厚生労働科学研究費補助金（医療機器開発推進研究事業）

分担研究報告書

「MRIを用いた非侵襲的生体イメージングによる糖尿病の超早期診断法の開発」

研究分担者　富樫かおり　　所属　京都大学　画像診断学・核医学

研究要旨：膵島を直接的に定量する方法とは異なるアプローチとして、糖尿病へと到る過程で生じる膵島破壊に着目して、その病変に関与する貪食細胞の可視化による早期診断の検討を行っている。鉄剤にコーティングを施し、活性化した貪食細胞に取り込まれる数十nmサイズとしたUSPIO造影剤を使用して、膵島の変性過程を検出する手法を開発すべく研究を進めている。

A. 研究目的

糖尿病を発症するまでに膵島の70%以上が破壊されるが、その過程に貪食細胞の関与が指摘されている。残存膵島量を定量する以外にも、糖尿病の前段階で発生している破壊過程を特異的に検出することで、未発症段階での早期発見と予防・機能回復を図れる可能性がある。そこでこの細胞をターゲットとしたイメージングが可能であるかについて、微小超常磁性酸化鉄粒子(ultrasmall superparamagnetic iron oxide particles: USPIO)製剤を使用して、動物モデルを対象とした描出の研究を進めている。

B. 研究手法検討

FDG-PETなどの核医学的な膵臓可視化手法についても検討しているが(1)、その分解能はやはり十分ではない。現在の研究方向の中心は貪食細胞が本来有する機能を活用することであり、その目的でUSPIO造影剤を入手した。これは生体内の細網内皮系に集積する大きさの製剤でMRI画像上信号低下を示すFeを有する。その陰性コントラストを最大限に活用して膵島描出に近づくにはどのような撮像法を使用すればよいか、MRIによる基礎検討を行った。

C. 検討結果

膵島に取り込まれる微量なFeによる信号変化をより鋭敏に捉える手法としては、画像処理により位相変化を強調する手法(2)や撮像の段階で信号減衰を強調する(3)など、多様な手法が活用可能である。まずは呼吸などの動きの影響が少ない脳の構造を対象として、微細構造の描出能を検討した。人体レベルでも数百ミクロンレベルの構造が描出可能であった。

D. 実験計画

現在モデル動物を対象として、USPIO製剤により膵島内の賦活化された貪食細胞への集積と膵島の可視化研究を進めている。さらに糖尿病発症に至る過程での膵島破壊活動自体をどの程度鋭敏に反映するかを検討する予定である。

E. 結論

本アプローチの推進により、糖尿病発症までのより早期において潜在する疾患の進行過程を描出可能となり、新たなる診断手法の探索がすすむと考えられる。

F. 健康危険情報　特になし。

G. 研究発表

1.論文発表

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2.学会発表　なし

H. 知的財産権の出願・登録状況

1.特許取得　なし

2.実用新案登録　なし

3.その他　なし

厚生労働科学研究費補助金（医療機器開発推進研究事業）
総括研究報告書

「非侵襲的生体膵島イメージングに必要な超高磁場MRIによる膵島撮像法の開発」

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研究要旨：膵島量の定量化を行うためのMRI撮像技術の開発を目的とし、動物用の超高磁場MRI装置を用いて摘出膵組織片を対象に高空間分解能のMRI画像による膵島の描出を試みた。新たに構築した膵島部の位置の識別が可能な実験系を用いた予備的な実験結果から、一般的なT1強調像で膵島部が高信号領域として描出される可能性が示唆され検討を進めたが、一定の結果が得られなかったため、プローブを用いた撮像検討を開始した。

A．研究
目的
本研究では、糖尿病の超早期診断のために生体内の膵島量を非侵襲的な画像診断法を用いて定量化するための技術開発を行うことを目的としているが、本分担研究者は動物用の超高磁場 MRI 装置を用いた膵島イメージングを担当し、特に膵島量の定量化に関する MRI 撮像技術の開発を目的としている。

B．研究方法
MRIによる膵島量の測定では、小動物用の超高磁場 MRI 装置を利用して昨年度に構築したマウスの膵組織を対象とする MRI 画像評価系を用い、数種類の分子プローブ候補物質に関する画像評価実験を行った。本評価系ではin vitroからin vivoまでのマウス膵組織を対象に200μmから50μm程度までの高空間分解能 MRI 画像を様々なMRI撮像パラメタを用いて撮影することができ、in vitroの評価では組織片および固定標本の MRI 画像を蛍光あるいは特殊染色などによる標識で定量化した膵島量との比較が可能である。

昨年度の検討では、分担研究者豊田らの協力により GFP を発現する膵島をもつトランスジェニックマウスを利用し、摘出膵組織の一部をアガロースに包埋して蛍光顕微鏡下で膵島を３次元マッピングし、これを参照することにより MRI 画像における膵島部の位置を識別できるかどうかの予備実験を行い、T1 強調像で周囲の組織と比較して膵島部がわずかながら高信号領域として描出される可能性を示唆する結果を得た。そこで本年度は、予備実験の結果に基づいて確認実験を行った。さらに、Gd 造影剤を用いた検討を加えた。

C．研究結果
摘出膵組織片において膵島部の位置を識別できる上記のような実験系においてMRI撮影実験を繰り返した結果、T1強調像で膵島部が高信号像と一定して得られず、周囲と識別できないものも多く認められ、その他T2強調像、拡散強調像を取得しても周囲組織との明瞭な識別が困難であることが判明した。（またホルマリン固定摘出膵組織片および上記実験系のアガロース包埋摘出膵組織片を用い同条件で画像測定を行ったと

