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トピックス

IV. 最近の話題
3. 膵島移植と再生医療

佐々真理子　岩永　康裕　山田祐一郎

要　旨

血糖が不安定なインスリン依存状態の患者に，内因性のインスリンを分泌する膵β細胞を補充する方法の一つとして，ドナーの膵臓から分離した膵島を用いる膵島移植がある．わが国でも2004年に開始され，生体内にある一定量の膵β細胞が存在することは，無自覚低血糖や重症低血糖の軽減などに効果が得られることが報告されている．今後，iPS細胞などから分化させた膵β細胞やブタの膵島を用いた異種移植ができれば，糖尿病患者にとって福音になるであろう．

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Key words：膵島移植，インスリン依存状態，iPS細胞

はじめに

インスリン依存状態の糖尿病では，内因性インスリン分泌が枯渇している．したがって，刻々と変動する血糖に対しては，インスリン頻回注射又はCSII(continuous subcutaneous insulin injection)療法に血糖自己測定を併用した，強化インスリン療法を用いて治療している．

しかし，外からのインスリン注射をいかに最適化しても，高血糖と低血糖を繰り返す症例がある．低血糖発作は時に生命に危険を及ぼすものとなり，インスリン依存状態の糖尿病患者における血糖管理のlimiting factorとなっている．また長期にわたる血糖コントロール不良は，糖尿病合併症の発症・進展を引き起こす．このように血糖が不安定なインスリン依存状態の患者に，内因性のインスリンを分泌する膵β細胞を補充する方法の一つとして，膵島移植がある．

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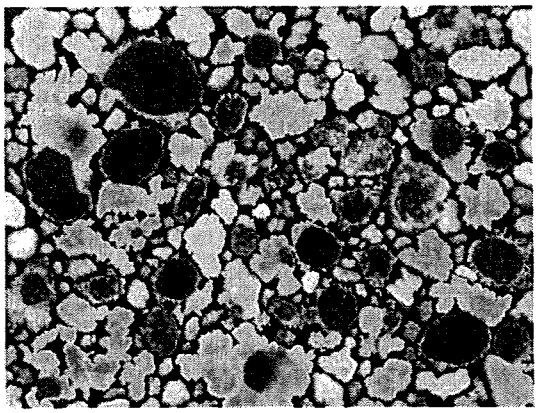
1. 膵島移植とは

膵島移植とは，提供された膵臓から特殊な技術を用いて膵島を分離し（図1），インスリン依存状態の糖尿病患者の門脈へ，点滴の要領で移植を行う細胞移植療法である．移植された膵島は，門脈の末梢又は類洞に生着する．生着した膵島は，血糖値に応じてインスリンを分泌し，血糖値のコントロールを行う．

膵島移植の最大の特徴は，移植にあたって全身麻酔や手術を必要とせず，移植に要する時間も10～20分と短く，侵襲が非常に小さいことである．また，臓器移植ではリンパ節なども一緒に移植されるが，膵島移植では単離の際に，膵



2005年1月生体膵島移植症例．ジチゾンで染色した単離膵島．赤く染色されているのが膵β細胞である．

図1．分離された膵島

島以外はできる限り除去されるため，感染源となりにくい．さらに，移植膵島が機能しない場合は自然に吸収され，再移植も容易である．しかし，膵島分離技術が難しいことや，拒絶反応を知るすべがないことから，最近まで普及しなかった．

2．エドモントンプロトコールと世界の膵島移植の現況

2000年にAlberta大学のShapiroらは，膵島移植の方法を確立し，7人の1型糖尿病患者に膵島移植を行い，すべての患者でインスリンから離脱することができたことを報告した[1]．この方法は，エドモントンプロトコールと呼ばれ，現在の臨床膵島移植の標準となっている．その内容とは，①膵島分離の方法を最適化し，②膵島分離後直ちに移植する，③免疫抑制薬として，耐糖能を悪化させる可能性のあるsteroidを使わず，sirolimusと少量のtacrolimusを使用する，④インスリン離脱するまで膵島移植を続ける（通常2～4回），というものである．

この方法は世界中に普及し，現在までに60以上の施設で約700人の患者が膵島移植を施行されている．最近のエドモントンの報告では，2～3人のドナーから1人の患者に移植することによって，一旦インスリン注射が不要となっても，それを持続できる症例は年々減少し，移植5年後のインスリン離脱率は7.5%であった．しかしながら膵島の生着率（C-peptideの陽性で判定）は移植5年後でも80%以上と良好である[2]（図2）．膵島が生着している患者では，血糖値の安定化が得られ，重症低血糖が消失し，HbA_{1c}も6.5～7%前後で推移している[2,3]．なお，Clinical Islet Transplantation Consortiumにより，臨床膵島移植のphase III trialが計画されており，一般医療に向けての準備が進められている．

3．本邦での膵島移植

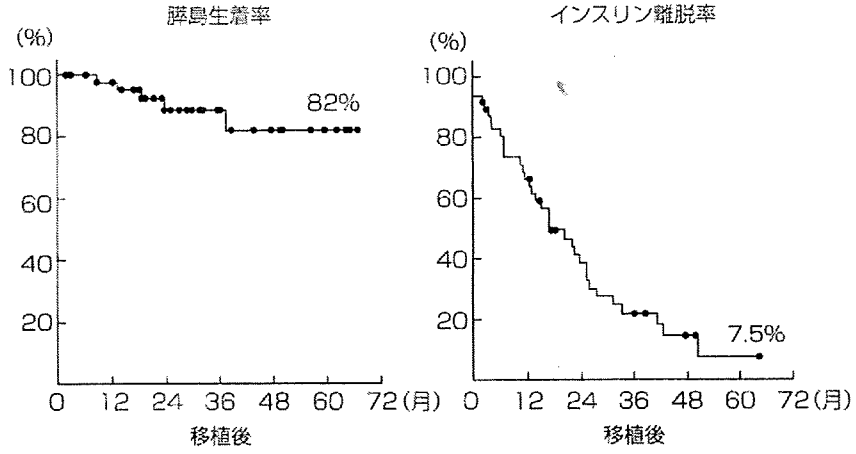
海外では，脳死ドナーから膵臓の提供を受けることが一般的であるが，本邦では臓器移植法により，脳死ドナーから提供を受けることはできないため，阻血のために膵島機能がより低下する可能性のある心停止ドナーからの提供が主体となっている．

