分離には，欧米では脳死ドナー，わが国では心停止ドナーより提供された膵臓を用いる。わが国では以前から心停止ドナーからの腎臓移植と角膜移植が行われているが，膵島移植のための膵臓提供は，法的に心停止ドナーより腎臓が提供されるときに限られている。膵島移植実施施設として，現時点では東北大学病院，福島県立医科大学病院，千葉東病院，京都大学

注）膵臓の外分泌細胞で産生された消化液の十二指腸への排出路



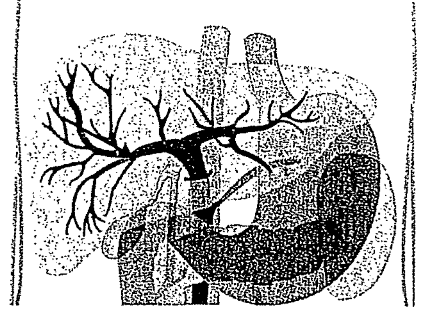
ヒト膵臓（臓器移植ドナー）





単離膵島　　位相差顕微鏡写真

図Ⅶ-5-2 膵臓と単離膵島
ヒトの膵臓は重さ約100g，100万個の膵島を含有する。膵島は容積にして膵臓の約1～2%を占めるに過ぎない。1個の膵島は大きさ約150～250 μM，2000～3000個の細胞より構成される。膵島の50～70%がインスリン産生細胞（β細胞）である。



図Ⅶ-5-3 膵島移植
局所麻酔下，超音波ガイド下に肝臓内血管（門脈）を穿刺，カテーテルを留置し，単離膵島を血液バックに入れ点滴の要領で移植（注入）する。

病院，大阪大学病院，福岡大学病院の6施設が認定されている。

膵島移植の成績に関して，多数例を経験しているアルバータ大学（カナダ）からの報告によると，1型糖尿病レシピエントの血糖は，1回の膵島移植後に安定はするが，正常血糖を維持するにはインスリン治療を継続しなければならない場合が大半である。これは移植後早期に膵島がなんらかの機序で障害され，機能している膵島の数が血糖を正常化するには不足しているためと判明している。膵島移植後にインスリン治療から離脱するためには2～3回の膵島移植，すなわち2～3人のドナーが必要である。わが国でも同様の成績が得られており，1回の膵島移植でインスリン治療から開放できないことが現在の膵島移植の最大の課題である。また，移植後時間の経過とともに移植膵島の機能が低下し，インスリン治療を再開せざるを得ないことも問題である。しかしこのような現状にあっても，移植後に確実に血糖の安定化が得られており，糖尿病専門医の治療をもってしても血糖コントロールに難渋する1型糖尿病患者の血糖管理，特に低血糖発作の消失が膵島移植の利点として広く認識されている。

④ 膵島移植の今後

1人のドナーより1人のレシピエントへの膵島移植でインスリン治療から離脱でき，さらに長期にわたって膵島機能が維持できれば，膵島移植は1型糖尿病の新しい治療法として確立さ

れよう。前述のように，移植後早期（24時間以内）に50%以上の移植膵島が破壊されることが知られているが，筆者らは新たな視点よりこの機序を解析し，その制御により，1回の移植でインスリン依存糖尿病が根治できることを動物モデルで実験的に証明した。現在，ヒト膵島を用いた研究を米国施設と共同で進めており，臨床治験を検討している。

移植医療におけるドナー不足は深刻な問題で，その解決策のひとつとして，ヒトではなく動物の膵島，中でもブタ膵島をドナーとする異種膵島移植の研究が進められている。ブタインスリンは構造上ヒトインスリンに近く，ブタ膵臓より抽出したインスリンが治療に用いられた臨床経験があること，供給に問題がないこと，大量膵島単離が可能であることなどが利点として挙げられる。最近，糖尿病のサルにブタ膵島を異種移植し，免疫抑制薬の投与下に移植膵島が移植後100日以上機能し，サルは正常血糖であったとの報告がなされ，臨床応用への可能性が示唆されている。しかしながらブタには内因性レトロウイルスが存在し，ブタ膵島をヒトに移植した場合，ウイルスが移植後に病原性を獲得し，エボラ出血熱，重症急性呼吸器症候群，トリインフルエンザなど，人類が遭遇したことがない新たな感染症を発症する危険性が指摘されている。この問題を克服できれば，ブタ膵島を用いた異種膵島移植が臨床導入される可能性がある。