ころ、両サンプルで膵島部と周囲組織が異なるコントラストを示したことから、この実験系を用いたin vivo膵島イメージングに向けた実験系確立は困難であると判断した）以上より、非造影での膵島イメージングは困難と判断した。

そこで、市販のGd造影剤を投与して膵島描出可能かどうかを検討した。しかしながら、造影剤投与でも同様の結果であり、一定した結果は得られなかった。

以上より、非プローブ存在下でのMRI撮像による膵島イメージングは困難と判断し、膵島特異的プローブ存在下での撮像条件検討を行うことにした。現在、GPR40のリガンドである化合物H1を用いた初期検討開始している。

D．考察

非プローブ存在下での MRI 撮像による膵島イメージングでは一定した結果が得られなかった。その原因は不明であるが、三次元情報構築のために膵臓を小切片化する必要性があり、実際の膵臓と撮像条件が乖離している可能性がある。また膵島部と外分泌など周囲組織間に緩和時間など MRI 画像コントラスト源となる性質に差がほとんどない可能性が考えられる。よって、非プローブ存在下での膵島イメージングは困難と判断した。

E．結論

膵島部の位置の識別が可能な実験系を構築し、高空間分解能 MRI を用いて、非プローブ存在下で T1 強調像を中心とする撮像検討を行ったが、一定した結果が得られなかったため、非プローブ存在下での膵島イメージングは困難と判断した。MRI による膵島イメージングにおいて、プローブを用いた検討の必要性が示唆された。

F．健康危険情報　特になし。

G．研究発表

1.　論文発表

（発表誌名巻号・頁・発行年等も記入）

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H．知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得　なし

2. 実用新案登録　なし

3. その他　なし

厚生労働科学研究費補助金（医療機器開発推進研究事業）
総括研究報告書

「非侵襲的生体膵島イメージングのためのグルコース誘導体の開発」

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研究要旨：糖尿病の超早期診断法開発のためには、GLUT2 の動的機能に着目し、膵島における GLUT2 の分布状態のみならず、そのグルコース輸送制御機能について、高い解像度で生細胞リアルタイム解析を行うことが重要である。本研究は、そのために必須のツールである蛍光グルコースの合成収率改善、純度検定、保存安定性の検討など、実用上重要な課題について検討することを目的としている。

A．研究目的

糖尿病の超早期診断法の開発の成否はその指標選択に負うところが大きいと考えられる。既に GLUT2 に着目した試みもなされており、グルコース輸送体に着目した考えは合理的ではあるが、GLUT2 のどのような機能に着目するかについては、超早期の観点から見直す必要があると考えられる。すなわち、グルコース濃度の変化に応じてインスリンの合成、あるいは分泌量が変化することは良く知られているが、グルコース輸送体自体の自己制御性については必ずしも明らかではない。しかも、超早期の指標として考えるならば、グルコース輸送体自体の動的性質を高い解像度で調べなおす必要があると考えられる。そのためには、膵島細胞におけるグルコース輸送のダイナミズムを細胞が生きた状態でリアルタイム解析することが必要と考えられる。本研究は、そのような観点から必須のツールである蛍光グルコースの作製と利用法の開発を目的としている。適用対象は今のところマウスなどの実験動物を想定しているが、上記の理由により、マウス実験のみからでも有用な知見が得られるはずである。

蛍光グルコースの開発は古いが、実用的には、その合成収率の低さや使用時の不安定性が問題になっていた。そこで、昨年度は反応材料を従来の Cl-体から F-体に変えることで劇的な改善効果を得ることができた。本年度は、そうして合成された新規化合物が、本当に、以前の蛍光グルコースと同一物質であるか、ということの詳しい検証、不純物の有無、さらに実用上懸案となっていた保存条件の最適化などについて調べることとした。

B．研究方法

(1)蛍光グルコースの合成

昨年度の報告書に記載の方法で合成、分離した。

(2)合成品の分光学的特性解析

蛍光スペクトル、励起スペクトル、NMR スペクトルなどを計測し、比較した。

(3)構造異性体の確認

グルコースの 1 位の炭素に結合した水酸基の向きによってα体とβ体が混在するとされてきた。しかし、それぞれに対応する HPLC でのピークの高さが、調製の度に異なっていたため、不純物の混入が懸念された。そこで、HPLC で得られた 2 つのピークのそれぞれを個別に取得した後、再度、HPLC に注入してピークの位置および形状を調べた。

(4)保存条件の検討

保存時の温度、pH、置換気体の３つの条件について検討した。

(5)細胞への取り込み活性測定

グルコーストランスポーター、マンノーストランスポーター、などの糖輸送体を介した取り込み機能について詳しい解析データのある大腸菌生細胞を用いて、新規合成品と従来の蛍光グルコースとの比較をした。

C．研究結果

(1)新規合成された蛍光グルコースの分光特性

蛍光スペクトル、励起スペクトル、NMR スペクトルのいずれにおいても、以前の方法で得られた蛍光グルコースと同じ分光特性を示した。

(2)構造異性体の確認結果

フラクションコレクターを用いて2本のピークのそれぞれを個別に取得した後、時間変化とともにピーク変化を測定したところ、どちらのピークも単離直後は1本であったが、30分後には2本のピークが見られ、60分後には合成時と同様の2本のピークが見られた。

(3)最適保存条件

保存条件は、4℃、pH4.5～7.0、遮光下が最適であることがわかった。

(4)細胞への取り込み活性評価結果

新規合成品の細胞への取り込み活性は、従来の蛍光グルコースと同様に、生細胞のみ取り込み、死細胞では取り込まなかった。

D．考察

新規合成化合物は、従来と同様な特性を有することが示された。今後、マウス実験への適用を検討する必要がある。合成および精製時の溶媒の種類によって、2-NBDGのピークが2本になることがわかった。