1）心停止ドナーからの膵島移植

（1）レシピエントの適応

本邦では，膵・膵島移植研究会・膵島移植班[4]によって膵島移植の適応基準ならびに禁忌が定められている（表1）．膵島移植の適応は，内因性インスリン分泌が枯渇（高感度測定法によっても血中C-peptideが0.1 ng/ml以下で，グルカゴン負荷試験でも反応が認められない）し，糖尿病専門医の治療努力によっても血糖コントロールが困難な，75歳以下の患者であり，本人，家族，主治医の同意が得られていることである．無自覚低血糖や，第三者の介助が必要な重症低血糖の有無，低血糖の頻度などが，血糖コントロールの困難性の判定に重要である．また重度の心疾患，肝疾患，アルコール中毒，感染症，5年以内の悪性腫瘍の既往，未処置の網膜症，肥満などの禁忌事項のない患者となっている．

なお，腎不全のレシピエント候補は当初エド



(文献2より)

図2. 膵島移植の成績(エドモントン)

表1. 心停止ドナー膵島移植レシピエント条件

[適応]
1. 内因性インスリンが著しく低下し，インスリン治療を必要とする
2. 糖尿病専門医の治療努力によっても，血糖コントロールが困難
3. 原則として75歳以下
4. 膵臓移植，膵島移植につき説明し，膵島移植に関して，本人，家族，主治医の同意が得られている
5. 発症後5年以上経過していること

[禁忌]
1. 重度の心疾患，肝疾患(心移植または肝移植と同時に行う場合には考慮する)
2. アルコール中毒
3. 感染症
4. 悪性腫瘍(5年以内に既往がないこと)
5. 重症肥満(BMI 25以上)
6. 未処置の網膜症(ただし失明例は除く)
7. その他移植に適さないもの

表2. 新鮮膵島移植基準

分離膵島が以下の条件を満たすときに，新鮮膵島を移植する．
1. 膵島量5,000 IE/kg(患者体重)以上
2. 純度30%以上
3. 組織量10 ml以下
4. Viability 70%以上
5. Endotoxin 5 EU/kg(患者体重)以下
6. グラム染色陰性

(文献4より)

モントンプロトコールに則って選択しないことになっていたが，欧米で，腎移植後の膵島移植によって移植腎機能が保護されることが示され，腎移植後の膵島移植症例が増加していることを受けて，本邦でも2006年9月より腎移植後膵島移植が開始された．レシピエントの条件は，腎移植後6カ月以上経過しており，血清Cre 1.8 mg/dl以下で，直近6カ月の血清Creの上昇が0.2 mg/dl以下で，持続上昇を認めないことである．またステロイドは内服量が10 mg/日以下であることが望ましい．

(2) 申請と登録

膵島移植の申請[1]の手順は，主治医が当該患者に膵島移植の説明をして上記のような評価を行い，膵・膵島移植研究会・膵島移植班事務局に膵島移植適応判定申請書を請求してこれを作成し，「膵島移植適応判定に関する承諾書」を添えて，膵島移植班事務局に送付する．膵島移植班事務局は，膵島移植適応検討委員会に適応判定を依頼する．この際に，患者は膵島移植を受ける移植希望施設を選択する．適応検討委員会の審査の結果「適応あり」とされた場合，患者の膵島移植希望の意志を確認し，膵島移植班事務局にてレシピエント登録を行う．

現在の膵島分離・移植施設は，7施設(東北大学医学部附属病院，福島県立医科大学医学部附

属病院，国立病院機構千葉東病院，京都大学医学部附属病院，大阪大学医学部附属病院，神戸大学医学部附属病院，福岡大学医学部附属病院）である．移植の機会を増やすために，患者は7施設のうち複数の施設に登録することが可能である．

(3) 膵島移植の工程

ドナーから膵臓を摘出し，二層法の溶液に浸して膵島分離実施施設に運ぶ．膵島分離実施施設のクリーンルームにて，膵臓をコラゲナーゼを用いて消化し，その後純化行程を経て膵島を回収する．分離膵島が，新鮮膵島移植基準（表2)を満たす場合に，膵島は輸血用バッグに詰められ，経皮経肝的に門脈に挿入されたカテーテルを通して，点滴の要領で移植される．

移植手技自体の合併症では，穿刺部位からの出血と，門脈内血栓が報告されている．

なお，リベレース®（コラゲナーゼ）に狂牛病の原因プリオンが混入している可能性が完全には否定できないため，膵島移植は2007年3月下旬より一旦中断している．新たなコラゲナーゼが開発され，効果や安全性が確認されており，安定供給できるようになれば再開が可能となる．

(4) 膵島移植後の管理

移植された膵島は，門脈の末梢部にとどまり，肝臓からの新生血管が，膵島の血管網と吻合することで生着する．

免疫抑制薬は，当初エドモントンプロトコールに従って，IL-2レセプターのモノクローナル抗体（basiliximab）による免疫抑制の導入と，sirolimus, tacrolimusによる維持療法が行われていた．しかし，エドモントンプロトコールの血中濃度では，sirolimusの副作用として，口腔内潰瘍，下肢の浮腫，高コレステロール血症，卵巣嚢腫，蛋白尿などが高頻度に生じることが判明し，最近では，sirolimusを減量あるいは削除し，mycophenolate mofetil (MMF) を使用している施設が多い．

糖毒性で移植膵島が障害されないように，移植直後から厳格に血糖コントロールを行う．京都大学医学部附属病院では食前血糖100 mg/dl以下，食後血糖120 mg/dl以下になるようにインスリン量を調節している．また，移植後約1週間は，移植膵島に少しでも酸素を供給するために，ベッド上にいる間は酸素投与が行われている．免疫抑制薬の血中濃度が安定化し，インスリンの必要量が漸減して一定となる移植後約1カ月後に退院となる．