もうひとつは再生医療の分野で，胚性あるいは体性幹細胞を用いてインスリン産生細胞を新たに創出しようとする研究である。胚性幹細胞（ES細胞）は発生初期の段階である胚盤胞の一部の細胞塊より樹立され，生体を構成するすべての細胞へ分化することができるという多能性を有し，かつ半永久的に増殖可能な細胞株である。このES細胞よりインスリン産生細胞を創出しようという試みが行われており，最近ではヒトES細胞よりβ細胞への分化誘導が成功したという報告がなされている。しかしながら，受精卵を用いることに対する倫理的問題など，解決すべき課題が数多く存在する。受精卵からではなくレシピエントの体細胞よりES細胞と同等の多能性幹細胞を作製し，その幹細胞より必要な細胞，たとえばインスリン産生細胞を分化誘導し，治療に用いる研究も進められている。最近，マウス，それに引き続きヒトでそのような体性多能性幹細胞が樹立できたとの報告がわが国の研究者よりなされた。この研究成果は画期的で，この細胞よりインスリン産生細胞を分化誘導できれば，まったく新しい細胞移植医療への展開となる。また，本来体内に存在する幹細胞よりインスリン産生細胞を誘導する研究も行われている。体内では個々の細胞には寿命があり少しずつ入れ替わっているが，どのような細胞がインスリン産生細胞になるのかなど，その詳細は解明されておらず，現在の研究課題となっている。多くの研究の成果が臨床応用されて，1人のドナーより多数例の膵島移植が実現し，あるいは創生β細胞が移植医療に応用される日も近いと思われる。さらには，インスリン産生細胞が破壊された1型糖尿病患者の膵臓で，再度膵島細胞の再生が誘導できれば，移植なしに糖尿病が根治できよう。

（安波洋一・小野順子）

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Prevention of Early Loss of Transplanted Islets in the Liver of Mice by Adenosine

Tomoyuki Nitta,[1] Takeshi Itoh,[1] Nobuhide Matsuoka,[1] Toshiyuki Mera,[1] Daibo Kojima,[1] Masahiko Nakano,[1] Yuichi Yamashita,[2] and Yohichi Yasunami[1,3]

Background. The low efficiency of islet transplantation necessitating sequential transplantations with the use of 2 to 3 donors for a recipient has been a major obstacle facing clinical islet transplantation. We determined whether adenosine has any beneficial effects on preventing early loss of transplanted islets in the liver, thereby facilitating successful islet transplantation from one donor to one recipient in mice.
Methods. Two hundred islets, the number of islets from a single mouse pancreas, were grafted into the liver of streptozotocin-induced diabetic C57BL/6 mice. Adenosine was administered once at the time of islet transplantation. Mononuclear cells in the liver of mice receiving islets were isolated and examined by flow cytometry.
Results. A single injection of adenosine at the time of transplantation ameliorated hyperglycemia of diabetic mice receiving 200 syngenic islets with suppression of interferon (IFN)-γ production of hepatic NKT cells and neutrophils, while that of control did not. The IFN-γ production of NKT cells and neutrophils in the liver of mice treated with α-galactosylceramide, a synthetic ligand of NKT cells was suppressed by adenosine. The beneficial effect of adenosine was also observed for BALB/c islet allografts when alloimmune rejection was prevented by anti-CD4 antibody.
Conclusions. Adenosine suppresses the NKT cell-mediated IFN-γ production of neutrophils in the liver of mice receiving islets, thus leading to prevention of early loss of transplanted syngenic and allogenic islets. The findings indicate that adenosine may improve efficiency of clinical islet transplantation.

Keywords: Islet transplantation, Adenosine, Early graft loss, NKT cells, Neutrophils.

(*Transplantation* 2009;88: 49–56)

Pancreatic islet transplantation has now become a procedure of choice for the treatment of insulin-dependent diabetes mellitus (*1*). Currently, however, pancreatic islet transplantation has limited success in achieving insulin independence of a diabetic patient after transplantation of islets from a single donor (*2*), and therefore sequential transplantations of islets with the use of 2 to 3 donor pancreases are required for the treatment of a single recipient (*1*). Therefore, the inability to produce successful islet transplantation from one donor to one recipient has been a major obstacle facing clinical islet transplantation.

This work was supported by a grant of Global FU Program from Fukuoka University (Y.Y.), a Health Science Research Grant from the Ministry of Health, Labor and Welfare, Japan (Y.Y.), a Grant-in-Aid for Scientific Research (Y.Y., M.N.) from the Japan Society for the Promotion of Science, and Shimura Memorial Foundation (Y.Y.).
There authors declare no conflict of interest.

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Received 26 January 2009. Revision requested 22 February 2009.
Accepted 30 March 2009.

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ISSN 0041-1337/09/8801-49
DOI: 10.1097/TP.0b013e3181aa6c9b

The necessity of sequential transplantations of islets to achieve insulin independence from a single donor after transplantation may imply that transplanted islets are lost after transplantation and that the amount of insulin produced from transplanted islets is not enough to maintain glycemic control without an exogenous insulin treatment. Ryan et al. (*3*) recently reported that an islet graft mass of insulin-independent diabetic patients after having received sequential transplantations of islets is only 36% even though they received a total of 1 million islets, which is the equivalent number of islets in a single human pancreas. Therefore, the islet graft loss after transplantation seems to be a major limiting factor for successful islet transplantation.

There are several major factors responsible for the islet graft loss after transplantation including the toxic effects of calcineurin inhibitors as immunosuppressive agents such as FK506 and cyclosporine A (*4*) and of sustained hyperglycemia (*5*) on transplanted islets and allo- and auto-immune rejection. We have previously shown another novel mechanism that is involved in islet graft loss after transplantation, namely an early loss of transplanted islets within 24 hr after transplantation, in which NKT cell-dependent interferon (IFN)-γ production of Gr-1^{+}CD11b^{+} cells (neutrophils) plays an essential role (*6*). These previous findings indicate that NKT cells and Gr-1^{+}CD11b^{+} cells may be targets for intervention to improve efficiency of islet transplantation.

