移植膵の内分泌機能の評価法として，われわれは人工膵島を用いた高血糖クランプ検査を実施している（図5）。この方法はグルコース応答性インスリン分泌の第1相と第2相の評価が可能であり，術後の侵襲による影響や腎機能，消化管機能の影響を受けないという特徴がある。Gorogawa ら[9]は，インスリン分泌第2相 IRI（90）が，糖尿病患者における治療選択のための主要因子であることを報告している。さらに，移植後72時間でのインスリン必要量は，術後1ヵ

表2　膵移植後の血糖管理指針

目的：	感染予防，全身状態の改善 膵グラフトの 糖毒性からのβ細胞保護 内分泌機能評価（急性グラフト障害の早期発見）
術後72時間まで	人工膵島による血糖モニターおよびインスリン持続静注 血糖管理目標 100＜随時血糖＜150 mg/dL
術後72時間以降	頻回血糖測定およびインスリン投与（持続静注，皮下注） 血糖管理目標 空腹時血糖＜120 mg/dL 食後血糖（2時間値）＜170 mg/dL

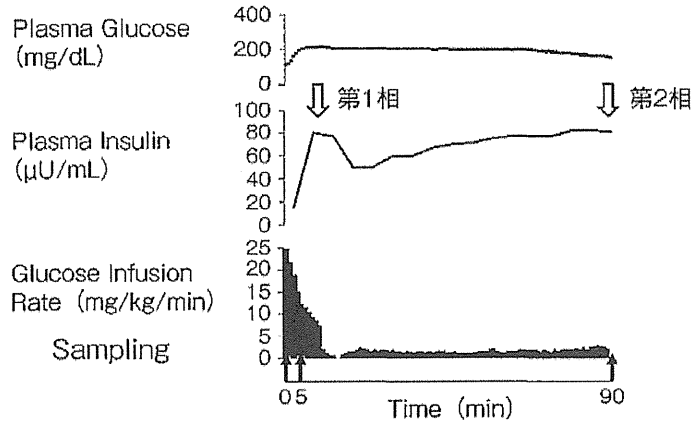

図5　高血糖クランプ検査

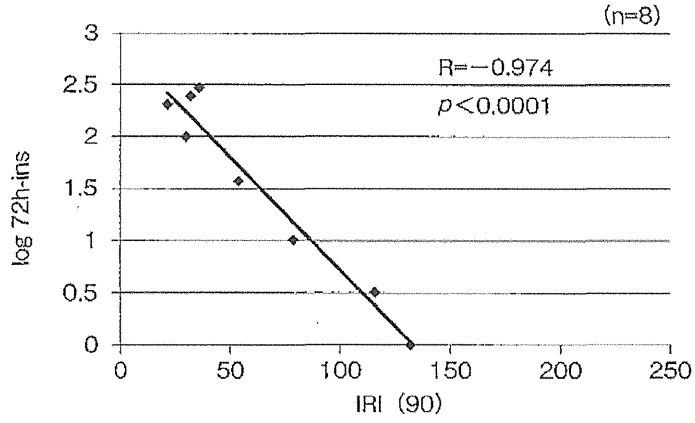

図6 log (72h-ins) と1ヵ月後IRI (90)

月で実施した高血糖クランプ検査での膵グラフトの内分泌機能をよく反映していた（図6)。この結果は移植後1年での検討においても再現された。したがって，術後72時間に血糖を維持(100～150 mg/dL）するために要したインスリン量はその後の膵グラフト機能の良い指標となると考えられた。

おわりに

当院における脳死下膵臓移植の現況と移植後の血糖管理について述べた。本邦での臓器移植は一昨年の臓器移植法の改正を受けて臓器提供数の増加がみられ，やっと軌道に乗ろうとしている。そうした現況下で，条件の厳しいマージナルドナーが多くを占めているが，移植成績は欧米と比べて決して遜色はなく，むしろそれを凌駕する結果が得られている。

これは本邦における木目の細かい周術期管理が功を奏しているものと考えられる。そうした中で，われわれは，種々の障害を受けている膵β細胞を可及的に温存した血糖管理を行っているが，こうした戦略が遠隔成績にどう影響するか，あるいは，さらなる成績の向上のために障害となる因子を明らかにしていくことが今後ますます必要になってくる。

③ points lessons

① 本邦の臓器（膵臓）移植の特徴として，ドナーが高年齢，死戦期の血行動態が不安定，脳死の原因として動脈硬化性疾患が多い，などのいわゆる“マージナルドナー”が多く（約75%）を占めている。

② 移植の周術期において，膵グラフト内の膵島は，冷保存や虚血再灌流による障害，免疫抑制剤，高インスリン血症など，さまざまな影響を受け，機能発現が遅延する可能性がある。

③ 移植後，72時間に血糖の維持（100～150mg/dL）に要したインスリン必要量は，術後1ヵ月でのグラフトの内分泌機能をよく反映しており，同機能の良い指標となると考えられた。

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報告

本邦膵移植症例登録報告（2012）

日本膵・膵島移植研究会膵臓移植班

The Registry of Japanese Pancreas and Islet Transplantation 2012

The Japan Society for Pancreas and Islet Transplantation

【Summary】

One hundred and forty eight cases of pancreas transplantation from deceased, non-heart beating and living-related donors have been performed in 17 institutions in Japan, since April 2000 as of the end of 2010. The following donor－and recipient－related factors were analyzed; sex and age of donor and recipient, cause of barin death, histories of diabetes and dialysis, waiting period, total cold ischemic time, operative procedure, immunosuppression and survival rates of patient and graft.

In spite of donor poor conditions which were mostly marginal in Japan, the outcome of pancreas transplants was considered comparable to that of the US and Europe.

Keywords: simultaneous pancreas and kidney transplantation (SPK), pancreas after kidney transplantation (PAK), pancreas transplantation alone (PTA), deceased donors (DD), non-heartbeating donors (NHBD), living-related donors, marginal donor, bladder drainage (BD), enteric drainage (ED), tacrolimus (TAC), anti IL-2 receptor monoclonal antibody, mycophenolate mofetil (MMF)

I. はじめに

膵・膵島移植研究会では，2007年以降，膵臓移植登録委員会において，毎年1回，本邦膵臓移植の現状ならびにその成績を報告している[1-5]。

1997年，「臓器の移植に関する法律」が実施されて以降，2011年末までに，本邦で実施された脳死下，心停止下での膵臓移植ならびに生体膵臓移植の全症例について，解析結果を報告する。

II. 対象と方法

「臓器の移植に関する法律」実施後，2000年4月に第1例目の膵腎同時移植が行われてから，2011年末までに，本邦で実施された脳死下，心停止下での膵臓移植121例，ならびに生体膵臓移植25例，計146症例につき，患者数の推移，ドナー・レシピエント関連因子（ドナーの性差と年齢，ドナーの死亡原因，レシピエントの性差と年齢，透析歴と糖尿病歴，待機期間，総冷阻血時間，手術術式，免疫抑制法），移植成績（生存率，移植膵・移植腎生着率）を解析し治療成績を検討した。なお，累積生存率，膵および腎の生着率はKaplan-Meier法で算出した。

1. 膵臓移植認定施設

現在，認定施設は北海道大学（3），東北大学（5），福島県立医科大学（3），獨協医科大学（0），新潟大学（1＋2*），東京女子医科大学（19），東京医科大学八王子医療センター（0），国立病院機構千葉東病院（10＋18*），名古屋第二赤十字病院（3），藤田保健衛生大学（15＋2*），京都府立医科大学（6），京都大学（0），大阪大学（24＋1*），神戸大学（5），広島大学（2），香川大学（1），九州大学（24＋2*），以上，計17施設である（括弧内は2011年末までの実施移植数で，*は生体膵臓移植数である）。