E．結論

新規に合成した化合物は、従来と同様の特性を有しており、以前作製した蛍光グルコースと同一であった。また、合成した2-NBDGをHPLCで分析した場合の2本のピークは、グルコースのα体とβ体に由来するものであることがわかった。

F．健康危険情報

特になし

G．研究発表

1.論文発表

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(2)末崎拓広, 島北寛仁, 斉藤美佳子, 松岡英明, "密度勾配遠心分離法に基づく食品中微生物のバイアブルセパレーション", **日本防菌防黴学会第３５回年次大会**, 浜松（2008 年 9 月 12 日）

H．知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし

3.その他

なし

厚生労働科学研究費補助金（医療機器開発推進研究事業）

分担研究報告書

「非侵襲的生体膵島イメージングに必要なプローブの作製」

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研究要旨：糖尿病の発症過程では、膵島量が耐糖能異常に先行して減少する。この知見に基づき、本研究では糖尿病超早期診断を目的とした、膵島量を測定する非侵襲的画像診断技術の開発を目標とする。このための新規分子プローブの開発を行う。標的分子の探索から開始し、それに基づく分子プローブの設計、作製と実験動物を用いた評価を行う。それに並行して、分子プローブの量産化検討及び、測定・解析システムの開発を行う。

A．研究目的

現在、我が国における2型糖尿病は推定740万人を越えて増加し続けており、この対策として耐糖能検査を基準とした糖尿病発症前の介入が行われているが、充分な成果が得られていない。その原因として、機能異常が明らかとなる境界型糖尿病の段階では膵島の障害はすでに高度に進行しており、介入開始時期としては遅い可能性がある。よって、適切な時期の介入を行うための超早期診断の必要性が求められている。

よって、本研究の目的は、糖尿病の超早期診断のために生体内の膵島量を非侵襲的な画像診断法を用いて定量化するための技術開発を行うことである。

B．研究方法

非侵襲的膵島定量に必要な分子プローブの開発と画像診断法の検討を、以下の4つの手順で行う。なお、京都大学分担分については、詳細を割愛した。

1. 膵島イメージング標的分子の選定
2. イメージング分子プローブの設計・開発
3. 標識分子プローブの基礎的評価（*in vitro* ～*ex vivo* まで）
4. In vivo における分子プローブの有効性と画像撮像条件の検討

本年度、Exendin-(9-39)の誘導体3種、GW9508の誘導体3種、Mitiglinideの、計7種類の分子プローブ標識化を終了し、基礎的評価及び *in vivo* での有効性検討を行った。

今後、GW1100誘導体の標識化を早急に行い、評価を行った後、分子プローブの絞り込みを行い、性能向上のための改良を加える予定である。

（倫理面への配慮）

動物を用いた実験については、京都大学動物実験に関する指針に基づいて施行する。

C．研究結果

下記6種類の分子プローブ合成を行なっている。（平成21年2月現在）

・GPR40/GPR120のリガンドGW9508
　：誘導体 H1、H2、H3を合成終了。
　　評価実験中。
・GPR40のリガンドGW1100
　：標識化検討中。
・GLP-1受容体のリガンドExendin-(9-39)
　：誘導体 E1、E2、F合成終了。
　　評価実験中。
・K_{ATP}チャンネル のリガンドMitiglinide
　：合成終了。
　　評価実験中。

D．考察

Exendin-(9-39)、GW9508においては、複数

の誘導体で有効性検討を行った。
その結果、標識部位や修飾の変化により分子プローブとしての性能に大きな差が認められた。
来年度行う予定の、分子プローブの絞り込みと性能向上のための修飾等による最適化に期待が持てる結果であった。

E．結論

早急にGW1100の標識化を実現し、評価を行い、分子プローブの絞り込みを行う。
絞り込まれた分子プローブの最適化を行うことにより、目的を達成できる分子プローブの開発ができると確信している。

F．健康危険情報　特になし。

G．研究発表

1.論文発表　なし。

2.学会発表　なし。

H．知的財産権の出願・登録状況

1.特許取得

出願番号：特願2008-241889

発明の名称：膵島イメージング用分子プローブ前駆体及びその使用

2.実用新案登録　なし。

3.その他　なし。

研究成果の刊行に関する一覧表

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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GLP-1 receptor signaling protects pancreatic beta cells in intraportal islet transplant by inhibiting apoptosis

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Abstract

To clarify the cytoprotective effect of glucagon-like peptide-1 receptor (GLP-1R) signaling in conditions of glucose toxicity *in vivo*, we performed murine isogenic islet transplantation with and without exendin-4 treatment. When a suboptimal number of islets (150) were transplanted into streptozotocin-induced diabetic mice, exendin-4 treatment contributed to the restoration of normoglycemia. When 50 islets expressing enhanced green fluorescent protein (EGFP) were transplanted, exendin-4 treatment reversed loss of both the number and mass of islet grafts one and 3 days after transplantation. TUNEL staining revealed that exendin-4 treatment reduced the number of apoptotic beta cells during the early posttransplant phase, indicating that GLP-1R signaling exerts its cytoprotective effect on pancreatic beta cells by inhibiting their apoptosis. This beneficial effect might be used both to ameliorate type 2 diabetes and to improve engraftment rates in clinical islet transplantation.