Adenosine is a purine nucleoside and is emerging as a key regulatory molecule which has anti-inflammatory, anticoagulatory, and pro-angiogenic effects in hypoxic and inflamed tissues (*7*). From an immunological point of view, adenosine has been reported to attenuate neutrophil and macrophage functions (*8*, *9*), and it is a potent inhibitor of T cell-mediated immune responses (*10*, *11*). Recently, adenosine has been found to be a key molecule that is responsible for the suppressive function of regulatory T cells (*12*). Moreover, adenosine has been reported to have an inhibitory effect that is mediated by NKT cells on the ischemia-induced reperfusion injury of the liver in mice (*13*).

On the basis of these previous reports, we hypothesize that adenosine may have a beneficial effect on preventing early loss of transplanted islets by improving the efficiency of islet transplantation because the NKT cell-mediated IFN-γ production of Gr-1$^+$CD11b$^+$ cells (neutrophil) is an essential component of the early loss of transplanted islets (*6*) and because adenosine is expected to have an inhibitory effect on NKT cells. Importantly, adenosine and an adenosine transporter inhibitor that increases the extracellular levels of adenosine such as dipyridamole have already been used in the clinical practice for the examination and the treatment of heart function and disease, respectively. Therefore, the safety issue related to the clinical use for islet transplantation has already been cleared.

The present study demonstrates that adenosine and an adenosine transporter inhibitor, dipyridamole can suppress the NKT cell-mediated IFN-γ production of Gr-1$^+$CD11b$^+$ cells, enabling islet transplantation from one donor to one recipient in mice, and thus suggesting that adenosine may improve the efficiency of clinical islet transplantation.

MATERIALS AND METHODS

Animals

Male BALB/c (H-2d) and C57BL/6 (H-2b) mice were purchased from Charles River Japan (Kanagawa, Japan) and used for the experiments. Because the severity of diabetes made with streptozotocin (STZ) injection differed depending on the weight of mice, only the mice weighing 23 to 25g were used as recipients. Mice weighing 25 to 30g served as donors. Diabetes was induced in the recipients by the intravenous injection of STZ (180 mg/kg) (Sigma, St. Louis, MO). The plasma glucose levels of the mice exceeded 400 mg/dL at 2 to 3 days after the STZ injection, and the mice remained hyperglycemic at the time of islet transplantation. All experiments were performed in accordance with the Institutional Animal Care and Use Committee of Fukuoka University.

Islet Isolation and Transplantation

Islets were isolated by the static digestion method using collagenase (*14*) and then separated by centrifugation using Ficoll-Conray gradients (*15*). Islets of 150 to 250 μm in diameter were hand-selected using Pasteur pipette with the aid of a dissecting microscope, because it was critical to minimize the size variation of individual islets to compare the effects of the difference in the number of donor islets. The size of individual islets in each islet isolation procedure was confirmed by using a phase-contrast microscope equipped with a scale in the eyepiece. Hand-picked islets were transplanted into the liver through the recipient's portal vein (*16*) at 3 days after the induction of diabetes with STZ injection.

Monitoring Plasma Glucose and Body Weight

The nonfasting plasma glucose levels and body weight were monitored three times a week in all the recipients for 60 days after islet transplantation. The plasma glucose was measured using a Beckman glucose analyzer (Beckman Japan, Tokyo, Japan). Normoglycemia after transplantation was defined as two consecutive plasma glucose levels with a reading below 200 mg/dL.

Administration of Adenosine, α-Galactosylceramide, Dipyridamole, Nitrobenzylthioinosine, and Anti-CD4 Antibody

Adenosine (Daiichi Pharmaceutical Co., Tokyo, Japan) and dipyridamole (Boehringer Ingelheim, Ingelheim, Germany) were dissolved in saline and were administered intravenously; nitrobenzylthioinosine (NBTI) (Sigma Aldrich, St. Louis, MO) was dissolved in 10%DMSO and administered intraperitoneally (IP) into appropriate groups of diabetic recipient mice, once at the time of islet transplantation.

α-Galactosylceramide (α-GalCer) was a generous gift from Dr. Masaru Taniguchi (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan) and was administered intravenously to naïve mice.

Anti-CD4 antibody (200 μg/injection/mouse, YTS177, rat IgG1; R&D, Minneapolis, MN) was administered IP to appropriate groups of diabetic recipient mice receiving allogenic islets into the liver.

Intraperitoneal Glucose Tolerance Test

Intraperitoneal glucose tolerance test (IPGTT) was performed in recipient mice at 60 days after the islet transplantation. The mice were fasted for 8 hr before the start of the examination. Blood samples were obtained from the orbital sinuses of recipient mice at 0, 30, and 120 min after the IP injection of glucose (1 g/kg body weight), and the plasma glucose was measured as previously described.

