な場合には USPIO を使用して同様の実験を行う。USPIO は細網内皮系での取り込みが少なく、より生理的な状態での観察が可能となる。

D. 考察

理想的な超早期診断は、膵β細胞以外に病態の進行から行うことも重要であるが、これまでそのようなアプローチは存在しない。よって、本研究の意義は大きい。

E. 結論

本アプローチの推進により、糖尿病の超早期診断における新規標的の探索がすすむと同時に、超早期診断をβ細胞量と病態の両面からとらえることができると考えられる。

F. 健康危険情報　特になし。

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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超高磁場MRIによる膵島撮像法の開発」

分担研究者　名前　松田 哲也　　所属　京都大学情報学研究科

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。膵島部の位置の識別が可能な実験系の構築により、様々な撮影法の適用とその結果の確認が容易となった。また、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、実際のMRI撮像を通じて、Mn や Gd をはじめとする様々なMR造影核種を利用した新規標識プローブの評価を行うことになるが、このような研究の最終目標に到達するため、1）摘出膵組織を対象とした高空間分解能 MRI 画像の撮影法の確立、2）標識プローブのMRI造影効果に関する *in vitro* 評価、3）標識プローブ投与後の膵組織を対象とする膵島量の定量化に最適な撮像法・撮影パラメタの検討、4）標識プローブを用いた *in vivo* の MRI 撮影の4段階に分けて研究を進めることとした。

本年度前半は、本学に新しく導入された超高磁場 MRI 装置の環境整備を行い、動物や植物の組織片および固定標本を対象に200mmから50mm程度までの高空間分解能 MRI 画像の撮影に関する基礎的検討を行った。本年度後半にはマウスの摘出膵組織を対象とする撮影を開始し、MRIに関する本研究課題の基礎となる1）摘出膵組織を対象とした高空間分解能 MRI 画像の撮影法の確立について検討した。

既に臨床用 MRI で利用されている様々な撮影法に超高磁場の特性を考慮した撮影パラメタを適用して撮影を繰り返したが、マクロスコピック(巨視的)には形態的な特徴に乏しい摘出膵組織片では、得られた MRI 画像中で膵島部の特徴を判定することが困難であり、しかも高空間分解能で信号/雑音比の低いMRI画像においては画像のノイズ成分との明確な判別ができず、膵島部を識別することは困難であることが明らかとなった。

そこで、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する実験系を構築した。

（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した。

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を構築することにより、MRI 画像の撮影パラメタ選択の成否が画像から判定できるようになった。現在は、まだ数個の摘出膵組織片を対象とした予備実験段階であるが、MRI 撮影で一般的な T1 強調像でも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ている

D．考察
摘出膵組織片を対象とした MRI による膵島イメージングは、米国糖尿病学会で 14T の高磁場における T2 強調像を用いたアプローチが報告されているが、われわれの予備検討ではより簡便な T1 強調像による描出の可能性が示唆された。未だ確定的な結果ではないものの、膵島部の位置の識別が可能な実験系の構築により、様々な撮影法の適用とその結果の確認が容易となり、*in vitro* の膵島イメージング法として最適な撮影法の確立が期待できる。

E．結論
高空間分解能 MRI を用いて *in vitro* の膵島イメージングを行うために、膵島部の位置の識別が可能な実験系を構築した。また、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た。

F．健康危険情報　特になし。

G．研究発表
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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）

分担研究報告書

「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」

分担研究者　名前：斉藤 美佳子　　所属：東京農工大学　生命機能科学部門

研究要旨：糖尿病は代表的な生活習慣病の一つであり、我が国において増加し続けている。この対策には、早期診断のため、非侵襲的に生体内の膵島量を定量化するイメージング技術が必要である。本研究では、グルコース輸送担体 2 の基質として 2-デオキシグルコースに着目し、この標識方法について検討することを目的とする。特にβ細胞を識別できるような高感度のプローブを設計することで、生体内の膵島の識別が可能となると思われる。

A．研究目的

糖尿病を早期に診断することができれば、効果的な糖尿病発症予防が可能となる。そこで本研究は、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ことを究極の目的とし、標的候補としてグルコース輸送担体 2 を選び、特に基質として 2-デオキシグルコースを基質とした標識方法について検討することを目的としている。