2. 膵臓移植実施体制

本邦における膵臓移植は中央調整委員会の下に，認定18施設の代表からなる実務者委員会が組織され，そこで作成された実施のためのガイドライン『膵臓移植に関する実施要綱，2010年版』（12月，改訂）に従って運用されている[6]。とりわけ，膵臓移植の特徴は，他の臓器と異なり，移植施設が近隣の認定施設と連携

をとりながら，対応している点が挙げられる。特に，経験の多い実務者委員が中心となり，手術ならびに術後管理に対応している。

3. レシピエントカテゴリーと登録システムとレシピエントの選択基準

膵臓移植には3つのレシピエントカテゴリーがあり，腎不全がある場合には膵臓と腎臓を同時に移植する膵腎同時移植(simultaneous pancreas and kidney transplantation：SPK）と先に腎臓移植を先行させ，後に膵臓移植を行う腎移植後膵移植（pancreas after kidney transplantation：PAK）とがあり，さらに腎不全のない場合には膵単独移植（pancreas transplantation alone：PTA）がある。

膵臓移植の適応基準に従い，レシピエント候補者の主治医が地域の膵臓移植適応評価委員会にデータを添えて申請して，その結果が中央調整委員会へ送付される。最終的に中央調整委員会から移植施設に対して，移植可能の是非が確認され，日本臓器移植ネットワークへ登録となる。

ドナー（脳死下，心停止下）発生時には，登録されたレシピエントの中から，選択基準に従って選択される。

III. 結果と考察

1. 膵移植新規登録患者数

膵移植の日本臓器移植ネットワークへの登録は1999年10月より開始され，それ以降の新規登録患者数の推移を**図1**に示した。2011年末までに，日本臓器移植ネットワークに新規登録された患者数は計384名であった。2001年以降は毎年25名程度の新規患者が登録されている。2010年は臓器移植法の改正の影響もあって40例と増加した。なお，登録後5名が生体膵移植を受け，また，糖尿病性合併症などの理由により35名が死亡し，25名が登録を取り消した。

2011年12月末日の時点で，移植後，死亡および取り消しを除いた登録待機中の患者198名について，性別，年齢ならびに待機期間につき検討した。性別では約67%が女性で，年齢では40歳代が44%，次いで50歳代が28%，30歳代が20%であった（**図2**）。

待機期間では法改正に伴って，新規症例が増加（1年未満が前年の40から64例へ）した。一方，3年以上待機例が52.5%であり，とりわけ5年以上が30.3%を占めていたが，法改正後は移植数の増加と相まって，前年度の報告と比較して，やや減少した（**図3**）。