Keywords: Exendin-4; Glucagon-like peptide-1; Cytoprotection; Apoptosis; Enhanced green fluorescent protein; Islet transplantation; Islet engraftment

Glucagon-like peptide-1 (GLP-1) is a physiological incretin, an intestinal hormone released in response to nutrient ingestion that stimulates glucose-dependent insulin secretion [1,2]. Recent studies have demonstrated that GLP-1 has beneficial effects on pancreatic beta cells [3–6], one of which is inhibition of apoptosis of native beta cells. *In vitro* studies have shown that GLP-1 receptor (GLP-1R) signaling has various beneficial actions such as ameliorating ER stress [7,8] and oxidative stress [9]. However, demonstration of the *in vivo* cytoprotective effect in an animal model of type 2 diabetes (T2DM) is problematic because enhancement of GLP-1R signaling reduces blood glucose levels due to its insulinotropic action [4,5], glucagonostatic action on alpha cells [10], and improvement of insulin sensitivity [11], which makes it difficult to evaluate the cytoprotective effects in the same conditions of glucose toxicity.

To clarify the cytoprotective effect of GLP-1R signaling *in vivo*, we used a murine isogenic islet transplantation model using a suboptimal number of islets together with exendin-4 treatment, a degradation-resistant GLP-1 analog [12]. As isogenic islet grafts in the natural course of the early posttransplant period are easily lost due to various physiological stress [13], various suboptimal number of islet transplantation can lead proper engraftment during the transplantation process without regard for the effects of improved blood glucose levels following transplantation

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of an optimal number of islets. When a higher suboptimal mass of islets is transplanted, blood glucose levels remain high during the early posttransplant period, changing to normoglycemic only during the late posttransplantation period if the engrafted mass is sufficient but remaining in the hyperglycemic state if the engrafted mass is insufficient. Thus, when a suboptimal number of islets are transplanted together with exendin-4 treatment in the early posttransplant period when the recipient is hyperglycemic, its indirect action on glucose tolerance can be excluded and its cytoprotective effect can be evaluated by monitoring the blood glucose levels. In addition, bio-imaging technology permits comparison of the number and mass of islets before and after transplantation.

In the present study, we evaluated the cytoprotective effect of GLP-1R signaling *in vivo* in pancreatic beta cells using a murine isogenic islet transplantation model. We used a suboptimal mass of transplanted islets with and without exendin-4 treatment, and monitored blood glucose levels. We also compared the number and mass of islet grafts with and without exendin-4 treatment under conditions of hyperglycemia.

Materials and methods

Animal care. All experiments were approved by the Kyoto University Animal Care Committee.

Animals. Male C57BL/6JJcl mice (CREA, Japan) aged 8–10 weeks were used as recipients and donors. Male transgenic C57BL/6-EGFP mice aged 8–10 weeks were also used as donors. The mice were obtained from Dr. Masaru Okabe (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) [14]. Recipient animals were rendered diabetic by a single intraperitoneal injection of streptozotocin (Sigma–Aldrich, USA), 120 mg/kg body weight, freshly dissolved in 10 mM citrate buffer (pH 4.2). Mice with a blood glucose concentration greater than 20 mmol/l for 2 consecutive days were used as recipients. Blood glucose concentrations were determined by glucose meter (Glucocard, Arkley, Japan).

Islet isolation, islet transplantation, and exendin-4 treatment. Islets were isolated, as previously described [15]. Recipient mice were anesthetized by isoflurane (Forane, Abott, Japan). Fresh islets in a volume of 400 μl PBS solution were injected into the portal vein and transplanted into the right hepatic lobe as previously described [15,16]. Exendin-4 at a dosage of 1.0 nmol/kg body weight was administered intraperitoneally once daily in the morning for 14 days.

Oral glucose tolerance test (OGTT). After fasting for 16 h, a basal blood sample was collected and the mice received glucose (1.5 g/kg body weight) orally; additional blood samples were collected at 15, 30, 60, 90, and 120 min after glucose loading.

Evaluation of number and mass of EGFP-expressing islet grafts. Islets isolated from transgenic C57BL/6-EGFP mice were first observed by fluorescence microscope BZ-8000 (KeyEnce, Japan) before transplantation; the area of fluorescence was measured using Image J software (National Institute of Mental Health, USA). Livers bearing islet grafts were removed and sectioned into 500-μm slices and serialized; digitalized photographs of all sections were taken. The number of EGFP-positive islets in each liver section was then counted, excepting those appearing by their position to be part of an islet in an adjacent section. The total area of fluorescence of all islets was then measured.

Measurement of beta-cell mass using immunohistochemistry. The right hepatic lobes were fixed, embedded in paraffin, cut in blocks at regular intervals, and sectioned into 5-μm sections. Deparaffinized sections were incubated with a polyclonal guinea pig anti-insulin antibody (Dako, USA), then with a biotinylated goat anti-guinea pig antibody (Vector, USA), and then with a streptavidin peroxidase conjugate and substrate kit (Dako). The total liver area and total insulin-positive beta-cell area were quantified using Image J software.

Apoptosis detection. TUNEL staining was performed using Apoptosis detection Kit (Takara Bio, Japan).

Statistical analyses. All data are presented as means ± SEM. Statistical analyses were performed by an unpaired *t*-test. p value of less than 0.05 was considered significant.

Results

Exendin-4 decreased the number of islet grafts required to restore normoglycemia

To evaluate the cytoprotective effect of GLP-1R signaling during the early posttransplant phase, we performed isogenic islet transplantation and observed blood glucose levels during the late posttransplant phase. Previous reports have shown that transplantation of only 75 islets can normalize blood glucose levels if the majority becomes engrafted [17], but because many islets are lost due to various stress such as glucotoxicity, transplantation of 75 islets is insufficient for restoration of normoglycemia. In our preliminary experiments, while some recipients showed improved blood glucose levels when 200 islets were transplanted (data not shown), no recipients showed any change in blood glucose levels when 150 islets were transplanted (Fig. 1A). Thus, 150 islets was chosen as an appropriate suboptimal number for use in these transplantation experiments. In addition, all mice transplanted with 150 islets together with exendin-4 treatment became hyperglycemic soon after transplantation but became normoglycemic approximately 14 days after transplantation (Fig. 1A). The responsibility of the islet grafts in exendin-4-treated mice in maintenance of glucose tolerance is demonstrated by the immediate return to hyperglycemia after removal of the right hepatic lobe (Fig. 1B). In addition, OGTT was similar in mice receiving 150 islets with exendin-4 treatment and sham-operated control mice (Fig. 1C). These results indicate that exendin-4 treatment played a crucial role in the restoration of normoglycemia by protecting the transplanted islets from damage during the early posttransplant phase.