蛍光グルコース（2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D- glucose (2-NBDG) は、トランスポーター変異株を用いた実験により、大腸菌ではマンノーストランスポーター、及びグルコーストランスポーターを介して取り込まれることが示されている（unpublished data)。一方、哺乳動物細胞によっても取り込まれることが示されており、D-グルコースによる競争的阻害反応によってGLUT系のトランスポーターを介して取り込まれていることが推定されている。これらの結果に基づき、2-NBGDを哺乳動物細胞におけるグルコース代謝活性の可視化に利用する試みが報告されている。しかし、哺乳動物細胞では非特異的取り込み、あるいは吸着によると思われる蛍光がしばしば観測されるため、実験条件の最適化が困難であった。そのため、コントロールとしての L-グルコース誘導体の 2-NBDG（2-NBD-LG）の開発が望まれていた。幸い、㈱タンパク研の山本によって、2NBD-LG 合成の原料である L-グルコサミンが新規合成され、それを用いた2-NBD-LGの合成にも成功したとの報告がなされた（私信)。L-グルコサミンは極めて貴重な試薬であるので、これとNBD-Cl とを反応過程、および生成物の精製過程の効率を向上させることが重要となった。本研究は、以上の背景により、NBD-Cl に代えて NBD-F を用いることによって合成収率の向上を図ろうとして実施されたものである。

B．研究方法

始めに反応時の溶媒の種類を検討した。メタノール、アセトニトリル、酢酸メチル、酢酸エチル、エタノールについて比較検討した。D-グルコサミン 200 mg、$NaHCO_3$ 100 mg、H_2O 4 mL を混合し溶液 A とした。50 mL ナスフラスコに NBD-F 23 mg

を入れ、これに上記の溶媒のいずれかを 2 mL を加えて溶解したのち、溶液 A を 400 mL 加え、37℃、暗所で攪拌しながら反応させた。一定時間毎に反応液 10 mL を分取し、アセトニトリルで 1/20 に希釈して、HPLC で分析した。

C．研究結果

HPLC 分析チャートより、2-NBDG と思われるピークの高さから反応量を見積もった。メタノール、アセトニトリル、酢酸メチル、酢酸エチル、エタノールについて 2-NBDG の生成量を調べたところ、エタノールの場合が最大で、以下、エタノール、アセトニトリル、酢酸エチルの順になり、酢酸メチルでは殆ど生成されなかった。従来から用いていたエタノールが最適であった。

次に反応の原料として NBD-Cl より反応性が高いと予想される NBD-F を用いることとした。NBD-Cl の場合は従来どおり 3 時間反応させた結果、最大生成収率は 10-12%であった。これに対して、NBD-F の場合は、30 分で最大生成収率 40-50%に達した。すなわち、反応速度、および反応効率ともに顕著な向上が認められた。

D．考察

2-NBDG のさらなる合成収率の向上とともに、今後 in vivo での評価が必要である。

E．結論

今後 L-グルコサミンと反応させる場合は NBD-Cl よりも、NBD-F を用いる方が有利であることが分かった。

F．健康危険情報

特になし。

G．研究発表

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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」

分担研究者　名前 平尾 佳　所属 アークレイ株式会社 研究開発本部第5チーム

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 5 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

本年度は、共同事業先の京都大学における、膵島イメージング標的分子探索が行われ、標的分子が具体化するまでは、分子プローブ作製のための基礎技術及び作製のための準備段階にあたる。

まず、この段階で、現状考えられる標的分子に対応した分子プローブを想定し、その分子プローブを確度高く作製ができる生産システムを構築し、選定された分子プローブを迅速に供給できる体制を確立する。

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

（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 5 種類の分子プローブ合成を行なっている。（平成 20 年 2 月現在）

・GPR40/GPR120 のリガンド GW9508
　：作製終了。
・GPR40 のリガンド GW1100
　：作製終了。

・GLP-1 受容体のリガンド Exendin(9-39)
：作製終了見込。
・小胞体モノアミノトランスポーター
VMAT2 のリガンド DTBZ
：標識法の決定終了。
・K_{ATP} チャンネル のリガンド Mitiglinide
：作製終了見込。

D．考察

Exendin(9-39)の ^{125}I 放射線標識を用いた、マウスでの臓器特異性と膵島細胞選択性の評価結果から、Exendin(9-39)は、臓器特異性、膵島細胞選択性を認めており、プローブとしての有用性が示されている。今後、現在合成中の各分子プローブを用いた評価を行う予定である。

E．結論

現在合成中の各分子プローブを用いた評価を行い、最適分子プローブの探索と、効率的な合成手法開発を続けて行く。最終的に、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　なし。
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

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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 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).