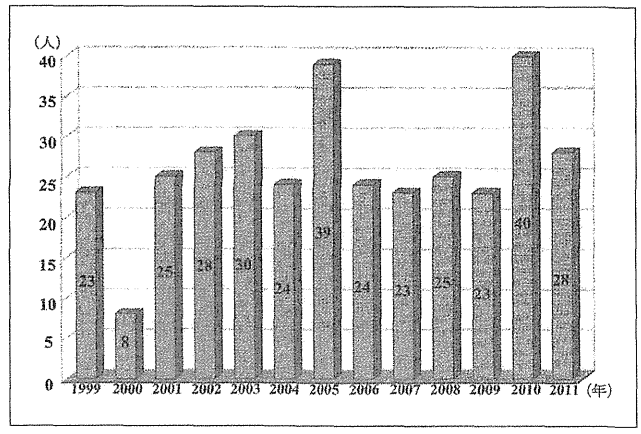

図1　膵移植新規登録患者数の年次推移

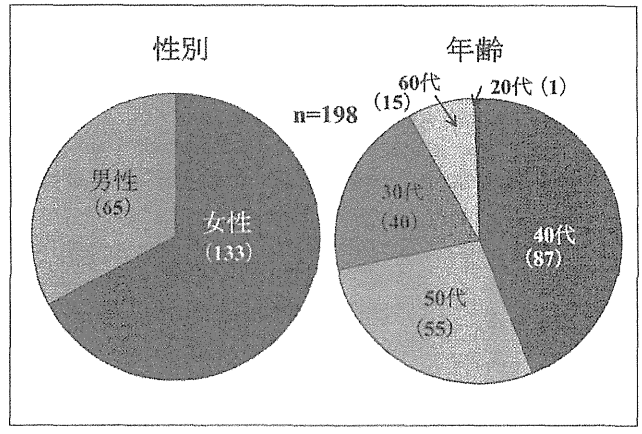

図2　膵臓移植待機登録者の性別と年齢

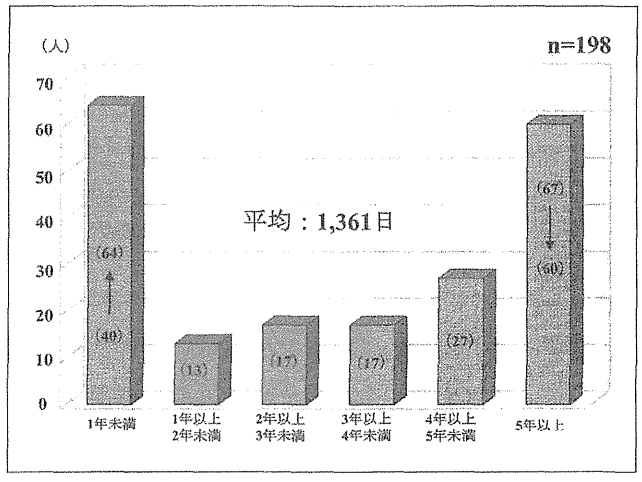

図3　膵臓移植登録者の待機期間

2. 膵移植症例数

1997年10月「臓器の移植に関する法律」の施行後，2011年末までの脳死下での臓器提供の承諾は159例

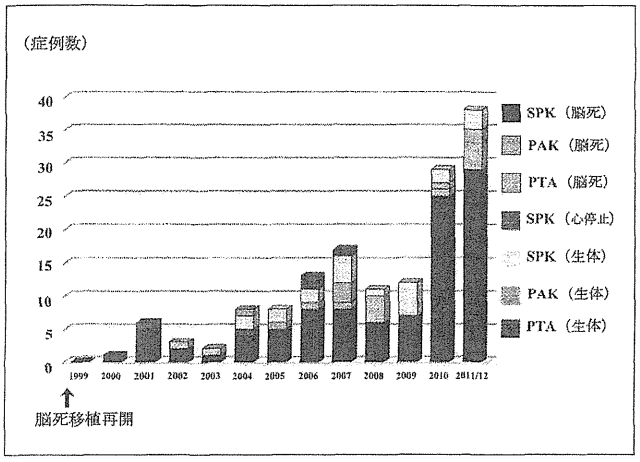

図４　膵移植症例数の年次推移

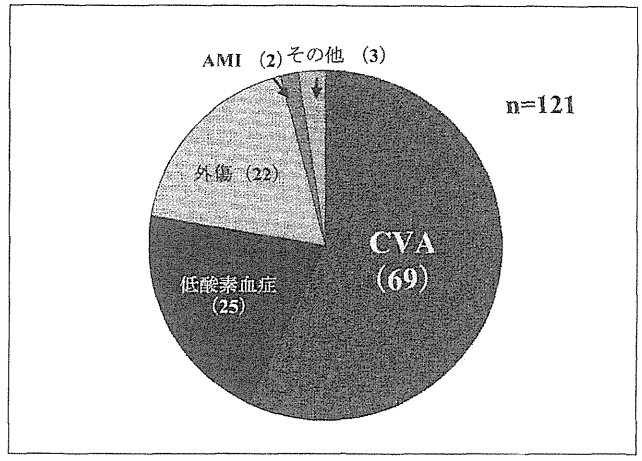

図６　ドナーの死亡原因

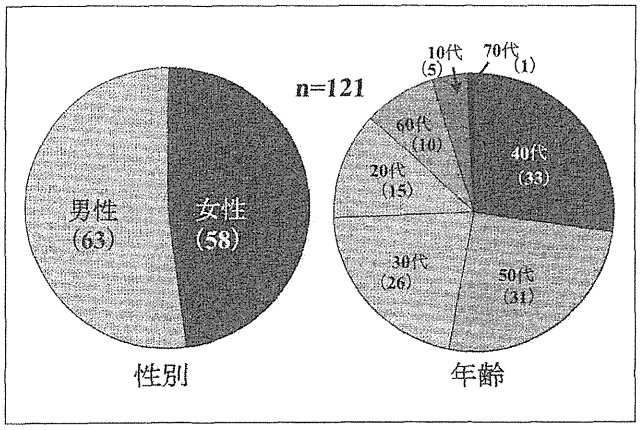

図５　ドナーの性別と年齢

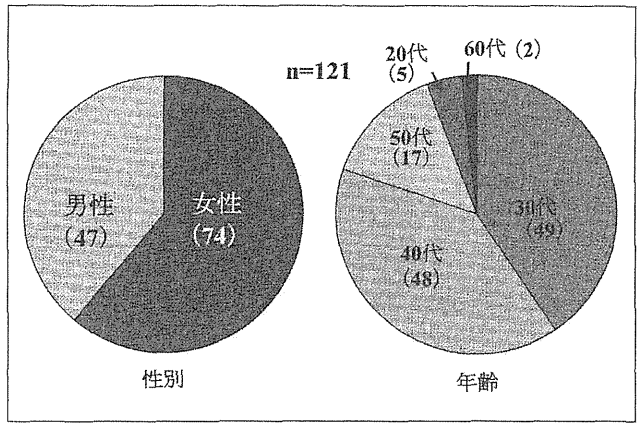

図７　レシピエントの性別と年齢

あり，そのうち，膵臓の提供に至ったのは119例（74.8%）であった。その内訳はSPKが99例，PAKが14例，およびPTAが6例であった。なお，提供されなかった40例の内訳は医学的理由が28例，未登録時期（～1999年9月）での提供が4例，意思表示カード上での未承諾が4例，適合者不在が3例，クロスマッチ陽性が1例であった。また，同期間中に2例の心停止下での膵臓移植（SPK）が行われた。さらに，生体ドナーからの膵臓移植も25例行われた。移植症例数の年次推移が示されている（**図4**）。

3. ドナー・レシピエント関連因子（脳死下・心停止下）

脳死・心停止下で行われた膵臓移植症例121例のドナーならびにレシピエントの関連因子について，解析した。

1）ドナー年齢・性差

男女比は63：58と男性がやや多く，年齢は40歳代が33名と最も多く，50歳代の31名に続き，30歳代，20歳代，10歳代がそれぞれ，26名，15名，10名であり，70歳代も1名みられた（**図5**）。本邦では40歳以上の高齢ドナーが75名と62.0%を占めていた。

2）ドナーの死亡原因

死因は脳血管障害が69名（57.0%）と最も多く，なんらかの動脈硬化性変化が否定できない。他に，低酸素血症が25名，外傷が22名，心筋梗塞が2名，その他が3名であった（**図6**）。

3）レシピエント年齢・性差

男女比は47：74で女性に多く，年齢は30・40代がそれぞれ49名（40.5%），48名（39.7%）と最も多く，ついで，50代が17名，20代が5名，60代が2名であった（**図7**）。