Morphological Study

The livers bearing islet grafts and pancreas were examined morphologically at 60 days after transplantation in appropriate groups of mice, and the pancreases of recipient mice were also examined simultaneously. The liver and pancreas were fixed with Bouin's solution, processed, and then were embedded in paraffin. The sections were prepared for light microscopy and stained with hematoxylin-eosin, and aldehyde and fuchsin.

Preparation of Hepatic Mononuclear Cells

Hepatic mononuclear cells (MNCs) were prepared as described previously (*17*). In brief, an excised liver was pressed through a stainless steel mesh, and the resulting dissociated liver tissues were suspended in Dulbecco's modified Eagle medium (D-MEM/F-12, Life Technologies, Tokyo, Japan) and washed twice. The mixture was resuspended in an

isotonic 33% Percoll solution containing heparin (67 U/mL) and centrifuged at 2000*g* at 4°C for 15 min. The resulting pellet was suspended in a 0.83% ammonium chloride solution to lyse erythrocytes. After counting, these MNCs were washed twice in PBS and used for further analysis.

Antibody and Flow Cytometry Analysis

The antibodies (Abs) used for the flow cytometry analysis were as follows: Fc block (anti-mouse FcRγIII/II mAb, 2.4G2), fluorescein isothiocyanate-conjugated anti-CD3ε (clone 145–2C11, American Hamster IgG1κ), phycoerythrin-CY5-conjugated rat anti-mouse CD4 mAb (clone RM4–5, Rat IgG2a), allophycocyanin-conjugated anti-IFN-γ mAb (clone XMG1.2, Rat IgG1), fluorescein isothiocyanate-conjugated anti-CD11b (M1/70), PerCP-conjugated anti-Gr-1 (RB6–8C5), and their isotype control (clone R3–34, Rat IgG1) were purchased from Pharmingen (San Diego, CA). Phycoerythrin-conjugated α-GalCer-loaded CD1d tetramers were prepared as described (*18*). For intracellular staining, cells were incubated with anti-FcRγIII/II and neutravidin (Invitrogen), surface stained, fixed, permeabilized, stained with mAbs, and analyzed on a flow cytometer (FACS Calibur; Becton Dickinson). A total of 10,000 viable cells were analyzed.

Statistical Analysis

The statistical significance with respect to the rate of euglycemia in streptozotocin-induced diabetic mice after transplantation and to the plasma glucose levels during IPGTT was determined by Fisher's exact test and Student's *t* test, respectively. Differences were considered significant when the *P* values were less than 0.05.

RESULTS

Early Loss of Transplanted Syngenic Islets in the Liver of Mice is Prevented by Adenosine

First, we determined whether adenosine has any beneficial effect on preventing early loss of transplanted syngenic islets in the liver of STZ-induced diabetic mice. Previously, we have shown that hyperglycemia of diabetic recipient mice is ameliorated after the transplantation of 400 syngenic islets into the liver, but not of 200 islets (*6*), the number of islets isolated from a single mouse pancreas. In this study, we determined whether hyperglycemia of diabetic mice that received 200 syngenic islets into the liver is ameliorated by adenosine. When diabetic mice received 200 islets and were treated with saline as a control, all the recipient mice (n=5) did not become normoglycemic after the transplantation (Fig. 1, upper panel). When diabetic mice received the same number of islets and were treated with 0.5 or 5 mg/kg adenosine once at the time of islet transplantation, 0 of 4 or 5 of 5 recipient mice became normoglycemic after the transplantation (Fig. 1, middle and lower panels). A histologic study revealed that intact or degenerated islets with well or poorly granulated β cells were seen in the liver of the normoglycemic or hyperglycemic recipient mice, respectively (histology not shown). The findings show that the amount of insulin produced from islet grafts was sufficient to ameliorate hyperglycemia of the diabetic recipient mice treated with 5 mg/kg adenosine but not that of the mice treated with saline or 0.5 mg/kg adenosine, indicating that early loss of transplanted islets is prevented by 5 mg/kg adenosine. Therefore, 5 mg/kg adenosine was used for the following studies.

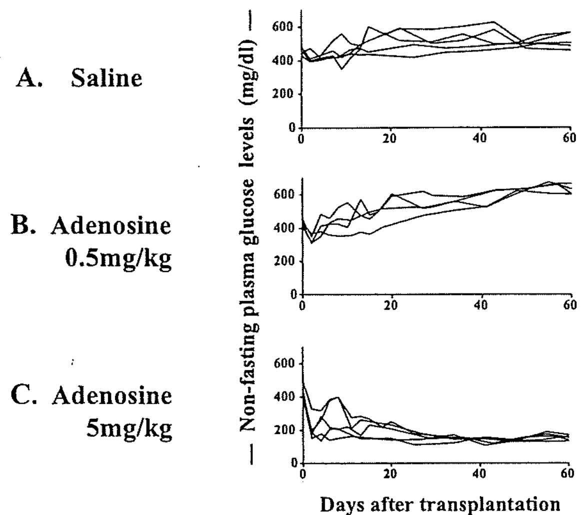

FIGURE 1. Plasma glucose levels of streptozotosin-induced diabetic mice receiving 200 syngenic islets into the liver. Diabetic mice receiving 200 syngenic islets were treated with saline (A), 0.5 mg/kg (B), or 5 mg/kg adenosine (C) once at the time of islet transplantation. Individual line represents the nonfasting plasma glucose levels of each animal.