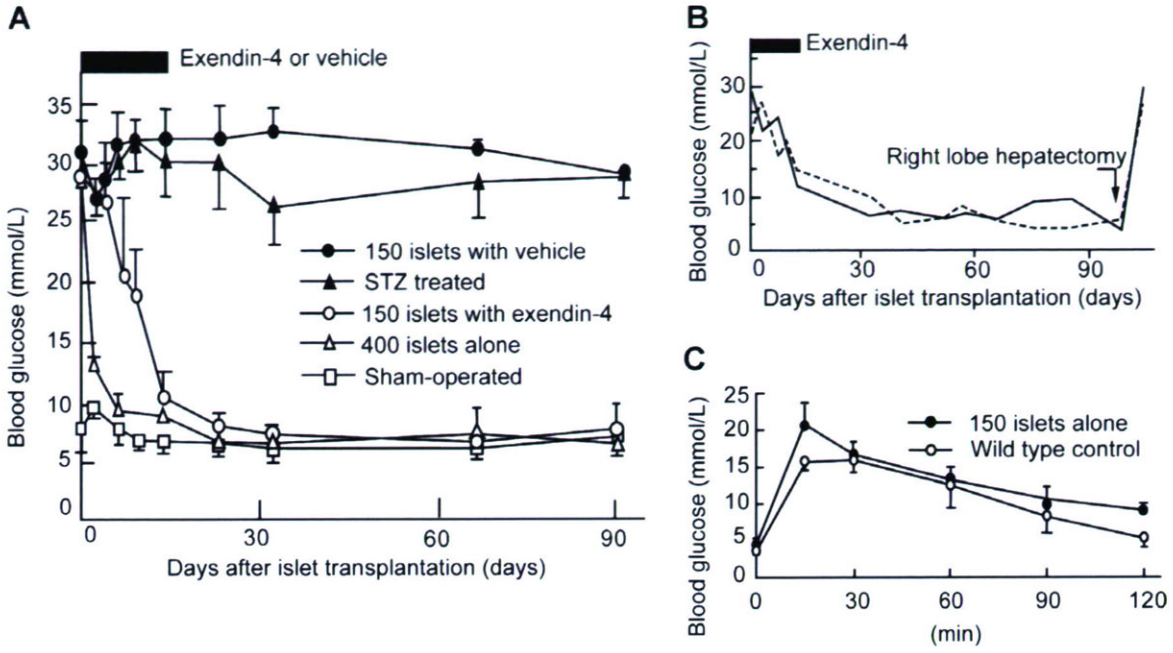

Fig. 1. Exendin-4 reduced the number of islets required for transplantation to restore normoglycemia in STZ-induced diabetic mice. (A) Blood glucose concentrations were measured in mice transplanted with 150 islets together with 1 nmol/kg exendin-4 treatment (open circles, $n = 4$), 400 islets alone (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(−).