Detection of fluorescence of transplanted Islets of transgenic C57BL/6-EGFP mice

To clarify the cytoprotective effect of exendin-4 *in vivo*, we established a novel system whereby the total number and the total mass of islets can be compared before and after transplantation by using fluorescent islets isolated from transgenic C57BL/6-EGFP mice. These mice exhibited normal pancreas and islet morphology and well as normal glucose tolerance by OGTT (data not shown).

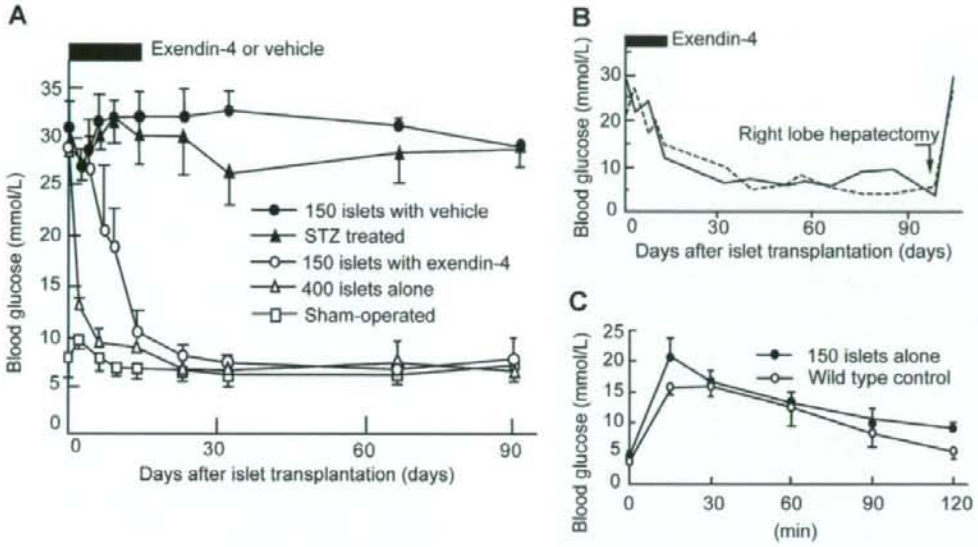

Fig. 1. Exendin-4 reduced the number of islets required for transplantation to restore normoglycemia in STZ-induced diabetic mice. (A) Blood glucose concentrations were measured in mice transplanted with 150 islets together with 1 nmol/kg exendin-4 treatment (open circles, $n = 4$), 400 islets alone (filled triangles, $n = 5$), 150 islets alone (filled circles, $n = 3$), STZ-treated only (filled triangles, $n = 5$), and Sham-operated C57BL/6 mice (open squares, $n = 5$). (B) Right hepatic lobe was resected from two recipients transplanted with 150 islets together with exendin-4 treatment on Day 90 to clarify the effect of the islet grafts on glycemic control. (C) OGTT was performed on Day 30 in recipients transplanted with 150 islets together with exendin-4 treatment and in sham-operated wild-type C57BL/6 mice ($n = 3$ for each).

Transplanted islets of transgenic C57BL/6-EGFP mice are traceable and measurable in both number and mass

To confirm traceability and measurability of the transplanted islets, intraportal transplantation of islets isolated from transgenic C57BL/6-EGFP mice was performed. One day and three days after transplantation, the right hepatic lobe was resected and sliced, and each slice was photographed by fluorescence microscope (Fig. 2A–C). Liver slices containing islet grafts were then immunostained for insulin. The area of fluorescence (Fig. 2A) coincided with that of the islet beta cells stained for insulin (Fig. 2B), demonstrating traceability of the islets. The number of islet grafts in the liver after transplantation was then compared. When 25, 50, or 75 islets were transplanted, the total number of islet grafts detected in the liver was 24.3 ± 0.3, 48.7 ± 0.8 and 73.3 ± 0.3, respectively ($n = 3$ for each), demonstrating a significant ($p < 0.0001$), strong correlation ($r = 1.000$) between the number of detected islet grafts in the liver and the number of transplanted islets (Fig. 2E). In addition, because the area of fluorescence coincided with that of immunostained islets (Fig. 2A–C), the total area of fluorescence reflected the total area mass of the islets, allowing comparison of total islet mass before and after transplantation. When 25, 50, and 75 islets were transplanted, the total area mass of islets before transplantation was 2.01 ± 0.04, 4.11 ± 0.01, and 5.89 ± 0.09 (mm^2), respectively, while that of islet grafts in the liver were 2.00 ± 0.02, 4.28 ± 0.07, and 6.08 ± 0.03 (mm^2), respectively ($n = 3$ for each), demonstrating a significant ($p < 0.0001$), strong ($r = 0.998$) correlation between before and after transplantation (Fig. 2F).