(5) 結果

本邦初の膵島移植は，京都大学医学部附属病院で2004年4月7日に施行された[5]．

現在までに心停止ドナーからの膵島分離が65回行われ，このうち34回で移植の条件を満たしていたため18名（男性5名，女性13名）に対して膵島移植が施行されている．

京都大学医学部附属病院では，心停止ドナー膵からも膵島が確実に分離できるKyoto Islet Isolation Method[6]を開発し，膵島分離24例のうち20例が移植基準を満たし，膵島分離成功率は83%と上昇した．そのうち，19例を1型糖尿病患者9名に移植できた．移植後血糖値は安定化し，第三者の介助を必要とする重症低血糖は消失した（図3a)．また必要インスリン量も徐々に減少し（図3b)，このうち2回移植および3回移植を受けた3症例では，インスリン注射が不要となった．

また，1回の膵島移植により，たとえインスリン注射が必要でも，全症例で，基礎インスリン分泌に相当する補充量が減少し，血糖の不安定性の定量的な指標であるM値（日内の血糖値が100あるいは120 mg/dlなどの基準値からどれぐらいかけ離れているかを示す）やMAGE（日内の血糖値の変動幅の大きさを示す）は著明に低下し（図4)，重症低血糖が消失し，血糖値の安定化が得られた[7]．

また我々は，膵島移植後に生着した膵島量を反映する指標として，SUIT（secretory unit of insulin in transplantation)指数を開発した(図5)[8]．

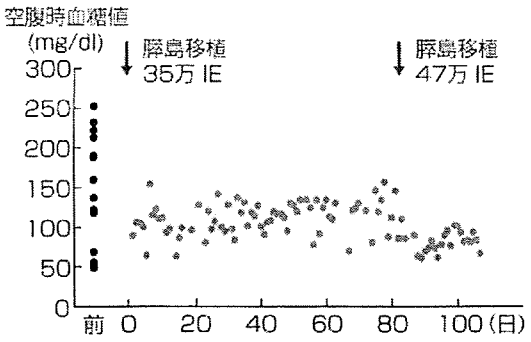


図 3a. 膵島移植前後の空腹時血糖値の推移(第 1 症例)

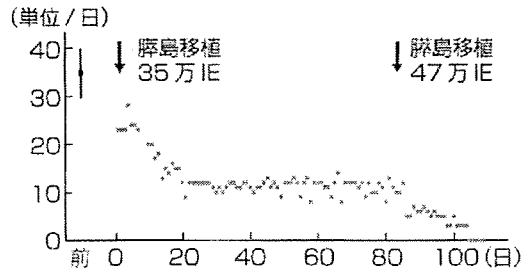


図 3b. 膵島移植前後のインスリン必要量(第 1 症例)

$$\text{SUIT 指数} = 1{,}485 \times \frac{\text{空腹時 C-peptide 値 (ng/ml)}}{\text{空腹時血糖値} - 61.8\ (\text{mg/dl})}$$

健常人の膵島を 100 として，大体どれくらいの膵島が生着しているかを表している．SUIT指数は，比較的早期から移植の成否を判定でき，インスリン治療中でも可能であり，移植した膵島に負荷を与えず，早朝 1 回の採血で評価可能である．京都大学医学部附属病院の結果から，SUIT 指数がおよそ 25 以上となると，インスリン離脱が可能であった．

2) 生体ドナーからの膵島移植

本邦では，ドナー不足は深刻である．京都大学医学部附属病院での膵島移植施行数が年間 10 例程度に対して，膵島移植を希望して受診する患者は年間 100 人以上であるため，生体ドナー膵島移植が研究され，2005 年 1 月世界で初めて成功した[9]．

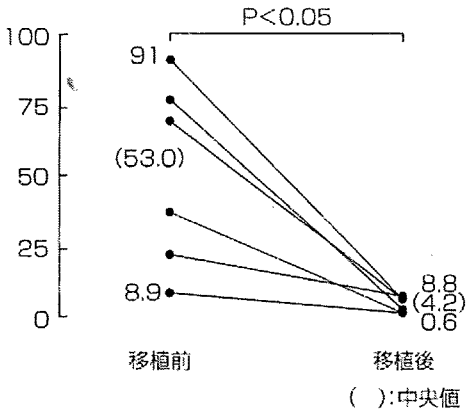


図 4a. 膵島移植前後の M 値

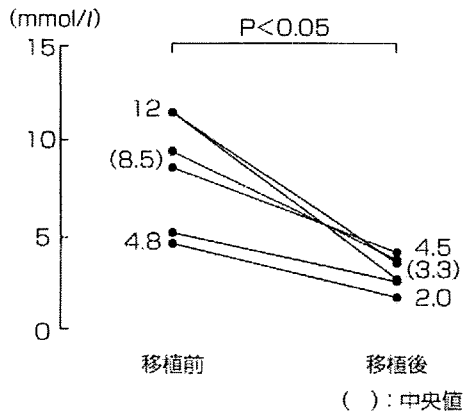
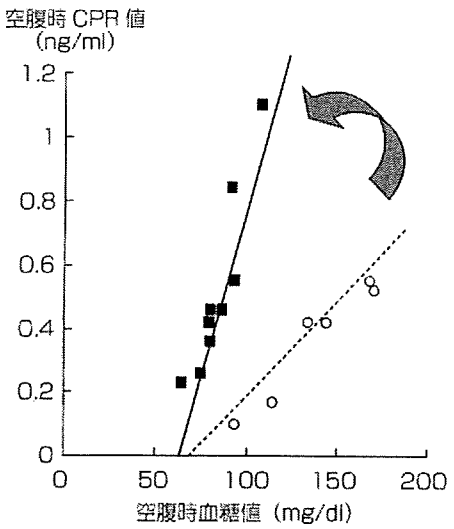


図 4b. 膵島移植前後の MAGE

生体ドナー膵島移植とは，健常なドナーの膵臓の体尾部を切除し，その膵体尾部から膵島を分離し，移植する治療法である．健常者であるドナーには，手術後の耐糖能の低下と周術期のリスクを伴う可能性があり，それを含めても移植による利益が上回ることが必要で，レシピエントの生命の危機の可能性のある重症低血糖発作の頻発などからの回避が目的となる．生体ドナー膵島移植では，脳死や心停止ドナー膵島移植と比べて死戦期の膵の傷害がなく，質の高い膵島を分離することができ，また移植時期を選べるため，レシピエントに免疫抑制薬の導入を行ったりインスリン量を最適にするなど，移植に最適な状態に準備することが可能である．