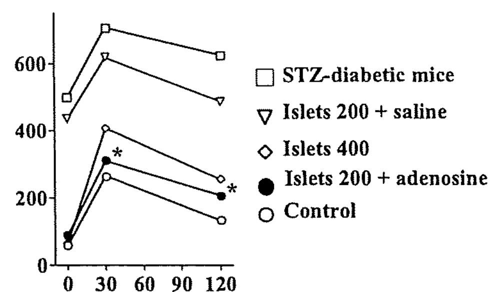

FIGURE 2. Intraperitoneal glucose tolerance test of diabetic mice receiving syngenic islets at 60 days after transplantation. Recipient mice were fasted for 8 hr before the examination, and plasma glucose levels were determined at 0, 30, and 120 min after the glucose injection (intraperitoneally, 1 g/kg). Experimental groups included diabetic mice without islet transplantation (□, n=4), those receiving 400 islets without treatment (◇, n=5), 200 islets treated with adenosine (5 mg/kg) (●, n=5), or saline (▽, n=4). Untreated mice served as control (○, n=4). The plasma glucose levels at 30 and 120 min after the glucose injection between the mice receiving 400 islets without treatment and those receiving 200 islets and treated with adenosine were significantly different (*$P<0.05$ by the Student's-*t* test).

Increase in Functional Islet Mass in the Liver of Recipient Mice by Adenosine

To evaluate the extent that adenosine improves the efficiency of islet transplantation, IPGTT was performed in appropriate groups of recipient mice. As shown previously (*19*), the plasma glucose levels of diabetic mice (n=5) receiving

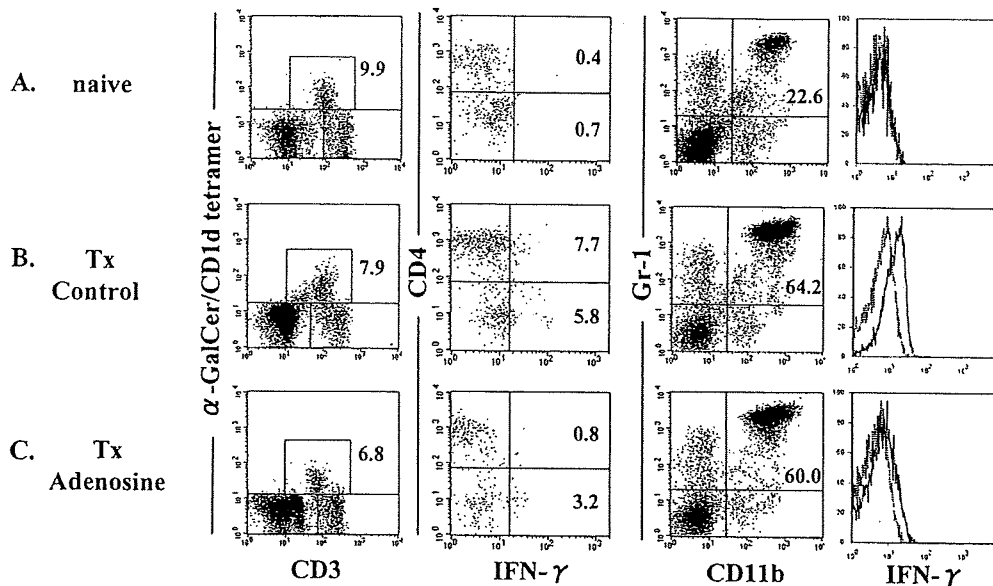

FIGURE 3. Flowcytometry of mononuclear cells in the liver of mice. Mononuclear cells in the liver of diabetic mice receiving 200 syngenic islets and treated with saline (B) or adenosine (5 mg/kg) (C) were isolated at 6 hr after islet transplantation and examined by the flow cytometry. NKT cells defined as the cells expressing α-galactosylceramide/CD1d tetramer and CD3 on their cell surface were further analyzed with respect to the CD4 expression and the interferon-γ production (second column). Gr-1$^+$CD11b$^+$ cells were gated to examine the interferon-γ production (4th column). The numbers in the figures represent the percentage of cells in the corresponding areas. Representative data from the three experiments are shown.

200 islets at 0, 30, and 120 min after the IP injection of glucose (1g/kg) were similar to those of diabetic hyperglycemic mice without islet transplantation (n=5; Fig. 2). In marked contrast, the plasma glucose levels of the normoglycemic mice received 200 islets and treated with 5 mg/kg adenosine were significantly lower in comparison with those of the normoglycemic mice receiving 400 islets at 30 and 120 min after the IP injection of glucose (1 g/kg; Fig. 2). The findings indicate that the treatment with adenosine (5 mg/kg/injection) prevented a significant decrease of the islet mass in the liver of recipient mice after transplantation.