Exendin-4 reduced loss of transplanted islets from transgenic C57BL/6-EGFP mice during the early posttransplant phase

To exclude the indirect effect of exendin-4 through its effect on blood glucose levels, we reduced the number of the transplanted islets to 50. When 50 islets of transgenic C57BL/6-EGFP mice were transplanted with or without treatment of exendin-4 into STZ-induced diabetic mice, the blood glucose levels were not significantly different on 1 day (Day 1) ($n = 3$, 27.1 ± 0.3 vs 27.8 ± 0.1 (mmol/l), $p = 0.193$) or 3 days (Day 3) after transplantation ($n = 3$, 28.7 ± 0.2 vs 28.7 ± 0.3 (mmol/l), $p = 0.936$). The number and the total area mass of the islet grafts in livers resected on Day 1 (figure not shown) and Day 3 (Fig. 3A and B) were then examined. The number of islet grafts with treatment of exendin-4 (Ex(+)) showed 9.4% and 19.9% increases on Day 1 ($n = 3$ for each, 46.7 ± 0.51 vs 42.0 ± 0.33, $p < 0.05$) and Day 3 ($n = 3$ for each, 44.6 ± 0.36 vs 34.7 ± 0.84, $p < 0.01$) (Fig. 3C) compared to those without treatment (Ex(−)). Ex(+) islet grafts exhibited 29.0% and 31.9% more total area mass on Day 1 ($n = 3$ for each, $69.5 \pm 2.5\%$ vs $53.3 \pm 2.1\%$ (normalized to the total fluorescence area mass before transplantation), $p < 0.05$) and Day 3 ($n = 3$ for each, $64.5 \pm 2.6\%$ vs $26.9 \pm 1.1\%$, $p < 0.05$) (Fig. 3D), respectively, than Ex(−).

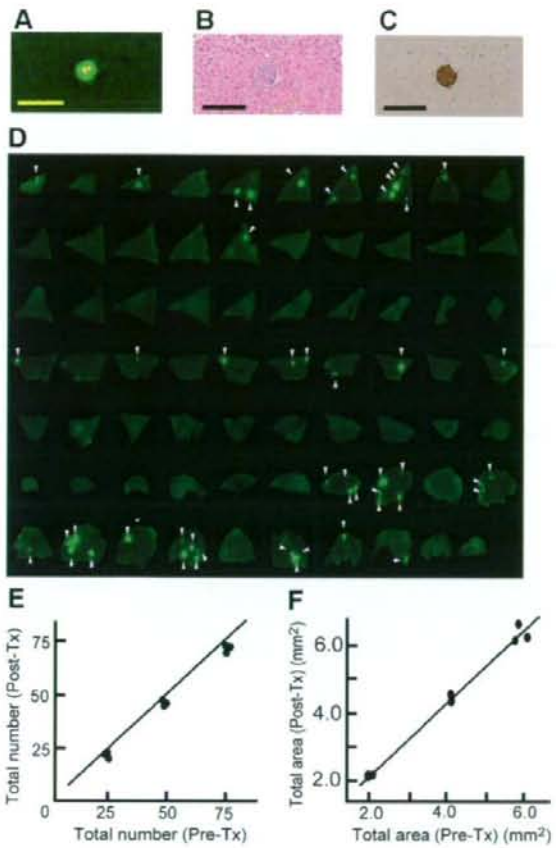

Fig. 2. Islets of transgenic C57BL/6-EGFP mice detected and measured by fluorescence microscopy. (A–C) Photographs of islet grafts in liver. Fluorescent islet (A), HE (B) and insulin immunostaining (C). Scale bar: 200 μm. (D) Representative photographs after transplantation with 50 islets of liver slices under fluorescence microscope. Fluorescent islets are indicated by arrowhead. (E, F) The total number (E) and the total area mass (F) of all EGFP-expressing islets before transplantation compared with fluorescent islet grafts in liver after transplantation ($n = 3$).

Area of islet grafts in liver with and without exendin-4 treatment compared by conventional immunohistochemical analysis

Conventional total area mass measurements, the ratio of the area of islet beta cells to that of the examined liver slice, was compared by immunohistochemical analysis using limited liver sections on Day 1 and Day 3 (Fig. 4A(a and c) and B (e and g)). The conventional relative area mass in Ex(+) was 32.0% and 44.7% higher on Day 1 ($n = 3$ for each, $0.07830 \pm 0.0003\%$ vs $0.0533 \pm 0.0003\%$, $p < 0.05$) and Day 3 ($n = 3$ for each, $0.0680 \pm 0.0009\%$ vs $0.0380 \pm 0.0043\%$, $p < 0.01$) than Ex(−) (Fig. 4C). The ratio of conventional relative area mass of Ex(+) to that of Ex(−) on Day 1 and Day 3 was comparable to the results of measurement of total area mass measured by our novel method.