4）透析歴と糖尿病歴

透析歴（SPK）は平均7.0（0～19）年で，糖尿病歴は平均26.1（9～45）年であった（**図8，9**）。

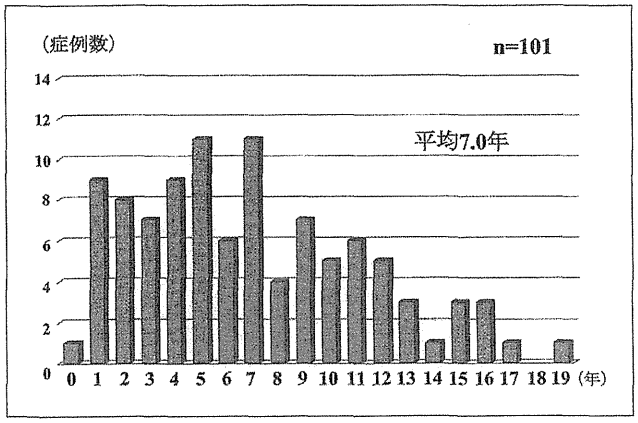

図8　レシピエントの透析歴

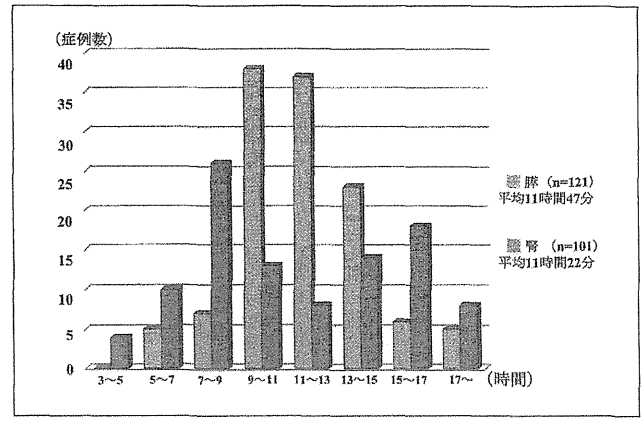

図11　移植膵・腎の総冷阻血時間

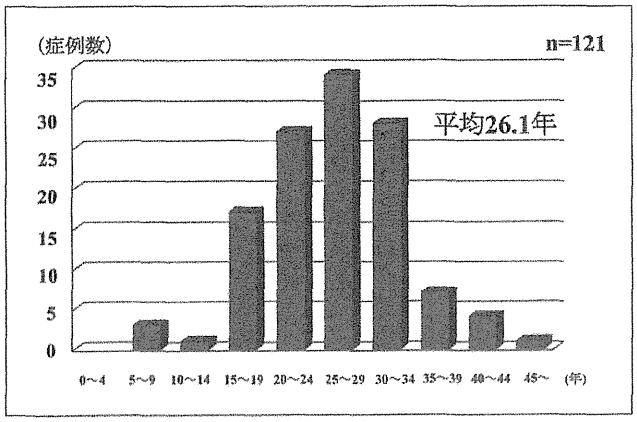

図9　レシピエントの糖尿病歴

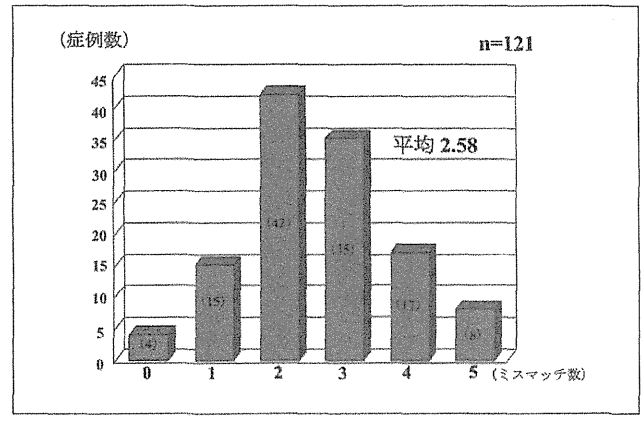

図12 HLAミスマッチ数

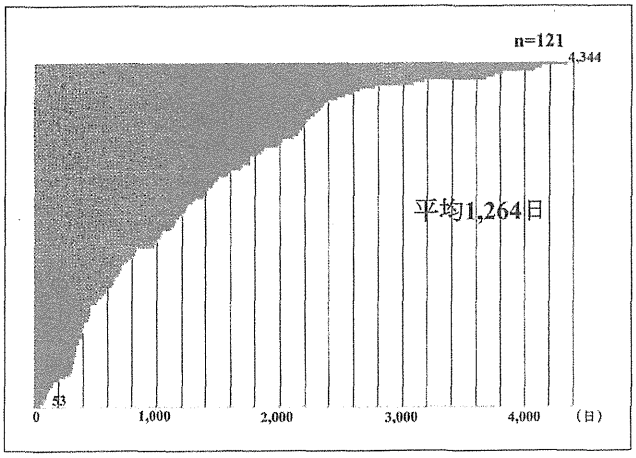

図10　レシピエントの待機期間

5）待機期間

移植を受けたレシピエントの平均待機期間は1,264（53～4,344）日と年々増加しており，約3年半であった（図10）。

6）総冷阻血時間（TCIT）

膵のTCITは平均11時間47分であった。腎のTCITは平均11時間22分であり，2峰性を示した。両臓器ともに十分許容範囲であった。これはSPKの場合，腎移植を先行させる場合と膵移植を先行させる場合があることによると考えられた（図11）。なお，臓器搬送に要する時間は平均3時間59分であった。

7）ミスマッチ

HLAミスマッチ数は平均2.58であった（図12）。

8）移植術式（膵液ドレナージ）

脳死下（DD）でのSPK 99例では当初は安全性，尿中アミラーゼモニターを考慮して，膀胱ドレナージ（bladder drainage：BD）が行われたが，最近ではもっぱら腸管ドレナージ（enteric drainage：ED）が82例（82.8%）と大半を占めている。なお，BD 17例のうち，尿路感染症や逆行性グラフト膵炎などの理由で3例はenteric conversion（EC）となった。また，心停止下（NHBD）での場合やPAKやPTA症例ではグラフ

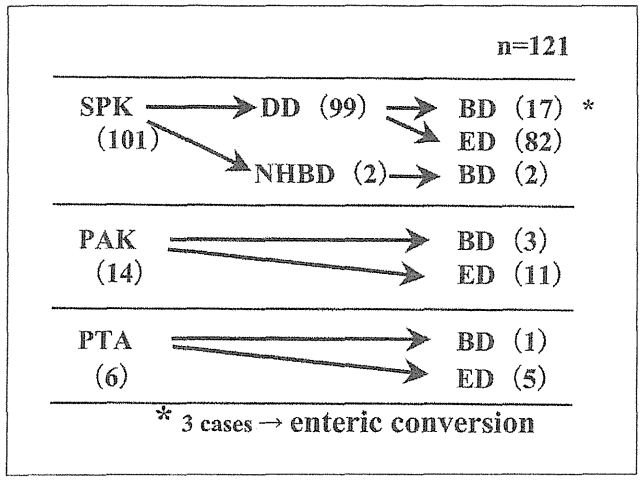

図13　手術術式

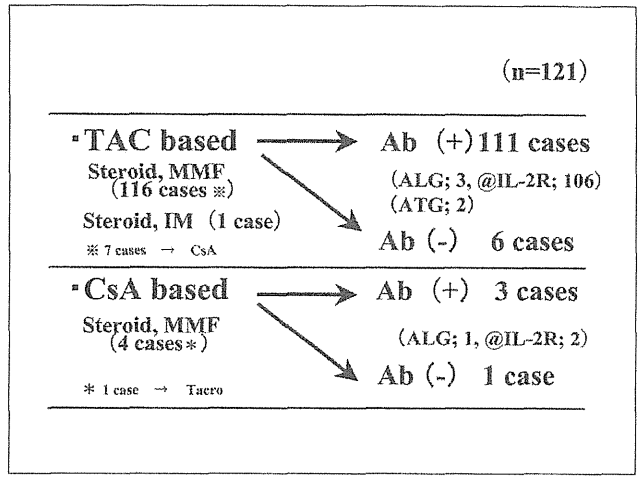

図14　免疫抑制法

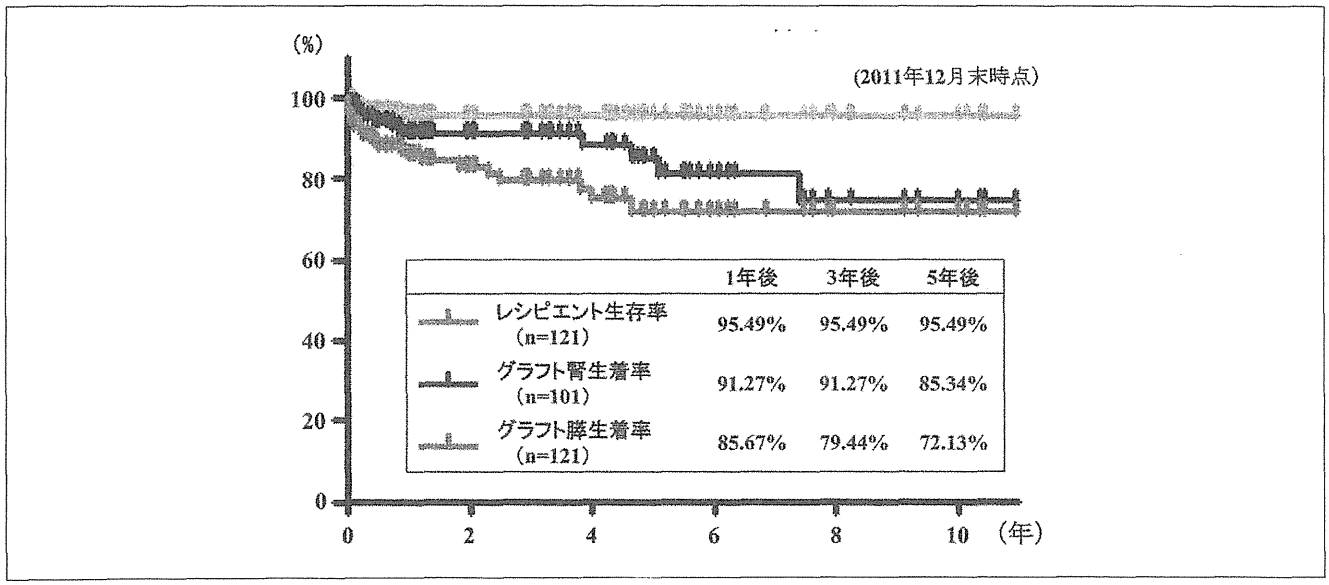

図15　膵臓移植後のレシピエント生存率とグラフト生着率

トの膵液をモニターする必要性から，22例中6例（27.3%）にBDが用いられた（図13）。

9）免疫抑制法

Tacrolimus（TAC）をベースとして，ステロイド，mycophenolate mofetil（MMF），抗IL-2R抗体（basiliximab）の4剤併用療法が87.6%と最も多く用いられている。うち，7例が毒性のためTACからcyclosporin（CsA）へ変更となっている。一方，CsAをベースとして，4剤併用療法が3例に行われ，うち1例はTACへ変更となった（図14）。

10）移植成績

本邦の膵臓移植はマージナルドナー（marginal donor）が多いことが特徴である。Kapurらによるmarginal donorの定義［①45歳以上，②不安定な血行動態（高用量のカテコラミンの使用），③心停止下での提供］[7]によると，生体を除く121例中88例（72.7%）がmarginal caseであった。

脳死・心停止下での膵臓移植症例121例のうち6例（すべてSPK）が死亡した。1例は移植後11カ月原因不明の心肺停止があり，その後蘇生後脳症にて，3例は敗血症にて，1例は心原性にて，さらに1例はGVHDにて死亡した。