IFN-γ Production of NKT Cells and Gr-1$^+$CD11b$^+$ Cells in the Liver of Mice Receiving Syngenic Islets is Suppressed by Adenosine

Previously, we have shown that the NKT cell-mediated IFN-γ production of Gr-1$^+$CD11b$^+$ cells (neutrophils) is an essential component of the early loss of transplanted islets in the liver of mice (*6*). Therefore, in this study we determined whether adenosine has any effect on the NKT cell-mediated IFN-γ production of Gr-1$^+$CD11b$^+$ cells in the liver receiving islets. For these purposes, MNCs in the liver of mice receiving islets and treated with adenosine or with saline were isolated and examined by flow cytometry with respect to IFN-γ production of α-GalCer/CD1d-tetramer$^+$ CD3$^+$NKT cells and Gr-1$^+$CD11b$^+$ cells (neutrophils). NKT cells are composed of two major populations, namely CD4$^+$CD8$^-$ and CD4$^-$CD8$^-$ NKT cells. It was found that the IFN-γ production of both NKT cell populations in the liver of mice receiving syngenic islets and treated with saline was upregulated at 6 hr after transplantation (Fig. 3, II, 2nd column). The IFN-γ production of CD4$^+$NKT cells was significantly suppressed in the liver of the mice treated with adenosine. The IFN-γ production of Gr-1$^+$CD11b$^+$ cells accumulated in the liver of mice receiving islets and treated with adenosine was suppressed at 6 hr after transplantation (Fig. 3, II and III, 4th column). Interestingly, the number of accumulated Gr-1$^+$CD11b$^+$ cells in the liver of mice receiving islets was not altered irrespective of the treatment with adenosine (Fig. 3, 3rd column). Therefore, these findings show that adenosine has an inhibitory effect on the IFN-γ production of NKT cells and Gr-1$^+$CD11b$^+$ cells in the liver of mice after islet transplantation.

Adenosine Prevents α-GalCer-Stimulated IFN-γ Production of NKT Cells and Gr-1$^+$CD11b$^+$ Cells

To confirm further an essential role of adenosine in NKT cell activation, α-GalCer, a synthetic ligand of NKT cells was used in the experiments and determined whether the IFN-γ production of NKT cells in response to α-GalCer was suppressed by adenosine. As originally reported (*20*), it is well known that NKT cells produce large amount of IFN-γ in response to α-GalCer. The fluorescence-activated cell sorting analysis of MNC in the liver of mice at 2 hr after the IV injection of α-GalCer (10 μg/kg/injection/mouse) revealed a marked increase in IFN-γ production of α-GalCer/CD1d-tetramer$^+$ CD3$^+$NKT cells and Gr-1$^+$CD11b$^+$ cells (neutrophils) in comparison with control mice treated with vehicle (Fig. 4, I and II). In contrast, the IFN-γ production of NKT cells and Gr-1$^+$CD11b$^+$ cells in the liver of mice treated with adenosine in conjunction with α-GalCer was suppressed (Fig. 4, III). Thus, adenosine has an inhibitory effect on activation of NKT cell and Gr-1$^+$CD11b$^+$ cell activation in response to α-GalCer.