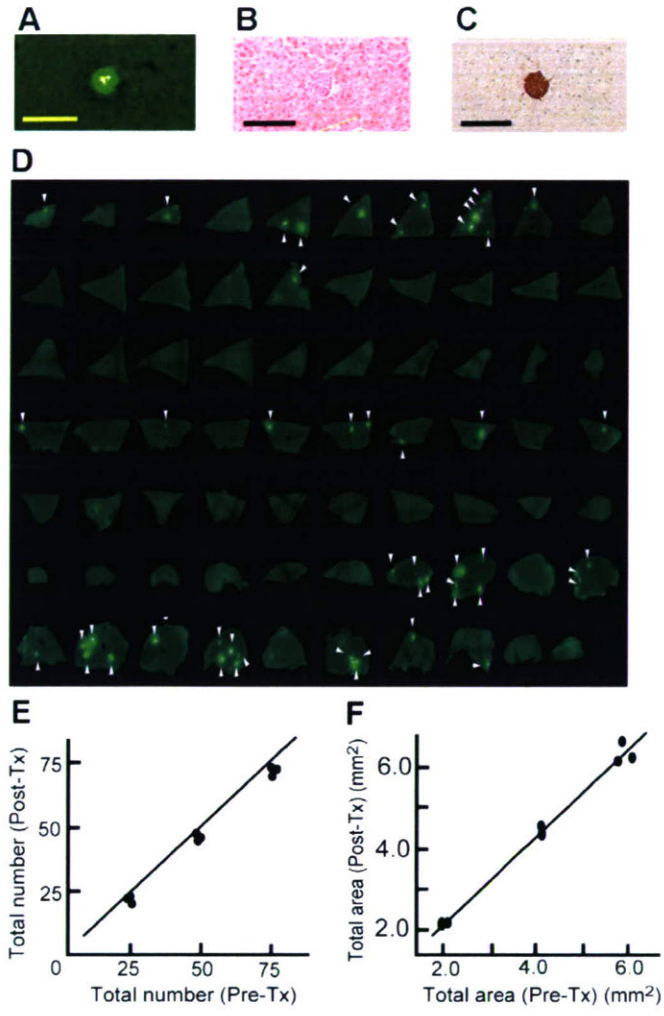

Fig. 2. Islets of transgenic C57BL/6-EGFP mice detected and measured by fluorescence microscopy. (A–C) Photographs of islet grafts in liver. Fluorescent islet (A), HE (B) and insulin immunostaining (C). Scale bar: 200 μm. (D) Representative photographs after transplantation with 50 islets of liver slices 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.