1回目の移植後（点線）ならびに2回目の移植後（実線）を示す．

図5．膵島移植後の空腹時血糖値とCPR値の相関

4．課題と展望

今後の主な課題として，ドナー不足の解消，長期成績の向上や免疫抑制薬の副作用軽減がある．

ドナー不足の解決策として，膵島分離法の改良によるmarginal donorからの移植や，新たな細胞源を用いるという方法がある．

ES細胞（embryonic stem cell）は，無限の増殖能とあらゆる細胞へ分化する多分化能をもち，膵β細胞への分化も報告されているが，奇形腫形成や免疫拒絶，倫理面などの問題がある．一方，山中らはヒトの線維芽細胞に4つの遺伝子を導入することにより，ES細胞とほぼ同等の多能性をもつiPS細胞(induced pluripotent stem cell)を作成することに成功し[10]，新たな細胞源として期待されている．また，Meltonらはマウスの膵外分泌細胞から，膵β細胞とほぼ同等の細胞にreprogramming（初期化）することに成功した[11]．

また，ドナーの供給源として，ブタの膵臓を用いた異種移植が研究されており，細胞性免疫を標的にした免疫抑制薬により，サルへの異種膵島移植における免疫拒絶反応を制御することができた[12]．ヒトへの異種膵島移植の臨床試験が米国で2，3年のうちに開始予定である．

抗胸腺細胞抗体（ATG）や抗CD20モノクローナル抗体であるrituximabによる導入療法[13]，tacrolimusを用いない新しい免疫抑制薬のプロトコールや，腸管内分泌細胞から分泌されインスリン分泌を促すホルモンであるGLP-1の長期作用アナログ（exendin-4）の使用により，膵島移植の長期成績の改善や免疫抑制薬の副作用の軽減が報告されている．また，現在は免疫抑制薬の全身投与が一生にわたって必要であるが，局所の免疫制御のみで免疫拒絶を回避できるよう研究が進められており，成功が待たれる．

おわりに

膵島移植は，血糖が極めて不安定なインスリン依存状態の糖尿病患者に対して，血糖値を安定化させ，重症低血糖を回避させる，安全で有効な治療法となり，移植を受けた患者のQOL（quality of life）は著明に改善されている．しかし，膵島移植の効果を長期に持続させたり，一人のドナーからの移植でインスリン離脱を目指すためには，慢性拒絶の回避や生着率の向上，より安全で副作用の少ない免疫抑制薬のプロトコールの開発，さらには免疫抑制薬からの離脱などが必要であり，精力的な研究が行われている．また，ドナー不足の解消のため，再生β細胞による移植や異種膵島移植が研究されており，臨床応用が期待される．これらの研究により，膵島移植はインスリン依存状態の糖尿病患者の根治療法となりうると考えられる．

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ISLETS
Human

A Novel Predictive Method for Assessing the Quality of Isolated Pancreatic Islets Using Scanning Electrochemical Microscopy

M. Goto, H. Abe, T. Ito-Sasaki, M. Goto, A. Inagaki, N. Ogawa, K. Fujimori, Y. Kurokawa, T. Matsue, and S. Satomi

ABSTRACT

Introduction. The current methods for evaluating islet potency are not useful in clinical transplantation. Therefore, we need reliable, rapid methods enabling accurate prediction of islet quality.

Materials and Methods. We evaluated respiratory activity using scanning electrochemical microscopy (SECM), glucose-stimulated respiratory activity, glucose-stimulated insulin release, ADP/ATP assays, insulin/DNA levels, and Trypan blue exclusion tests as predictive methods for the ability of isolated rat islets to cure syngeneic diabetic rats.

Results. Although glucose-stimulated respiratory activity, basal respiratory activity, ADP/ATP ratio, and glucose-stimulated insulin release were significantly correlated with the outcome of transplantation into diabetic rats, there was no correlation between outcomes, insulin/DNA ratios, and Trypan blue exclusion tests. The glucose-stimulated respiratory activity in islet preparations that could cure diabetic rats was significantly greater than those unable to cure diabetes. Rat islets with >1.5-fold glucose-stimulated respiratory activity consistently cured diabetic rats, whereas those with a value <1.5 hardly cured any rats.

Conclusion. Measurement of the glucose-stimulated respiratory activity using SECM technique is a novel method that may be useful as a rapid, potent predictor of the outcome of clinical islet transplantation.

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THE CURRENT standard methods to evaluate islet potency are not useful in clinical islet transplantation. Furthermore, most tests are relatively subjective and time-consuming.[1] We have thus far shown that the ADP/ATP ratio correlated with in vivo viability of isolated islets.[2] However, insulin release from isolated islets is not entirely related to the ADP/ATP ratio. Moreover, it is difficult to continuously measure the ADP/ATP ratio of the same islets. Therefore, we sought to establish a reliable, rapid method enabling accurate prediction of both islet viability and insulin release. Scanning electrochemical microscopy (SECM) is a technique in which the tip of a microelectrode monitors the local distribution of electro-active species near the sample surface. SECM has been used to investigate numerous biological molecules, including DNA,[3] enzymes,[4] and antigen-antibody interactions.[5] This technique noninvasively measures respiratory activity of isolated islets under physiological conditions. We have used SECM to examine islet viability and potency of insulin release.

MATERIALS AND METHODS

In the present study, we evaluated respiratory activity using SECM, glucose-stimulated respiratory activity, glucose-stimulated insulin release, ADP/ATP assays, insulin/DNA levels, and Trypan blue exclusion tests as predictive methods to evaluate the ability of isolated rat islets exposed to various degrees of heat shock stress (0, 40, 50, 60 or 80 seconds) to cure syngeneic Streptozotocin-induced diabetic rats (n = 7, 6, 6, 7, and 7, respectively). SECM was programmed to automatically measure the reduction current of far and near points of samples based on spherical diffusion theory.[6] The respiratory activity of 10 islets in each group was calculated by evaluating the difference of the reduction current around the samples using 2–4 μm platinum-coated microelectrode. The glucose-stimulated respiratory activity was indicated by the stimulation index of the respiratory activity, defined as the ratio of the respiratory activity in high-glucose concentration (16.7 mmol/L) against that in basal glucose concentration (1.67 mmol/L). The ADP/ATP assay, insulin/DNA levels, and Trypan blue exclusion tests were performed as previously described.[2,7] In islet transplantation, 6 islet equivalents/g of body weight were transplanted into recipient livers via the portal vein using a 24-gauge butterfly needle using the previously described method.[8] Heat shock stress was induced by placing the isolated islets at 60°C for 0, 40, 50, 60, or 80 seconds.