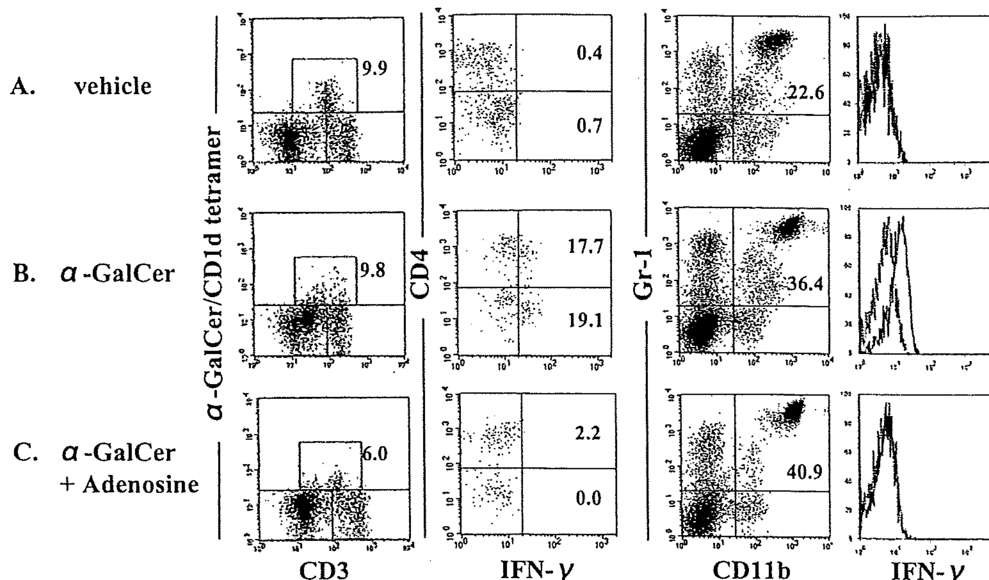

FIGURE 4. Flow cytometry of mononuclear cells in the liver of mice treated with intravenous administration of α-galactosylceramide (α-GalCer). Mononuclear cells in the liver of mice treated with (C) or without adenosine (5 mg/kg) (B) were isolated at 2 hr after the intravenous injection of α-GalCer (10 μg/kg) and examined by the flow cytometry. Mice treated with vehicle served as the control (A). The α-GalCer-CD1d tetramer$^+$CD3$^+$ NKT cells were further gated to examine the interferon-γ production of CD4$^+$ or CD4$^-$ NKT cells (second column). The Gr-1$^+$CD11b$^+$ cells were further examined with respect to the interferon-γ production (4th column). The numbers in the figures represent the percentage of cells in the corresponding areas. Representative data from the three experiments are shown.

Beneficial Effects of Adenosine Transporter Inhibitors, Dipyridamole, and Nitrobenzylthioinosine on Prevention of Early Loss of Transplanted Islets

Thereafter, we determined whether adenosine transporter inhibitors, such as dipyridamole and NBTI, which increase extracellular levels of adenosine have a similar effect to adenosine with respect to prevention of early loss of transplanted islets. When diabetic mice receiving 200 syngenic islets into the liver were treated with vehicle as controls, all the recipient mice remained hyperglycemic for more than 60 days after the transplantation (Fig. 5, A). In contrast, diabetic mice receiving 200 syngenic islets into the liver and treated with dipyridamole or NBTI became normoglycemic by 60 days after the transplantation (Fig. 5, B and C). Histologically, intact islets with well-granulated or degranulated β cells were identified in the liver of the normoglycemic or hyperglycemic mice, respectively at 60 days after the transplantation (histology not shown).

Adenosine Prevents Early Loss of Transplanted Allogenic Islets in the Liver of Recipient Mice Treated With Anti-CD4 Antibody

Finally, we determined whether the beneficial effect of adenosine on prevention of early loss of transplanted syngenic islets in the liver of mice is similar for islet allotransplantation. When 200 BALB/c islets were grafted into the liver of STZ-diabetic C57BL/6 mice treated with saline, recipient mice did not become normoglycemic and remained hyperglycemic by 60 days after the transplantation (Fig. 6, A, left panel). Histologic examinations revealed that foci of MNCs were seen in the liver of mice receiving islet allografts at 14 days after the transplantation, whereas only a few insulin producing cells were identified (Fig. 6, A, right panels). In contrast, when 200 BALB/c islets were grafted into the liver of STZ-diabetic C57BL/6 mice treated with adenosine once at the time of islet transplantation, hyperglycemia of recipient mice was ameliorated by 3 days and the mice became hyperglycemic again by 7 days after the transplantation (Fig. 6, B, left panel). Histologically, islet grafts infiltrated with MNCs were seen in the liver of the recipient mice at the time of rejection (Fig. 6, B, right panels). When diabetic mice received 200 allogenic islets and were treated with anti-CD4 antibody, the recipient mice remained hyperglycemic by 60 days after transplantation (Fig. 6, C, left panel). Histologically, islets with degranulated β cells were seen in the liver of the recipient mice at 60 days after the transplantation as reported previously (*19*; data not shown). When diabetic mice received 200 allogenic islets and were treated with adenosine in conjunction with anti-CD4 antibody, the recipient mice became normoglycemic by 5 days and remained in this state for more than 60 days after transplantation (Fig. 6, D, left panel). Histologically, intact islets with well-granulated β cells were seen in the liver of the recipient mice at 60 days after the transplantation (Fig. 6, D, right panels). These findings indicate that adenosine promotes early engraftment while anti-CD4 antibody prevents the alloimmune rejection of transplanted allogenic islets.

DISCUSSION

These findings clearly show that adenosine prevents the early loss of transplanted syngenic islets by suppressing the NKT cell-mediated IFN-γ production of Gr-1$^+$CD11b$^+$ cells in the liver of mice receiving islets, thus enabling islet trans-

plantation from one donor to one recipient in mice. Moreover, the beneficial effect of adenosine was also found in islet allotransplantation when alloimmune rejection was prevented under an immunosuppressive agent such as anti-CD4 antibody.