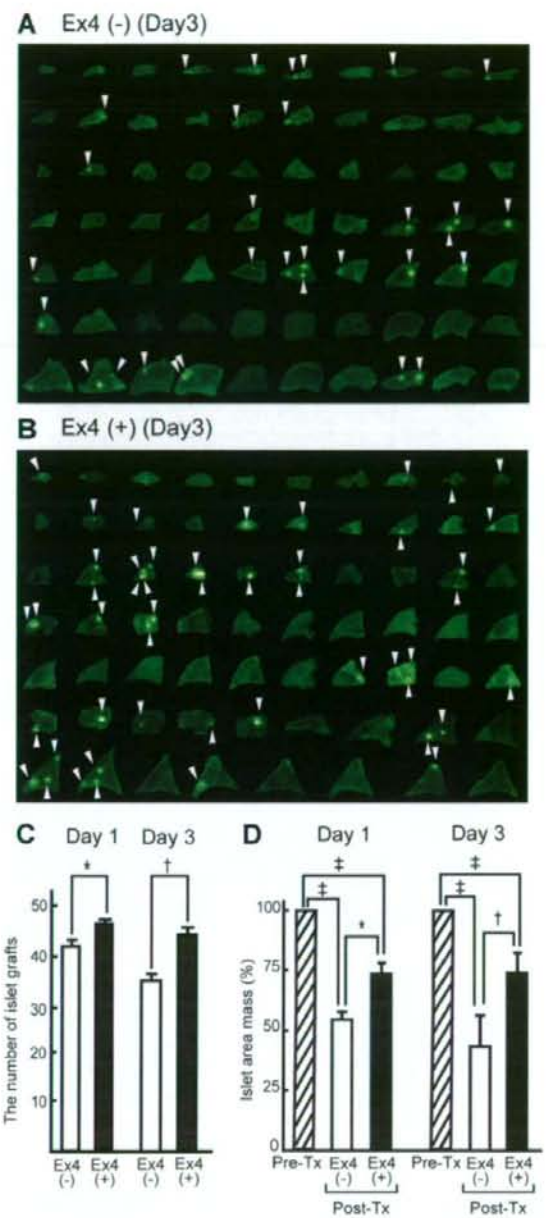

Fig. 3. Exendin-4 preserved transplanted islets during the early posttransplant period in number and total area mass. (A–B) Representative photographs of fluorescent islet grafts in all liver slices from exendin-4-treated mice (Ex4(+)) (A) and -untreated mice (Ex4(−)) (B) on Day 3. (C) Number of islet grafts in liver slices on Day 1 ($n = 3$) and Day 3 ($n = 3$) in Ex4(+) and Ex4(−). $^{*}p < 0.05$ and $^{\dagger}p < 0.01$ vs Ex4(−). (D) Total area mass of all fluorescent islet grafts in liver slices on Day 1 ($n = 3$) and on Day 3 ($n = 3$) in Ex4(+) and Ex(−). Data after transplantation (Post-Tx) and before transplantation (Pre-Tx) are also compared. $^{*}p < 0.05$ and $^{\dagger}p < 0.01$ vs Ex4(−), $^{\ddagger}p < 0.01$ vs Pre-Tx.

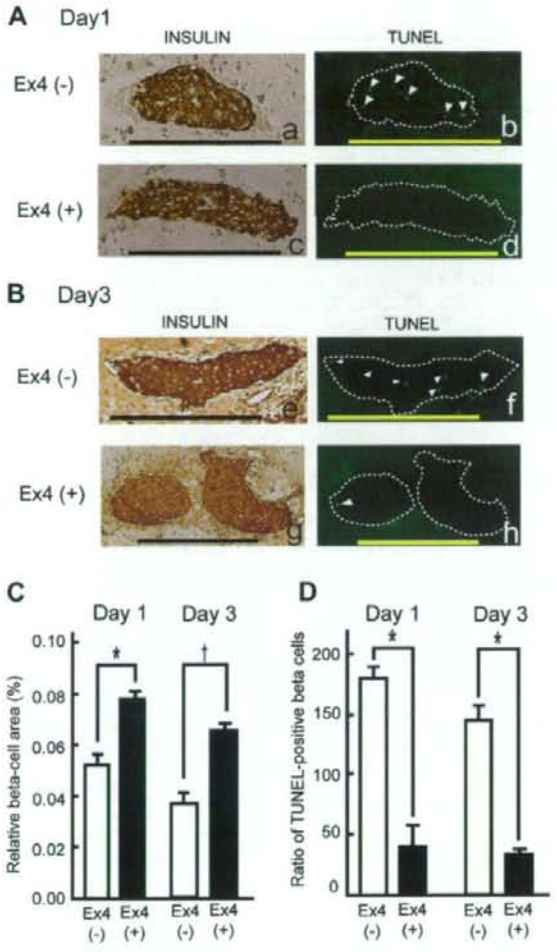

Fig. 4. Exendin-4 treatment reduced beta-cell apoptosis after intraportal islet transplantation. (A–B) Representative photographs of liver sections on Day 1 (A) and Day 3 (B) from Ex4(+) mice and Ex4(−) mice stained for insulin (a, c, e, and g) and TUNEL-assay (b, d, f, and h) are shown. TUNEL-positive cells are indicated by arrowhead. Scale bar: 200 μm. (C) Ex4(+) showed significantly greater beta-cell mass than Ex4(−) on Day 1 ($n = 3$ for each) and Day 3 ($n = 3$ for each). $^{*}p < 0.05$ and $^{\dagger}p < 0.01$ vs Ex4(−). (D) Ex4(+) showed a significantly greater decrease in the ratio of TUNEL-positive beta cells than Ex4(−) on Day 1 ($n = 3$ for each) (number/beta-cell area (mm^2)) and Day 3 ($n = 3$ for each) (number/beta-cell area (mm^2)). $^{*}p < 0.05$ vs Ex4(−).

Exendin-4 decreased the rate of apoptosis of beta cells introduced by intraportal islet graft after transplantation

To investigate the difference in area mass of transplanted islets in Ex(+) and Ex(−), the rate of apoptosis of beta cells of islet grafts on Day 1 and Day 3 was examined (Fig. 4A and B). The rate of apoptosis of TUNEL and insulin-double positive cells was significantly lower on Day 1 ($n = 3$ for each, 246.5 ± 5.5 vs 36.4 ± 3.6 (number/beta-cell area (mm^2), $p < 0.01$) and on Day 3 ($n = 3$ for each, 148.7 ± 17.7 vs 41.3 ± 1.3 (number/beta-cell area (mm^2), $p < 0.01$) with Ex(+) than Ex(−) (Fig. 4D).