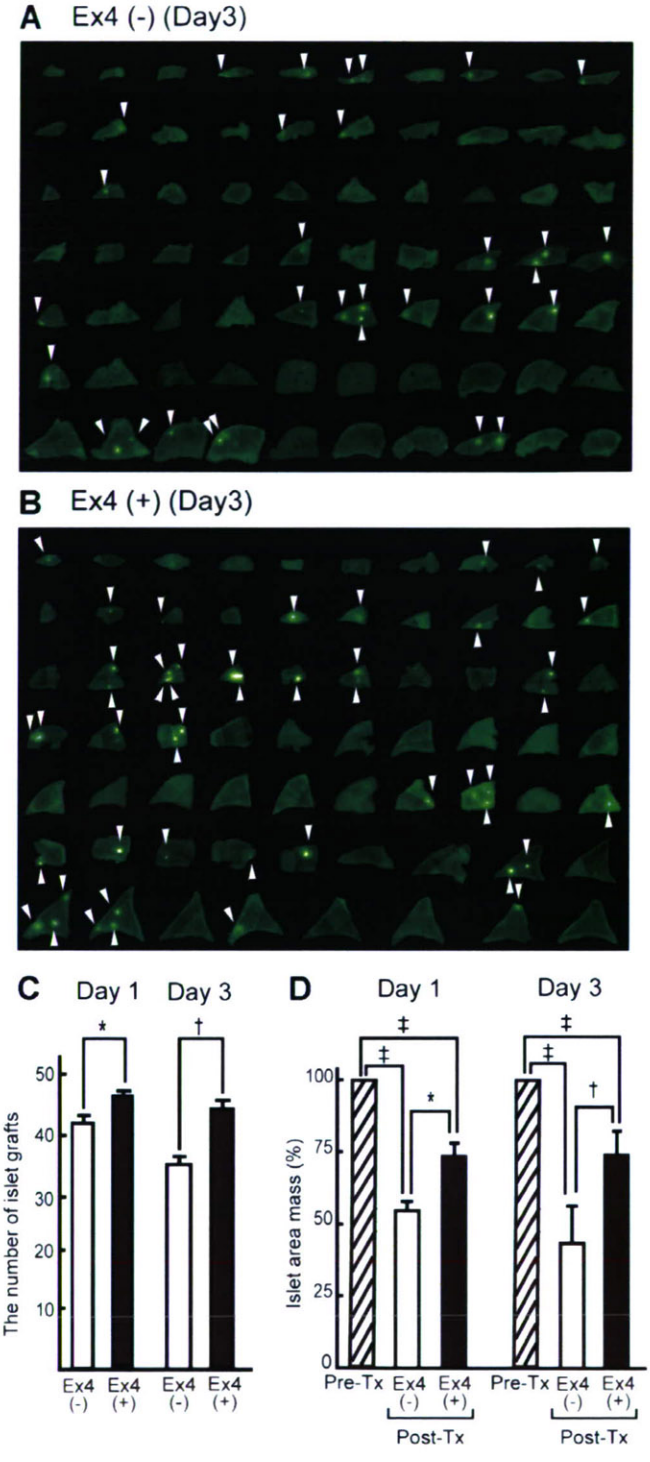

Fig. 3. Exendin-4 preserved transplanted islets during the early posttransplant period in number and total area mass. (A–B) Representative photographs of fluorescent islet grafts in all liver slices from exendin-4-treated mice (Ex4(+)) (A) and -untreated mice (Ex4(−)) (B) on Day 3. (C) Number of islet 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.

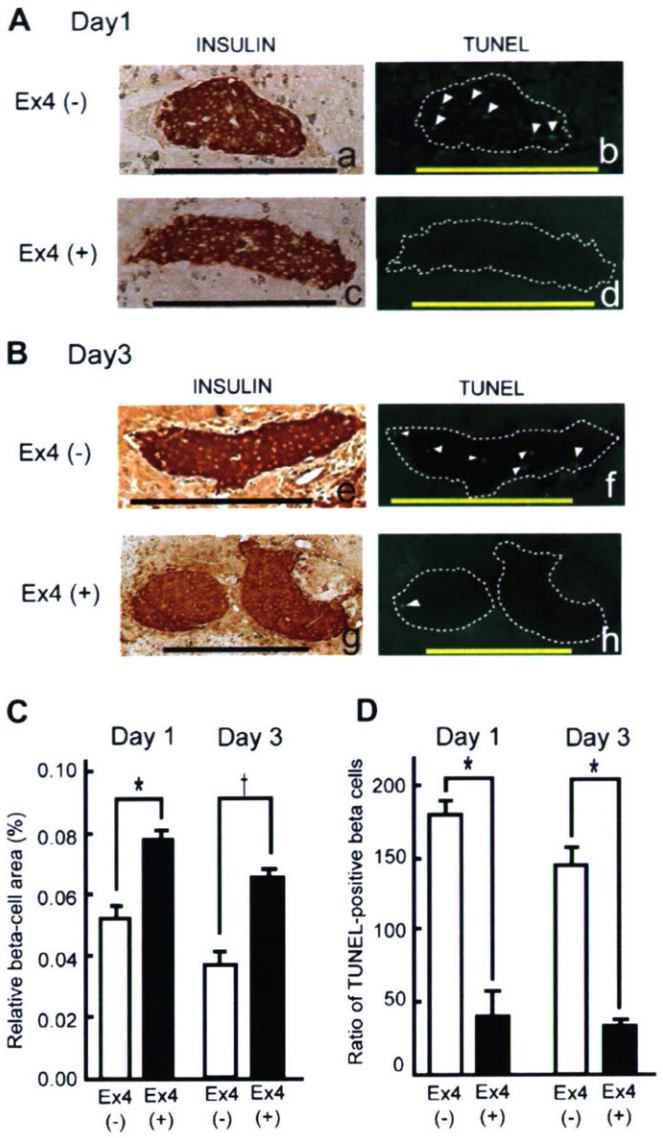

Fig. 4. Exendin-4 treatment reduced beta-cell apoptosis after intraportal islet transplantation. (A–B) Representative photographs of liver sections on Day 1 (A) and Day 3 (B) from Ex4(+) mice and Ex4(−) mice stained for insulin (a, c, e, and g) and TUNEL-assay (b, d, f, and h) are shown. TUNEL-positive 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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sensitivity are reported to decrease with age [12-14]. We have reported that some subgroups of Japanese NGT subjects show especially decreased β-cell function [15]. However, it is unclear whether deteriorated insulin secretion or insulin sensitivity is the primary factor in the increase in FPG during the period of development from NGT to IFG in Japanese.