Adenosine has long been used as a supplemental component of preservation solution for organ transplantation to minimize ischemic damage of donor organs during preservation (*21*). Accumulating evidences now show that adenosine is produced in various cells when they are placed under stress such as ischemia and inflammation, and it serve as a suppressive molecule through its receptor on cellular membrane, such as adenosine A2A receptor (*22*). Thus, the presence of adenosine outside of the cells seems to be essential for the inhibitory effect of adenosine. Adenosine is present in equal amounts between inside and outside the cells and its transport is controlled by the equilibrium nucleoside transporter (ENT) on the cellular membrane (*23*). Adenosine is produced from adenosine triphosphate/adenosine diphosphate and adenosine monophosphate mediated by CD39 (ectonucleoside triphosphate diphosphohydrolase-1) and CD73 (ecto-5-nucleotidase), respectively, and from *s*-adenosylhomosistein by adenosine hydrolase (*24*). Adenosine is degraded by adenosine deaminase (*25*). Therefore, there are several ways to increase the extracellular concentration of adenosine including the administration of adenosine itself, the blockade of ENT to prevent transportation of adenosine from outside to inside the cells, the prevention of adenosine degradation and the promotion of adenosine production. In this study, we focused on the former two interventions because adenosine and the ENT inhibitor, dipyridamole have already been used in the clinic and are ready to be applied to clinical islet transplantation. First, we determined whether adenosine has any beneficial effect on prevention of the early loss of syngenic transplanted islets and found that this was the case as shown in Figure 1. The flowcytometry of MNC in the liver of mice receiving islets and treated with adenosine revealed that the IFN-γ production of NKT cells and neutrophils infiltrated into the liver of mice after islet transplantation was suppressed. The finding suggests that the beneficial effect of adenosine was mediated through the inhibition of NKT cells and not though the activation of neutrophils, because the IFN-γ production of neutrohils in the liver of mice receiving islets, which is an essential component of the early loss of transplanted islets, was dependent on NKT cells (*6*). To examine further whether NKT cells were responsible for the inhibitory effect of adenosine, we determined whether IFN-γ production of NKT cells in response to α-GalCer, a synthetic ligand of NKT cells (*20*), was inhibited by adenosine. The flow

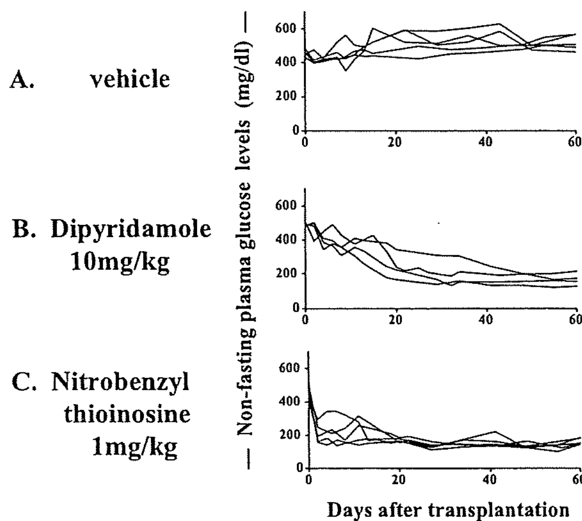

FIGURE 5. Beneficial effects of dipyridamole and nitrobenzylethioinosine on prevention of early loss of transplanted islets. Individual line represents the nonfasting plasma glucose levels of each diabetic mouse receiving 200 syngenic islets and treated with vehicle (A), dipyridamole (10 mg/kg) (B), or nitrobenzylethioinosine (1 mg/kg) (C).

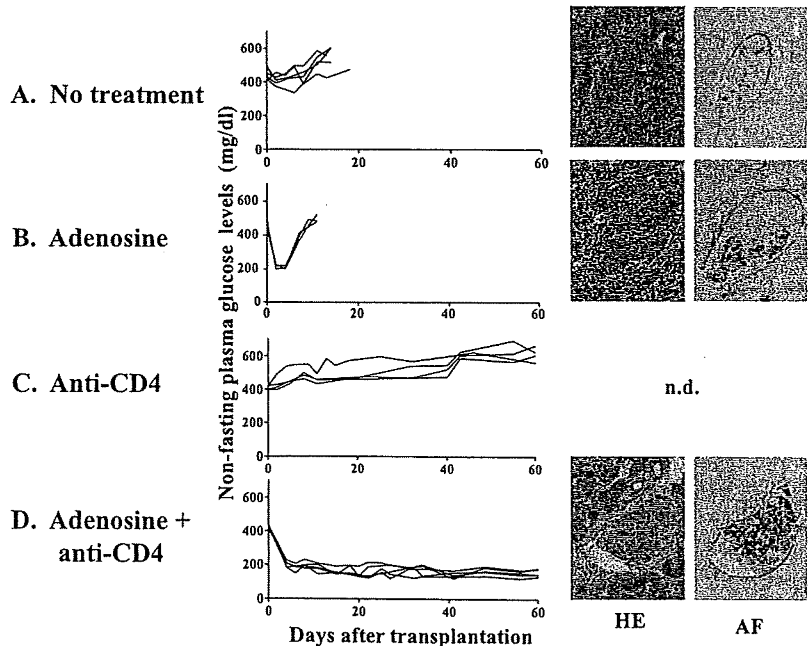

FIGURE 6. Early loss of transplanted allogenic islets was prevented by adenosine. The plasma glucose levels of diabetic mice receiving 200 allogenic islets and treated with saline or adenosine (5 mg/kg, administered intravenously) are shown in groups A or B, respectively. In groups D or C, recipient mice receiving 200 allogenic islets were treated with anti-CD4 antibody combined with and without the treatment by adenosine, respectively. The livers of mice receiving islet allografts were examined morphologically at 14 (A and B) or 60 days (D) after the transplantation (right columns). nd, not done; HE, hematoxylin-eosin stain; AF, aldehyde and fuchsin stain. Original magnification ×100.