Discussion

In the present study, we demonstrate that GLP-1R signaling has a cytoprotective effect in the posttransplant period using a murine islet transplantation model. Exendin-4 treatment during the early posttransplant hyperglycemic phase contributed to restore normoglycemia during the late posttransplant phase in STZ-induced diabetic mice receiving a suboptimal graft of 150 islets. In addition, the total number and total area mass of the islet grafts both on Day 1 and Day 3 was significantly greater in Ex(+) than in Ex(−). The finding that the rate of apoptosis was less in Ex(+) than in Ex(−) both on Day 1 and Day 3, when their blood glucose levels were yet unchanged, demonstrates that GLP-1R signaling inhibits apoptosis *in vivo* under conditions of glucose toxicity.

Murine islet transplantation is an ideal model for investigating the cytoprotective effect of exendin-4 on transplanted pancreatic beta cells *in vivo*. Although isogenic islets injected into the portal vein are spared rejection by the immune reaction, the cells may succumb to apoptosis due to various stress factors including hypoxia [18,19], inflammation [20,21], and mechanical shear stress [22,21] before engraftment. The efficacy of exendin-4 treatment on posttransplant hyperglycemic status in this transplantation model can be quantified using different suboptimal numbers of islets because the posttransplant glycemic condition directly reflects the mass of engrafted islets. The number and mass of transplanted islets can be traced because isolated islets can be labeled and examined before transplantation. Thus, this murine islet transplantation model allows observation of the direct effect of the cytoprotective effect on beta cells *in vivo*.

In this study, we established a method for tracing the transplanted islets of transgenic C57BL/6-EGFP mice in liver sections under fluorescence excitation. Our findings reveal that the area of fluorescence of islet grafts in liver coincides with that of insulin immunostaining (Fig. 2A–C), which areas before transplantation correlate highly with those after transplantation (Fig. 2F). Observation of each islet grafts before and after transplantation is definitive for evaluation of the cytoprotective action, which is not practicable by the conventional immunohistochemical method due to the necessarily limited observation of the organ.

We have also shown that the natural course of islet engraftment in the early posttransplant period can involve loss of about half of the transplanted beta cells. Recently, Eich et al. reported evaluation of islet mass by positron-emission tomography using islets labeled with ^{18}Fluorodeoxyglucose, and found that almost 50% of the transplanted islets in the graft were lost [23], which is comparable with our data. Although about 30% of the graft was found to be lost even with exendin-4 treatment on Day 1, the rate

of apoptosis remained lower, resulting in a mass of engraftment more than adequate for normoglycemia thereafter. This finding is encouraging regarding the possible clinical use of exendin-4 in islet transplantation therapy in human subjects [24,25].

Although exendin-4 is already in clinical use for treatment of T2DM [26], this cytoprotective effect on beta cells *in vivo* also certainly functions independently of other actions in T2DM. The mass of islets is usually already decreased when patients are diagnosed with T2DM [27]. Thus, exendin-4 treatment used in the early phase of development, when glycemic tolerance is yet normal, might hamper the progression of T2DM.

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Factors responsible for age-related elevation in fasting plasma glucose: a cross-sectional study in Japanese men

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Abstract

To evaluate the factors associated with age-related increase in fasting plasma glucose (FPG) in Japanese men with normal fasting glucose, we measured FPG, fasting immunoreactive insulin, glycated hemoglobin, total cholesterol, triglyceride, and high-density lipoprotein cholesterol levels in health check examinees. Subjects with FPG less than 6.1 mmol/L together with glycated hemoglobin less than 5.6% were enrolled in the study. The homeostasis model assessment of insulin resistance (HOMA-IR) and HOMA-β were used as the indices of insulin sensitivity and insulin secretion, respectively. Fasting plasma glucose increased significantly with age ($r = 0.30$, $P < .0001$), and HOMA-β decreased significantly with age ($r = 0.24$, $P < .0001$). The HOMA-IR had no significant relation with age ($r = 0.06$, not significant), whereas body mass index and serum triglyceride were associated with HOMA-IR ($r = 0.49$, $P < .0001$ and $r = 0.33$, $P < .0001$, respectively). Thus, in Japanese male subjects with normal fasting glucose, it is suggested that the FPG increment with age is associated with decreased β-cell function rather than with insulin resistance. Further analyses were performed by comparing 3 groups: low FPG (FPG <5.0 mmol/L), high FPG (5.0 ≤ FPG < 5.6 mmol/L), and mild impairment of fasting glycemia (mild IFG) (5.6 ≤ FPG < 6.1 mmol/L). The insulin levels in mild IFG and high FPG were significantly higher than in low FPG ($P < .001$), but those in mild IFG were similar to those in high FPG. Analysis of the 3 subgroups revealed that, whereas insulin sensitivity was impaired more in high FPG, there was little compensatory increase in insulin in mild IFG, suggesting that β-cell function is already deteriorated when the FPG level is greater than 5.6 mmol/L.

1. Introduction

Type 2 diabetes mellitus is characterized by both decreasing insulin secretion and insulin sensitivity, partly due to genetic factors [1-3]. Although diabetes is a worldwide health problem [4], it is clear that there are ethnic differences in the pathophysiology of the decreasing glucose tolerance characteristic of its development [5]. Factors responsible for glucose intolerance occur from a prediabetic state: impaired glucose regulation according to the World Health Organization classification. Impaired glucose regulation comprises 2 subgroups: impaired fasting glycemia (IFG) characterized by increasingly impaired fasting plasma glucose (FPG) with 2-hour plasma glucose (2h-PG) within normal limits and impaired glucose tolerance (IGT) characterized by increasingly impaired 2h-PG [6,7]. We previously reported that insulin secretory capacity and insulin sensitivity are both decreased in Japanese subjects with IFG [8-10]. Although β-cell function and insulin sensitivity may well begin to deteriorate earlier, there are few studies of the normal glucose tolerance (NGT) population. Fasting plasma glucose is known to increase with age [11], and both insulin secretory capacity and insulin

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