移植膵の生着については，移植後急性期に6例が血栓症にて急性期に移植膵が摘出され，1例は門脈血栓症が引き金となり6カ月後にインスリン再導入となった。移植後2年目に1例がイレウスからグラフト十二

指腸穿孔により摘出された。他に，1例が急性拒絶（POD；45日）で摘出，8例が慢性拒絶反応などの理由で，それぞれ移植後4カ月～4年7カ月でインスリン再導入となった。さらに，前記死亡例を含めると，計23例が移植膵の機能喪失となった。1年，3年，5年生着率はそれぞれ85.7%，79.4%，72.1%であった（**図15**）。

一方，SPK症例の移植腎の生着については，101症例中，1例がPNF（primary non-function），1例は急性拒絶（POD；51日）で摘出，他に6例がそれぞれ，10カ月から7年5カ月で透析再導入となった。前記死亡例を含めると，計13例が機能喪失となった。その結果，1年，3年，5年腎生着率はそれぞれ91.3%，91.3%，85.3%であった。

4. 生体膵臓移植について

生体ドナーから行われた膵臓移植症例25例における上記関連因子について解析した。

ドナーは20例が両親のどちらか（母；13例，父；7例）からであり，3例が兄弟から，2例が姉妹から提供された。多くは父母からの提供であるため，ドナーの平均年齢は56.6(28～72)歳と高齢であった。一方，レシピエントは男性10例，女性15例で，平均年齢は36.0（25～50）歳であった。カテゴリー別では，SPKが21例と最も多く，ついでPTAの3例，PAKが1例であった。術式別では，脳死・心停止下とは異なり，大半がBD（20例）でありEDは5例であった。免疫抑制療法は脳死・心停止下の場合と同様であった。

移植成績：1例が移植1年後，脳梗塞にて死亡した。これはPAKの1例で，移植膵は機能するも，膵移植後2カ月で移植腎の機能が増悪して透析再導入となった症例であった。SPKの4例で急性期に機能が喪失した。1例はPNFで，3例は血栓症にて移植膵を摘出しインスリン再導入となった。また，慢性期に3例がインスリン再導入となった。なお，PAKやPTAの場合には，膵臓移植前に移植腎の機能を慎重に評価しなければならない。

IV. まとめと今後の展望

以上，2011年末までの膵臓移植症例146例について，その解析結果を報告した。本邦ではmarginal caseが多く，ドナーの条件は良くはないが，移植成績は欧米のそれと比較して，決して遜色のない結果である。なお，2010年7月17日の臓器移植法改正後，2011年12月末までに73例の臓器提供（うち，膵の提供は57例）があり，このうち，多くが家族の承諾であった。今後はさらに，臓器提供の増加が見込まれ，それに向けたスムーズな対応とその体制が必要となる。

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膵臓移植症例登録委員会事務局
伊藤壽記

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Prevention of High-Mobility Group Box 1-Mediated Early Loss of Transplanted Mouse Islets in the Liver by Antithrombin III

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Background. The low efficiency of pancreatic islet transplantation mainly because of the early loss of transplanted islets hampers its clinical application. Previously, we have shown in mice that the early loss of transplanted islets in the liver is caused by innate immune rejection in concert with dendritic cells, natural killer T cells, and neutrophils to produce interferon (IFN)-γ, which is triggered by high-mobility group box 1 (HMGB1) released from transplanted islets. We herein determined whether the HMGB1-mediated early loss of transplanted mouse islets is prevented by antithrombin (ATIII).

Methods. The effect of ATIII on in vitro and in vivo HMGB1-stimulated IFN-γ production of hepatic mononuclear cells was examined. Then, the effect of ATIII on amelioration of hyperglycemia in streptozotocin-induced diabetic mice receiving 200 syngeneic islets from a single donor was determined.

Results. In vitro and in vivo IFN-γ production of mononuclear cells in the liver of mice in response to HMGB1 was suppressed by ATIII. Hyperglycemia of streptozotocin-induced diabetic mice receiving 200 syngeneic islets into the liver from a single donor was ameliorated with down-regulation of IFN-γ production of natural killer T cells and neutrophils in the liver when ATIII but not vehicle was administered once at the time of islet transplantation. The favorable effect of ATIII was similarly achieved in mice receiving islet allografts when rejection was prevented with anti-CD4 antibody treatment.

Conclusions. These findings demonstrate that ATIII prevents HMGB1-mediated early loss of transplanted islets caused by innate immune rejection, suggesting a potential application of ATIII to improve efficiency of clinical islet transplantation.

Keywords: Islet transplantation, Early loss of transplanted islets, Antithrombin III.

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Pancreatic islet transplantation is an attractive therapy for insulin-dependent diabetes mellitus (*1, 2*). Currently, however, islet transplantation has limited success in achieving insulin independence of a diabetic patient after transplantation of islets from a single donor due to islet graft loss soon after transplantation, and therefore, sequential transplantations of islets with the use of two to three donors are required for the treatment of a single recipient (*1*). Thus, the low efficiency has been a major obstacle facing islet transplantation and hampers its clinical application.

Previously, we have shown that Gr-1$^+$CD11b$^+$ cells (neutrophils) become accumulated in the liver of mice receiving islets soon after transplantation and that their interferon (IFN)-γ production, which is dependent on natural killer T (NKT) cells, is an essential component for early loss of islet grafts in association with engraftments (*3*). Subsequently, we have found that a large amount of high-mobility group box 1 (HMGB1) is released from islets soon after their transplantation and that this triggers innate immune rejection in concert with the activation of dendritic cells, NKT cells, and neutrophils to produce IFN-γ and finally leads to the early loss of transplanted islets (*4*). Thus, these previous findings suggest that the HMGB1-mediated pathways as to islet graft loss might be targets of intervention to improve efficiency of islet transplantation.

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The authors declare no conflicts of interest.

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D.K., N.M., and Y.Y. participated in research design; T.M., H.N., T.I., T.O., and S.K. performed the experiments; and D.K., S.K., and Y.Y. analyzed data and wrote the manuscript.

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There are accumulating evidences that the crosstalk between coagulation and inflammation exist, whereby inflammation not only leads to activation of coagulation but also coagulation affects inflammatory activity (*5–7*). Antithrombin (ATIII) is a 58-kD glycoprotein that functions as a potent natural anticoagulant and synthesized in the liver and circulated in the plasma (*8*). ATIII is a serine protease inhibitor that inactivates many enzymes in the coagulation cascade, although thrombin and factor Xa are its primary targets (*8*). In addition to potent anticoagulant activity, ATIII possesses antiinflammatory properties, many of which are mediated by its actions in the coagulation cascade (*9*).

On the basis of these findings, we hypothesize that ATIII may have a beneficial effect on inhibiting inflammatory responses in the liver of mice receiving islets soon after transplantation leading to protection of transplanted islets from innate immune rejection because there is an evidence that coagulation is involved in the early loss of transplanted islets in which tissue factor (TF), an initiator of coagulation, has been reported to become expressed in islets after transplantation (*10*) and because other anticoagulants including activated protein C (APC) and thrombomodulin (TM) have been shown to have protective effects of transplanted islets (*11, 12*).

Here, in this study, we demonstrate that ATIII produces prevention of early loss of transplanted mouse islets and that this inhibitory effect by ATIII is achieved through down-regulation of HMGB1-stimulated IFN-γ production of NKT cells and neutrophils in the liver receiving islets.