RESULTS

On the one hand, significant correlations with the outcome of transplantation into diabetic rats were observed for glucose-stimulated respiratory activity (heat shock stress; 0 seconds; 2.39 ± 0.08; 40 seconds, 1.85 ± 0.17; 50 seconds, 0.86 ± 0.08; 60 seconds, 0.49 ± 0.03; 80 seconds, 0.37 ± 0.07; cured group: 1.94 ± 0.18; noncured group: 0.57 ± 0.07, respectively), basal respiratory activity (heat shock stress: 0 seconds, 5.65 ± 0.15, 40 seconds, 5.31 ± 0.51, 50 seconds, 4.18 ± 0.58, 60 seconds, 1.83 ± 0.27, 80 seconds, 0.31 ± 0.05; cured group: 5.27 ± 0.26; noncured group: 1.98 ± 0.46, respectively), ADP/ATP ratio (heat shock stress; 0 seconds, 0.003 ± 0.003, 40 seconds, 0.05 ± 0.03, 50 seconds, 0.21 ± 0.05, 60 seconds, 0.30 ± 0.07, 80 seconds, 0.42 ± 0.05, cured group: 0.05 ± 0.03; noncured group: 0.30 ± 0.04, respectively), and glucose-stimulated insulin release (heat shock stress; 0 seconds, 11.0 ± 2.6, 40 seconds, 2.51 ± 0.76, 50 seconds, 1.12 ± 0.14, 60 seconds, 1.13 ± 0.21, 80 seconds, 1.40 ± 0.41; cured group: 6.59 ± 1.78; noncured group: 1.35 ± 0.18, respectively) P <.0001, <.0001, <.0001, and .002: ρ = .80, .71, −.66, and .53, respectively. On the other hand, there was no correlation between islet transplantation outcome and insulin/DNA ratio (heat shock stress: 0 seconds, 0.73 ± 0.05, 40 seconds, 0.99 ± 0.13, 50 seconds, 0.86 ± 0.10, 60 seconds, 0.91 ± 0.06, 80 seconds, 1.12 ± 0.06; cured group: 0.78 ± 0.04; noncured group: 1.03 ± 0.05, respectively), and Trypan blue exclusion test (heat shock stress: 0 seconds, 100.0 ± 0.0, 40 seconds, 98.8 ± 0.6, 50 seconds, 99.3 ± 0.5, 60 seconds, 99.9 ± 0.1, 80 seconds, 94.9 ± 1.8; cured group: 99.5 ± 0.3; noncured group: 97.8 ± 0.8, respectively). The glucose-stimulated respiratory activity in islet preparations that could cure diabetic rats was significantly higher than in those unable to cure diabetes (P < .0001). Rat islets with glucose-stimulated respiratory activity more than 1.5 consistently cured diabetic rats, whereas rat islets with a value <1.5 hardly cured any rats (P < .0001) (Fig 1). Notably, the predictive rate for curing diabetic rats was 91% when glucose-stimulated respiratory activity was used.

DISCUSSION

It is well known that unexpectedly poor effects of grafts are still seen in the field of islet transplantation even using the current refined procedures. Most likely, this is attributed to suboptimal quality of the isolated islets.

It has been reported that the current methods of islet quality assessment have only a limited ability to predict

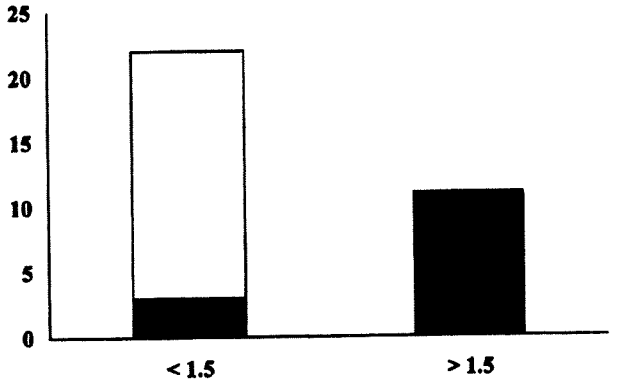

Fig 1. Streptozotocin-induced diabetic rats underwent intraportal transplantation with syngeneic islets that were exposed to various degrees of heat shock stress (0, 40, 50, 60, and 80 seconds). The X-axis indicates the glucose-stimulated respiratory activity, and the Y-axis indicates the number of animals. The black bar shows cured animals; the white bar shows noncured diabetic animals. Rat islets with glucose-stimulated respiratory activity >1.5 consistently cured diabetic rats, whereas rat islets with a value <1.5 hardly cured any rats (P < .0001).

outcomes after clinical transplantation.[1,9,10] In vivo bioassay has thus far been regarded as the most reliable assessment.[11,12] However, it is not clinically useful because several days are needed for evaluation. Therefore, we need establishment of reliable, rapid methods enabling accurate prediction of islet potency. This issue is crucial for Japan because only marginal organs from non–heart-beating donors are currently available for islet isolation.

In 2006, we reported that the ADP/ATP ratio was a useful predictive assay for isolated islets.[2] Although the ADP/ATP assay has many advantages as islet quality assessment, its limitation is the absence of a correlation with insulin release from the isolated islets, suggesting that it reflects islet viability rather than function.

As shown in the present study, glucose-stimulated respiratory activity strongly correlated with islet quality. This highly sensitive, noninvasive method made it possible to distinguish respiratory activity even in one islet by visualizing the reduction current in a simple form. Notably, the glucose-stimulated respiratory activity is expected to reflect not only islet viability but also function.

Taken together, measurement of the glucose-stimulated respiratory activity using SECM technique is a novel rapid, potent predictor of the outcome of clinical islet transplantation.

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

Y. Saito, M. Goto, K. Maya, N. Ogawa, K. Fujimori, Y. Kurokawa, and S. Satomi

ABSTRACT

Introduction. Tissue factor (TF) in islets has been identified as the main trigger of the instant blood-mediated inflammatory reaction. Because the crucial events that directly induce TF remain to be determined, we focused on the influence of brain death (BD) on TF expression in pancreatic tissues and isolated islets.