RESULTS

Inhibitory Effect of ATIII on IFN-γ Production of Hepatic Mononuclear Cells in Response to HMGB1

As an initial experiment, we determined whether ATIII has any inhibitory effect on in vitro HMGB1-stimulated IFN-γ production of hepatic mononuclear cells (MNC) of mice. For those purposes, hepatic MNC were isolated and cultured for 48 hr in the presence of HMGB1 with or without ATIII, and the amount of IFN-γ in the culture medium was measured. Consistent with the previous report (*4*), HMGB1 (20 μg/mL) induced the augmented production of IFN-γ from hepatic MNC, which was found to be suppressed by ATIII in a dose-dependent manner in which the maximum inhibition was achieved with 1 U/mL ATIII (Fig. 1A).

To determine whether the inhibitory effect of ATIII on IFN-γ production of hepatic MNC is also the case in vivo, ATIII was administered intravenously (IV) into mice 5 min before the administration of HMGB1 (100 μg/injection/mouse), and hepatic MNC were isolated at 2 hr after the HMGB1 injection and examined by fluorescence-activated cell sorting. As originally reported (*4*), a marked increase in IFN-γ production of hepatic α-GalCer/CD1d-tetramer$^+$ CD3$^+$NKT cells and Gr-1$^+$CD11b$^+$ cells (neutrophils) in comparison with control untreated mice was seen (Fig. 1B, first and second rows). In contrast, the IFN-γ production of NKT cells and Gr-1$^+$CD11b$^+$ cells in the liver of mice treated with ATIII (1 or 10 U/injection/mouse) in conjunction with

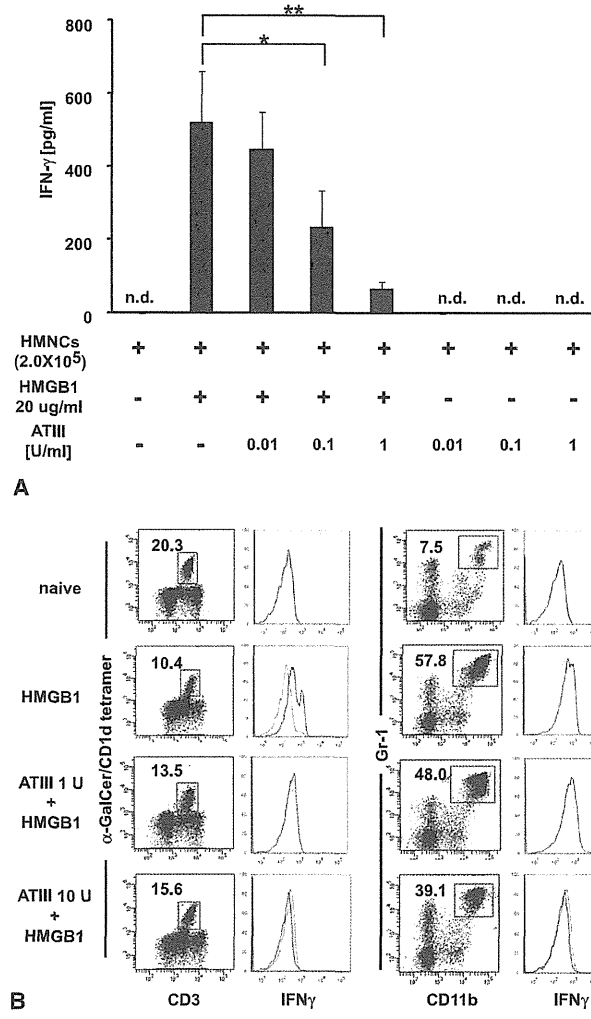

FIGURE 1. Inhibitory effects of antithrombin III (ATIII) on high-mobility group box 1 (HMGB1)-stimulated interferon (INF)-γ production of mouse hepatic mononuclear cells (MNC). A, Hepatic MNCs (2.0×10^5) of mice were isolated and cultured in vitro for 48 hr in the presence of HMGB1 (20 μg/mL) with or without ATIII. Subsequently, the media was collected, and IFN-γ levels were determined by enzyme-linked immunosorbent assay (ELISA). Representative data from two experiments are shown. *, $P<0.05$; **, $P<0.01$; n.d., not detected. B, Fluorescence-activated cell sorting (FACS) profiles of liver MNCs from naïve mice (*first row*) and from mice at 2 hr after the intravenous (IV) injection of HMGB1 (100 μg/injection/mouse) (*second row*) in conjunction with ATIII at the dosage of 1 (*third row*) or 10 U (*fourth row*), which was administered 5 min before the injection of HMGB1. Natural killer T (NKT) (α-GalCer/CD1d-tetramer$^+$ CD3$^+$) cells and Gr-1$^+$ CD11b$^+$ cells were gated and analyzed for their IFN-γ production (*second and fourth columns*). The numbers in each box represent the percentages of cells in the corresponding area. Representative data from two to three experiments are shown.

HMGB1was down-regulated (Fig. 1B, third and fourth rows) although the accumulation of Gr-1$^+$CD11b$^+$ cells was similarly seen irrespective of the treatment with ATIII.

Early Loss of Transplanted Syngeneic Islets in the Liver of Mice Is Prevented by ATIII

The afore-mentioned in vitro and in vivo findings suggest that ATIII might have a beneficial effect on prevention of early loss of transplanted islets. To address this question, we determined whether hyperglycemia of streptozotocin (STZ)-induced diabetic mice is ameliorated after transplantation of a marginal mass of syngeneic islets, namely 200 islets from a single donor when recipient mice were treated with ATIII. When diabetic mice received 200 islets and were treated with saline as a control, all the recipient mice remained hyperglycemic after the transplantation (Fig. 2A, upper panel). When diabetic mice received the same number of islets and were treated with ATIII (1 or 10 U) once at the time of islet transplantation, hyperglycemia of recipient mice was ameliorated, and all mice remained normoglycemic for more than 60 days after the transplantation (Fig. 2A, middle and lower panels), indicating that ATIII has no deleterious effect on survival of recipient mice and that importantly, ATIII improves efficiency of islet transplantation. A histological 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, at 60 days after transplantation (histology not shown), similar to the previous findings (*3*).

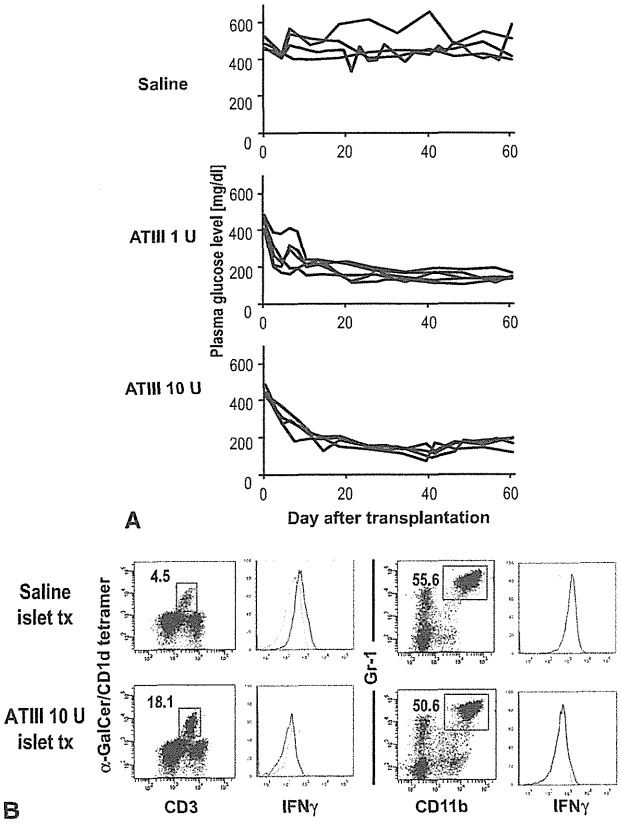

FIGURE 2. The beneficial effect of antithrombin III (ATIII) on prevention of early loss of transplanted islets. A, Nonfasting plasma glucose levels of streptozotocin (STZ)-induced diabetic mice (C57BL/6) received 200 syngeneic islets and treated with saline (*upper row*), 1 (*second row*) or 10 U (*third row*) ATIII once at the time of islet transplantation. Individual *lines* represent glucose levels of each animal. B, Mononuclear cells in the liver of mice receiving 200 syngeneic islets and treated with saline (*upper row*) or 10 U ATIII were isolated at 6 hr after transplantation and examined by flow cytometry. Natural killer T (NKT) (α-GalCer/CD1d-tetramer$^+$ CD3$^+$) cells and Gr-1$^+$ CD11b$^+$ cells were analyzed for their interferon (IFN)-γ production (*second and fourth columns*). Representative data from two experiments are shown.

IFN-γ Production of NKT Cells and Gr-1$^+$CD11b$^+$ Cells in the Liver of Mice Receiving Syngeneic Islets is Down-Regulated by ATIII

To determine whether IFN-γ production of NKT cells and neutrophils in the liver of mice after islet transplantation into the liver was actually inhibited by ATIII, fluorescence-activated cell sorting analysis of MNC in the liver of mice receiving islets was performed. As a result, the IFN-γ production of both NKT cells and neutrophils in the liver of mice receiving islets and treated with ATIII (10 U) was found to be down-regulated at 6 hr after transplantation (Fig. 2B, lower panel), whereas that of mice treated with saline was up-regulated, consistent with the previous report (*3*). Interestingly, the number of accumulated Gr-1$^+$CD11b$^+$ cells in the liver of mice receiving islets was not altered irrespective of the treatment with ATIII (Fig. 2B).

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

To validate the extent that ATIII improves the efficiency of islet transplantation, intraperitoneal glucose tolerance test (IPGTT) was performed in appropriate groups of recipient mice. Previously, we have shown that 400 islets isolated from two donor mouse pancreases are required to ameliorate hyperglycemia of STZ-induced diabetic mice without any treatment after their transplantation into the liver (*3, 13*). Thus, normoglycemic mice after transplantation of 400 islets were added to the experimental groups of IPGTT to compare the glucose tolerance of normoglycemic mice receiving 200 islets and treated with ATIII. As shown previously (*13*), the plasma glucose levels of diabetic mice receiving 200 islets at 0, 30, and 120 min after the intraperitoneal (IP) injection of glucose (1 g/kg) were similar to those of diabetic hyperglycemic mice without islet transplantation (Fig. 3). In contrast, the plasma glucose levels of the normoglycemic mice received 200 islets and treated with ATIII (1 U) were significantly lower not only than those of mice receiving 200 islets without the treatment (Fig. 3; $P<0.05$) but also than those of mice receiving 400 islets without the treatment (Fig. 3; $P<0.05$).

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

It is important to determine whether the beneficial effect of ATIII obtained in syngeneic islet transplantation is applicable to allogeneic islet transplantation under immunosuppression as it is the case in clinical islet transplantation. 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

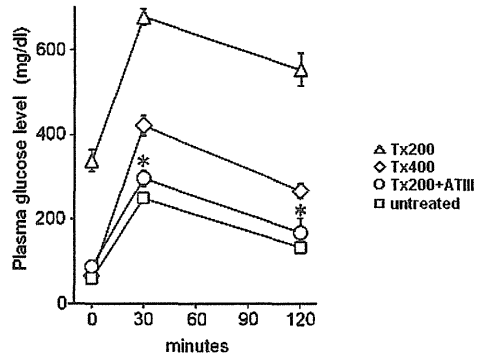

FIGURE 3. Intraperitoneal glucose tolerance test (IPGTT) of diabetic mice receiving syngeneic islets on day 60 after transplantation. Recipient mice were fasted for 8 hr before the examination, and plasma glucose levels were determined at 0, 30, and 120 min after the glucose injection (intraperitoneally [IP] 1 mg/kg). Experimental groups included diabetic mice receiving 200 (△, n=5) or 400 (◇, n=4) islets without treatment and those received 200 islets and treated with AT III (○, n=5). Naive untreated mice were served as control (□, n=5). 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 ATIII were significantly different (*P<0.05).

after transplantation (Fig. 4, left, first row). In contrast, when 200 BALB/c islets were grafted into the liver of STZ-diabetic C57BL/6 mice treated with ATIII (1 U) once at the time of islet transplantation, hyperglycemia of recipient mice was ameliorated by 3 days, and the mice became hyperglycemic again with 13.4±10.9 days (mean±SD, n=8) after transplantation. Histologically, islet grafts infiltrated with MNCs were seen in the liver of the recipient mice at the time of rejection (Fig. 4, right column). When diabetic mice received 200 allogenic islets and were treated with anti-CD4 antibody, the recipient mice remained hyperglycemic by 60 days after transplantation (left, fourth row), similar to the findings reported previously. Histologically, islets with degranulated β cells were seen in the liver of the recipient mice at 60 days after the transplantation as reported previously (*13*) (histology not shown), indicating that alloimmune rejection was prevented by anti-CD4 treatment, but the islet graft mass was not enough to ameliorate hyperglycemia after transplantation. When diabetic mice received 200 allogenic islets and were treated with ATIII 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. 4, left, third row). Histologically, intact islets with well-granulated β cells were seen in the liver of the recipient mice at 60 days after the transplantation (Fig. 4, right column). These findings indicate that ATIII promotes early engraftment, whereas anti-CD4 antibody prevents the alloimmune rejection of transplanted allogeneic islets.

DISCUSSION

This study clearly demonstrates that ATIII has a beneficial effect on preventing early loss of transplanted islets in the liver of mice by inhibiting HMGB1-mediated IFN-γ production of NKT cells and neutrophils, which is an essential component of early islet graft loss, thus facilitating to improve efficiency of islet transplantation.

Concerned with the link between coagulation and inflammation in islet transplantation, Moberg et al. (*10*) reported that TF, an initiator of the extrinsic pathway of blood coagulation, is synthesized in islets and secreted and that thrombin-antithrombin complex is elevated in recipients after islet transplantation. The findings indicate that the coagulation cascade is activated in the portal vein of the recipient liver, the site of islet transplantation, and may lead to inflammatory response which causes innate immune rejection of transplanted islets. As to inflammation after islet transplantation in relationship to islet graft rejection, we have previously shown that Gr-1$^+$CD11b$^+$ cells (neutrophils) accumulate in the liver of mice soon after islet transplantation into the liver and their IFN-γ production, which is dependent on NKT cells, plays an essential role in graft rejection within 24 hr after transplantation (*3*, *13*). Subsequently, we found that pancreatic islets contain abundant HMGB1 that is released from transplanted islets in the liver soon after transplantation and triggers NKT cell-dependent IFN-γ production of Gr-1$^+$CD11b$^+$ cells (*4*). Regarding a role of HMGB1 in coagulation, Ito et al. (*14*) demonstrate that the IV injection of HMGB1 promotes development of microvascular