Materials and Methods. BD was induced in male Lewis rats weighing 250–300 g by inflation of a Fogarty catheter placed intracranially. The rats were mechanically ventilated for 6 hours until removal of the pancreas. The expression of TF protein in pancreatic tissues was examined using Western blotting assay. Messenger RNA (mRNA) expressions of TF in pancreatic tissue and isolated islets were analyzed using real-time polymerase chain reaction (PCR) assay. The influence of BD on the isolation outcome was evaluated by islet yield, purity, viability, and function.

Results. TF protein and mRNA levels in the pancreatic tissues were similar between the groups. However, TF mRNA in the isolated islets of the BD group was significantly greater than that of the control group ($P = .04$). Islet yield was considerably lower, and purity significantly lower in the BD than the control group ($P = .002$). Unexpectedly, ATP/DNA ratio and respiratory activity were comparable between the groups.

Conclusions. Although BD per se was not sufficient to induce TF expression in pancreatic tissues, BD combined with subsequent warm ischemic damage during isolation procedures remarkably up-regulated TF expression in isolated islets, suggesting that BD is of great importance as an initiator of TF induction in the islet grafts. The present study demonstrated that the expression of inflammatory mediators rather than islet viability is more susceptible to BD.

TISSUE factor (TF), a 47-kd transmembrane glycoprotein, acts as the initiator of the extrinsic coagulation system. It is pivotal for activation of the intrinsic pathway as well. Pancreatic islets have thus far been reported to express TF.[1] It has been revealed that TF in islets has been identified as the main trigger of the instant blood-mediated inflammatory reaction.[1,2] Low expression of TF in the graft has been correlated with high C-peptide values after clinical islet transplantation.[3] However, the crucial procedures to directly induce TF remain to be determined.

It is well known that the outcome of organ transplantation is highly influenced by brain death (BD). The success rate of kidney transplantations derived from cadaveric

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donors remains significantly inferior to that from living donors regardless of their genetic relationship to the recipient.[4] Contreras et al presented data that demonstrated BD to reduce isolated pancreatic islet yield and function, as well as up-regulation of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 in the serum and pancreatic tissues from BD donors.[5] In the present study, therefore, we focused on the influence of BD on TF expression in pancreatic tissues and isolated islets.

MATERIALS AND METHODS

Rodent BD Model

BD was induced in male Lewis rats weighing 250–300 g by inflation of a Fogarty catheter placed intracranially. The rats were mechanically ventilated for 6 hours until removal of the pancreas as previously described.[5,6]

Western Blotting Assay of the Pancreatic Tissues

Pieces of pancreatic tissues from BD and control groups snap-frozen in liquid nitrogen were stored at −80°C until use (n = 5 and n = 4, respectively). Approximately 10 mg of pancreatic biopsy specimens prepared on dry ice were immediately transferred into phosphate-buffered saline containing 5 mmol/L EDTA, 10 mmol/L benzamidine (Merck-Schuchardt, Hohenbrunn, Germany), 0.1 g/L soybean trypsin inhibitor (Sigma-Aldrich, Steinheim, Germany), and 1 mmol/L phenyl methyl sulfonyl fluoride (Sigma). The samples were then homogenized using Polytron PT 1300D (Kinematica AG, Littau-Lucerne, Switzerland) and Vibra-Cell (Sonics & Materials Inc, Newtown, Conn, USA) for 30 seconds each. Thereafter the samples were centrifuged at 4°C at 10,000*g* for 30 minutes to collect the supernate. The samples, containing 2.5 mg/mL of protein measured by BCA Protein Assay kit (Thermo Prod, Rockford, Ill, USA), were separated using SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Invitrogen, Carlsbad, Calif, USA). Membranes were incubated with rabbit anti-rat TF polyclonal antibody (Hokudo, Sapporo, Japan) at 4°C overnight and subsequently with goat anti-rabbit immunoglobulin (Ig)G-horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, Calif) antibody for 1 hour at room temperature. TF antigen was visualized using enhanced chemiluminescence Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK).

Determination of TF mRNA in the Pancreatic Tissues

Pieces of pancreatic tissues from BD and control groups snap-frozen in liquid nitrogen were stored at −80°C until use (n = 6 and n = 4, respectively). Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. RNA concentrations were estimated from absorbance at 260 nm. First-strand complementary DNA (cDNA) was synthesized from 2500 ng total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, Ind, USA). The cDNAs were amplified by PCR using rat TF primer probe set (Nihon Gene Research Laboratories Inc., Sendai, Japan) and rat GAPDH primer probe set (Nihon Gene Research Laboratories Inc.) with a Lightcycler (Roche Diagnostics).

Islet Isolation and Culture

Before removal of the pancreas, the cannulated bile duct was injected with 10 mL of cold Hanks' Balanced Salt Solutions (HBSS) containing 1 mg/mL Coragenase (Sigma type V; Sigma Chemicals, St. Louis, Mo). After addition of 10 mL HBSS the pancreas was digested at 37°C for 14 minutes. Thereafter, density-gradient centrifugation was performed using Histopaque-1119 (Sigma Diagnostics) and Lymphoprep (Nycomed Pharma AS, Oslo, Norway) to isolate pancreatic islets. The islet count was performed as islet equivalents (IEQ) under a scaled microscope using diphenylthiocarbazone (Wako, Osaka, Japan) staining (BD, n = 8; control, n = 7). One IEQ was the islet tissue mass equivalent to a spherical islet of 150 μm in diameter. Islets were cultured in RPMI-1640 containing 5.5 mmol/L glucose and 10% FBS at 37°C in 5% CO_2 and humidified air before examination.

Islet Viability and Function

ATP/DNA ratio was measured to evaluate the energy status of isolated islets. Eighty islet equivalents of islets with overnight culture were used in both BD and control groups (BD, n = 6; control, n = 5). The ApoGlow kit (Lonza Rockland Inc, Rockland, ME, USA) was used for ATP measurement as described previously.[7] Using the same sample, the DNA content was measured using DNA Quantify kit (Primary Cell, Sapporo, Japan) as described previously.[8] We evaluated the respiratory activity of isolated islets with overnight culture using scanning electrochemical microscopy (BD, n = 6; control, n = 5). The stimulation index of the respiratory activity, defined as the ratio of the respiratory activity in high glucose (16.7 mmol/L) against that in basal glucose (1.67 mmol/L), is a novel marker that was applied as a rapid, potent predictor for the outcome of clinical islet transplantation.