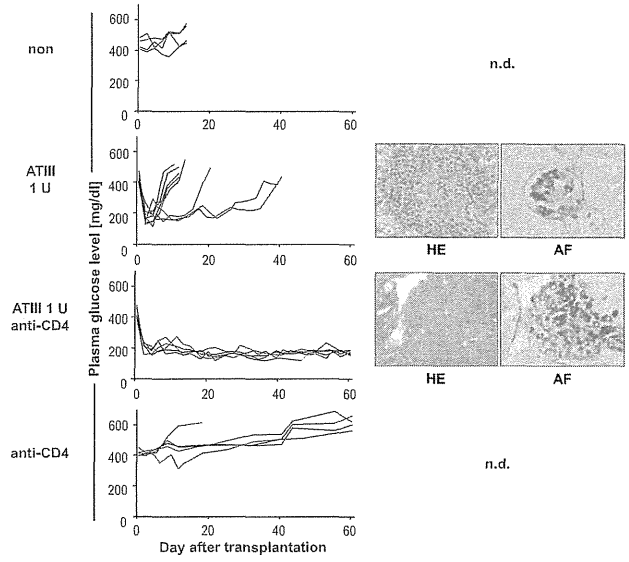

FIGURE 4. The beneficial effect of antithrombin III (ATIII) on functional outcome of islet allografts. Two hundred BALB/c islets were grafted into the liver of streptozotocin (STZ)-induced diabetic C57BL/6 mice. ATIII (1 U/injection) was administered intravenously (IV) once at the time of islet transplantation. Nondepleting anti-CD4 antibody (200 μg/injection/mouse) was administered intraperitoneally (IP) into appropriate groups of recipient mice once at the time of islet transplantation. Individual *lines* represent the nonfasting plasma glucose levels of each animal. In the right column, photomicrographs of islet allografts at 7 (*second row*) and 60 days (*third row*) after transplantation were shown. The sections were stained with hematoxylin-eosin (HE) and aldehyde-fuchsin (AF) (magnification: ×400). n.d., not done.

thrombosis in rat. These findings indicate that HMGB1 may bridge between coagulation and inflammation. Our initial experiments showed that the IFN-γ production of NKT cells and Gr-1$^+$CD11b$^+$ cells in the liver of mice treated with the IV injection of HMGB1 is suppressed by ATIII. Then, we found that the IFN-γ production of NKT cells and Gr-1$^+$ CD11b$^+$ cells in the liver of mice receiving islets is also suppressed by ATIII, leading to prevention of early loss of transplanted islets.

Regarding the effects of ATIII on immune cells, it has been reported that the antiinflammatory effects of ATIII is mediated by direct binding to neutrophil, lymphocyte, and monocyte cell surface receptors such as syndecan-4 (*15, 16*) and reduces production of interleukin-6 and TF (*17*) and inhibits activation of the transcription factor nuclear factor-κB (*18*). ATIII is also known to inhibit CD40L expression of platelets, essential for their activation (*19*). Although the exact mechanisms involved in the inhibitory effect of ATIII on HMGB1-mediated IFN-γ production of NKT cells and Gr-1$^+$CD11b$^+$ cells remains unclear, it is possible that the effect of ATIII is mediated through inhibition of HMGB1 release from transplanted islets since there is a report in which ATIII down-regulates LPS-stimulated HMGB1 release from macrophages (*20*).

Contreras et al. (*11*) reported that exogenous administration of recombinant murine APC significantly reduced loss of functional islet mass after intraportal transplantation into diabetic mice. Cui et al. (*12*) recently showed that TM affords improved efficiency of intraportal islet transplantation in mice. These studies revealed that the treatment with APC or TM ameliorates hyperglycemia of STZ-induced diabetic mice receiving a marginal mass of islets into the liver. As to inflammatory response, both studies showed that APC and TM inhibit the expression of proinflammatory cytokine messages including tumor necrosis factor-α and interleukin-1 in the liver of mice receiving islets. However, what produces inhibitory effect of APC or TM on proinflammatory cytokine production remains unclear. In this study, we showed that IFN-γ production of NKT cells and Gr-1$^+$CD11b$^+$ cells triggered by HMGB1 released from transplanted islets, which is an essential component of early loss of transplanted islets (*3*), is suppressed by ATIII. Thus, it is interesting to learn whether the similar suppressive effect of IFN-γ production is achieved by APC or TM. Notably, it has been reported that TM denatures HMGB1 to abolish its stimulatory effect (*21, 22*).

To validate the efficiency of islet transplantation, IPGTT was performed in recipient mice at 60 days after transplantation. It was found that glucose tolerance of diabetic mice receiving 200 islets from a single donor and treated with ATIII at the time of islet transplantation is superior to that of mice receiving 400 islets from two donors without the ATIII treatment, indicating that transplantation efficiency increased more than twofolds by the treatment with ATIII.

Regarding the effect of ATIII on engraftments of islet allografts in the liver of mice, the similar beneficial effect of ATIII to that obtained in syngeneic islet transplantation was achieved when alloimmune rejection is prevented by the use of anti-CD4 antibody as an immunosuppressive agent. The finding is important from the point of clinical application of ATIII, and however, it must be determined whether the beneficial effect of ATIII is also achieved in allogeneic islet transplantation under the immunosuppressive protocol which is currently used in clinical islet transplantation because the effect of ATIII may differ under different immunosuppressive agents.

Taken collectively, this study demonstrates that ATIII produces beneficial effects on prevention of early loss of transplanted islets. Because ATIII has already been used in clinics for the treatment of ATIII deficiency (*23*), the safety issue of its clinical use has been cleared. Thus, ATIII may improve efficiency of clinical islet transplantation when the beneficial effect of ATIII revealed by this study in mice holds true in humans.

MATERIALS AND METHODS

Animals

Male BALB/c (H-2d) and C57BL/6 (H-2b) mice were purchased from Charles River Japan (Kanagawa, Japan). 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 recipient mice remained hyperglycemic at the time of islet transplantation. All experiments were performed in accordance with the Institutional Animal Care and Use Committee.

Islet Isolation and Transplantation

Islets were isolated by the static digestion method using collagenase (*24*) and then separated by centrifugation on Ficoll-Conray gradients (*25*). Islets of 150 to 250 μm in diameter were hand-selected using a Pasteur pipette with the aid of a dissecting microscope. 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 (*26*) at 3 days after the induction of diabetes with STZ injection.

Monitoring Plasma Glucose

The nonfasting plasma glucose levels 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 level readings below 200 mg/dL.

Administration of HMGB1, ATIII, and Anti-CD4 Antibody

Bovine HMGB1 was purchased from Shino-test Co., Sagamihara, Japan and administered IV to untreated mice.

ATIII was kindly supplied by Mitsubishi Tanabe Pharma Co. (Osaka, Japan). ATIII or vehicle (saline) was administered IV into appropriate groups of mice 5 min before HMGB1 injection or islet transplantation.

Nondepleting anti-CD4 mAb (200 μg/injection/mouse, YTS177; R&D, Minneapolis, MN) was administered IP once at the time of islet transplantation into appropriate groups of mice.

Intraperitoneal Glucose Tolerance Test

IPGTT was performed in recipient mice at 60 days after islet transplantation. The mice were fasted for 8 hr before 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 were examined morphologically after transplantation in appropriate groups of mice. The livers were fixed with Bouin's solution, processed, and embedded in paraffin. The sections were prepared for light microscopy and stained with hematoxylin-eosin and aldehyde-fuchsin.

Flow Cytometry Analysis

Hepatic MNCs were prepared as described previously (*27*). The following monoclonal antibodies were used: anti-mouse FcR_II/III (2.4G2), fluorescein isothiocyanate-conjugated anti-CD3ε (145-2C11), fluorescein isothiocyanate- or PE-conjugated anti-CD11b (M1/70), allophycocyanin-conjugated anti-IFN-γ (XMG1.2), PerCP-conjugated anti-Gr-1 (Rb6-8c5), and isotype control (clone R3-34, Rat IgG1) and were purchased from BD Biosciences (San Jose, CA). PE-α-galactosylceramide (α-GalCer)–CD1d tetramers were prepared as previously described (*28*). Cells were analyzed on a flow cytometer (FACSCanto; Becton Dickinson). Ten thousand viable cells were analyzed.

Statistical Analysis

The statistical significance with respect to the rate of euglycemia in streptozotocin-induced diabetic mice after islet transplantation and that of 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.

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