Determination of TF mRNA in the Isolated Islets

Total RNA extracted from the 40 islets after 3-hour culture was prepared using RNeasy Micro Kit (Qiagen) according to the manufacturer's protocol (BD, n = 5; control, n = 8). RNA concentration was estimated from absorbance at 260 nm. First-strand cDNA was synthesized from 100 ng total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). The cDNAs were amplified by PCR, using rat TF primer probe set (Nihon Gene Research Laboratories Inc.) and rat GAPDH primer probe set (Nihon Gene Research Laboratories Inc.) with a Lightcycler (Roche Diagnostics).

Statistical Analysis

All data are expressed as mean values ± SD. Comparisons between groups were performed by student *t* test using Statcel 2nd Edition (Oms Publishing, Osaka, Japan). Statistical significance was established at $P < .05$.

RESULTS

Tissue factor protein and mRNA levels in the pancreatic tissues were similar between the groups (Fig 1 and 2). However, TF mRNA in the isolated islets of the BD group was significantly greater than that of the control group (TF/GAPDH BD, 0.169 ± 0.033; control, 0.119 ± 0.041; P = .04) (Fig 3). Islet yield was considerably lower (BD, 2110 ± 231 IEQs; control, 2390 ± 528 IEQs; P = .19), and purity was significantly lower in the BD than the control group (BD, 87.7 ± 7.5%; control, 97.0 ± 2.6%; P = .002). Unexpectedly, the ATP/DNA ratio and respiratory activity were comparable between the groups (ATP/DNA BD, 51.6 ± 12.8; control, 59.1 ± 3.47; P = .20; and BD, 2.39 ± 0.55; control, 2.58 ± 0.19; P = .45).

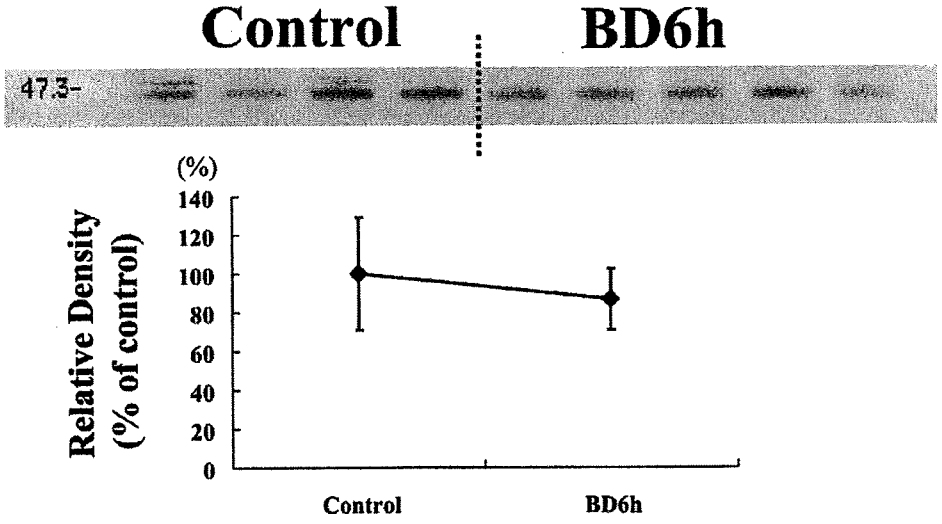

Fig 1. Protein expression of TF in the pancreatic tissues from the donors with/without BD was analyzed using Western blotting assay.

DISCUSSION

In the present study, our data showed that BD influenced TF expression in isolated islets but not in pancreatic tissues prior to the digestion procedure. It may be speculated that the difference was attributable to warm ischemic damage during the digestion procedure. In islet transplantation, unlike other organ transplantations, islet grafts are placed at 37°C during whole digestion procedure. This period could theoretically be considered as one kind of "warm ischemia," a concept that is supported by many investigators in the field of islet transplantation.[9–11] As shown in the present study, TF was not up-regulated in the isolated islets from the donors without BD, suggesting that warm ischemic damage during digestion procedure per se was not sufficient to induce TF in isolated islets. We therefore believe that the induction of TF from BD was accelerated by warm ischemic damage during the digestion procedure.

In the present study, islet yield and purity were certainly affected by BD. However, the difference was extremely small compared with a previous report,[5] moreover, almost no influence was observed in terms of islet viability. One possible explanation for this discrepancy is a difference in isolation procedures. In our isolation procedures, pancreatic tissues were kept on ice except during the digestion phase. Furthermore, at the density-gradient centrifugation phase, we applied Histopaque-1119 and Lymphoprep, in contrast, a dextran gradient separation was performed in the previous report.[5] Hence, the important message from our present study is that the expression of inflammatory mediators rather than islet viability is more susceptible to BD.

In conclusion, although BD per se was not sufficient to induce TF expression in pancreatic tissues, BD combined with warm ischemic damage during isolation procedures remarkably up-regulated TF expression in isolated islets, suggesting that BD is of great importance as an initiator of TF induction in islet grafts.

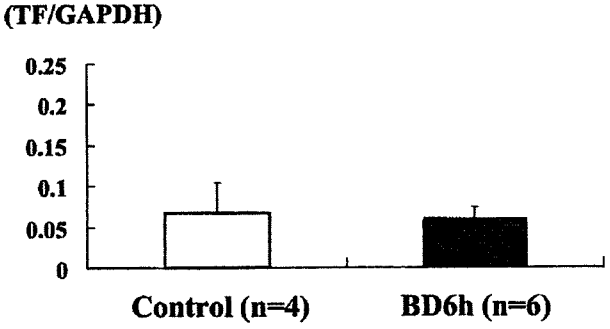

Fig 2. mRNA expression of TF in the pancreatic tissues from the donors with/without BD was analyzed using real-time PCR assay.

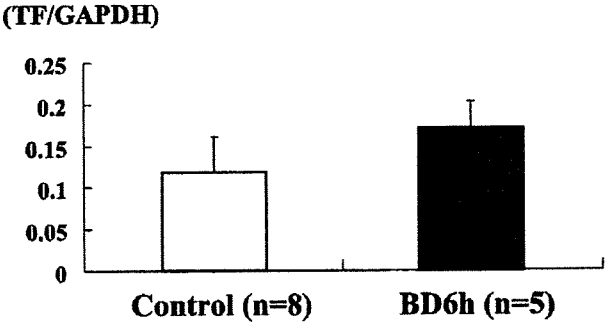

Fig 3. mRNA expression of TF in the isolated islets from the donors with/without BD was analyzed using real-time PCR assay.

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

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ELSEVIER

Superiority of Fresh Islets Compared With Cultured Islets

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ABSTRACT

Introduction. It has recently been reported that the outcomes of islet transplantation with short periods of culture are comparable with those of freshly isolated islets. To clarify the influence of culture, fresh islets were compared with cultured islets in terms of quality.

Materials and Methods. The quality of freshly isolated islets was compared with that of cultured islets with CMRL 1066 including 10% allogeneic serum, CMRL 1066 including 0.5% human serum albumin, or Miami medium. We evaluated static glucose stimulation tests, insulin/DNA contents, ADP/ATP ratios, and an intraportal transplantation model into syngeneic diabetic rats. The expression of inflammatory mediators in the islets was examined using Western blotting for tissue factor (TF), which is the initiator of detrimental instant, blood-mediated, inflammatory reactions (IBMIR).

Results. Although the survival rate was similar in all groups, the stimulation index upon glucose challenge and the insulin/DNA ratio were significantly higher among fresh islets. Most importantly, the expression of TF on islets was significantly lower in fresh islets, suggesting that culture enhanced TF-dependent IBMIR after transplantation. In an in vivo transplantation model, the curative rate and insulin production by the recipient liver was considerably greater in the fresh islet group.

Conclusions. Isolated islets without prior culture showed results superior to cultured islets.

ALTHOUGH one of the key factors of the Edmonton protocol is transplantation of fresh islets just after isolation, comparable outcomes of islet transplantation have recently been reported with a short-period culture. To clarify the influence of culture on isolated pancreatic islets, we compared fresh islets with those cultured using several current techniques, in terms of islet quality, including not only viability but also inflammatory mediator expressed on the islets. Also in this study, we examined the effects of correcting the islet dose just prior to rat islet transplantation.

MATERIALS AND METHODS

The quality of freshly isolated islets was compared with that of islets cultured using CMRL 1066 plus 10% allogeneic serum, CMRL 1066 plus 0.5% human serum albumin (HAS), or Miami medium (Miami). The evaluation used islets survival rates, visual scoring, static glucose stimulation tests,[1] insulin/DNA contents,[1] ADP/ATP ratios,[2] and intraportal transplantation models into syngeneic Streptozotocin-induced diabetic rats. The influence of culture on the expression of inflammatory mediators in the islets was examined using Western blotting assay for tissue factor (TF), which is the initiator of detrimental instant blood-mediated inflammatory reactions (IBMIR).[3,4] Statistical analyses were performed using analysis of variance (ANOVA).

RESULTS

Although the survival rate was similar in all groups, the visual scoring was lower among the Miami group. Stimulation index on glucose challenge tests was higher in the fresh group: fresh, 17.89 ± 4.93; serum, 13.69 ± 5.44; HSA, 5.36 ± 1.60; and Miami, 2.69 ± 0.82 (P = .008). Insulin/DNA ratios revealed a similar tendency as the glucose challenge tests: fresh, 1.02 ± 0.07; serum, 0.83 ± 0.11; HSA, 0.52 ± 0.07; and Miami, 0.37 ± 0.08 (P = .0001). The ADP/ATP ratios were lower for both the fresh and serum groups than the others: fresh, 0.047 ± 0.021; serum, 0.054 ±

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0.017; HSA, 0.086 ± 0.004; and Miami, 0.084 ± 0.026 (P = .38), suggesting a relatively lower number of apoptotic islets in both the fresh and the serum groups. Most importantly, the expression of TF on the islets was significantly lower in fresh islets (P = .01), suggesting that a current culture method could enhance TF-dependent IBMIR after transplantation. In an in vivo transplantation model, the curative rate and insulin amount in the recipient liver were considerably higher in the fresh islet than the other groups (Fig 1). Intravenous glucose tolerance was also ameliorated in the fresh and serum groups rather than the nonserum group: AUC of fresh = 25,376.71 ± 973.9; serum, 24,691.43 ± 1233.1; and Miami, 30,654.86 ± 2706.1 (P = .06) and the glucose disappearance rate (Kg) of fresh = 1.231 ± 0.101; serum, 1.203 ± 0.12, and Miami, 0.851 ± 0.174 (P = .114). Notably, the disadvantage of the nonserum culture groups was recovered by augmenting the graft amount just prior to transplantation (data not shown).

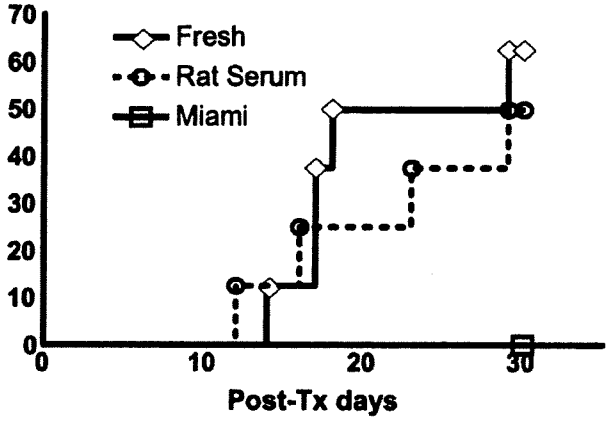

Fig 1. The influence of culture on islet quality was evaluated by intraportal transplantation of syngeneic islets into streptozotocin-induced diabetic rats. In an in vivo transplantation model, the curative rate was higher in the fresh islet group than in the others.

DISCUSSION

Isolated islets without prior culture showed results beneficial to transplantation compared with current culture methods. Further improvements are required to optimize a substitute for serum supplements using a clinically available model.

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

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

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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.

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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.

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.

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There authors declare no conflict of interest.

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Adenosine is a purine nucleoside and is emerging as a key regulatory molecule which has anti-inflammatory, anti-coagulatory, 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