In addition, the American Diabetes Association (ADA) lowered the cutoff value of IFG from 6.1 to 5.6 mmol/L [16]. Subjects with FPG from 5.6 to 6.1 mmol/L and with normal postprandial glucose level are categorized as having IFG in the ADA criteria, although they are categorized as having NGT in the criteria of the World Health Organization and the Japanese Diabetes Association. Thus, analysis of these subjects with mild IFG (mild impairment of fasting glucose) in view of insulin secretion and insulin sensitivity is crucial to elucidate the characteristic of subjects with borderline glucose dysregulation. To investigate the pathogenesis of prediabetes in Japanese, we compared insulin secretory capacity and insulin sensitivity in health check examinees exhibiting normal fasting glucose (NFG).

2. Subjects and methods

2.1. Subjects

Among health check examinees between 1993 and 2004 at Kyoto University Hospital, Kansai-Denryoku Hospital, and Kyoto Preventive Medical Center, 657 male subjects with FPG <6.1 mmol/L and glycated hemoglobin (HbA_{1c}) <5.6% were enrolled in the study (Table 1). Subjects with known history or signs of diabetes, previous gastrointestinal operation, liver disease, renal failure, endocrine disease, malignancy, hypertension, frequent heavy exercise, or history of medications before the study were excluded.

2.2. Measurements

Physical measurement (body height, body weight) and laboratory measurements (urine test, FPG, fasting immunoreactive insulin [F-IRI], HbA_{1c}, total cholesterol [TC], triglyceride [TG], and high-density lipoprotein cholesterol [HDL-C] level) were taken. The study was designed in compliance with the ethics regulations of the Helsinki Declaration. Blood samples were collected after overnight fasting for 16 hours [8]. Plasma glucose levels were measured by glucose oxidase method using the Hitachi Automatic Clinical Analyzer 7170 (Hitachi, Tokyo, Japan). Serum insulin levels were measured by radioimmunoassay (RIA beads II; Dainabot, Tokyo, Japan), which shows low cross-reaction with C-peptide of less than 0.005% and proinsulin less than 0.5% [8]. Glycated hemoglobin levels were measured by high-performance liquid chromatography methods. Serum TC, TG, and HDL-C levels were measured as reported previously [17]. To evaluate insulin resistance, we used the homeostasis model assessment of insulin resistance index (HOMA-IR) calculated by the formula FPG (in millimoles per liter) × IRI (in microunits per milliliter)/22.5. The HOMA-IR is a reliable measure of insulin resistance, correlating well with values obtained by glucose clamp and minimal model studies [18-20]. To calculate pancreatic β-cell function (HOMA β-cell), we used the formula 20 × IRI (in microunits per milliliter)/[FPG (in millimoles per liter) − 3.5] [18].

Table 1
Clinical characteristics of the subjects with NFG

	Data
n	657
Age (y)	44.9 ± 11.2
BMI (kg/m^2)	23.6 ± 2.8
HbA_{1c} (%)	4.8 ± 0.3
FPG (mmol/L)	5.1 ± 0.4
F-IRI (μU/mL)	5.2 ± 2.9
TC (mmol/L)	5.19 ± 0.88
TG (mmol/L)	1.45 ± 1.01
HDL-C (mmol/L)	1.45 ± 0.35

Data are mean ± SD.

2.3. Statistical analysis

Clinical data are expressed as mean ± SD. Analyses were performed using the STATVIEW 5 system (StatView, Berkeley, CA). Multiple regression analysis was used to compare age and FPG, HOMA-β, HOMA-IR, and body mass index (BMI). The same analysis was performed between HOMA-IR and BMI and TG. The NFG group was divided into low and high FPG and mild IFG, and the metabolic profiles were compared using analysis of variance. The data are expressed as mean ± SE. $P < .05$ is considered significant.

3. Results

3.1. Characteristics of the study population

As shown in Table 1, the mean age of the subjects is 44.9 ± 11.2 years and the mean BMI is 23.6 ± 2.8 kg/m^2. Among them, the number of subjects with BMI more than 30 are 22 (3.4%), concomitant with the representative epidemiologic studies in Japanese [21-23].

3.2. Correlation between age and FPG, HOMA-β, and HOMA-IR

Fig. 1A shows a positive relationship of FPG with age ($r = 0.30$, $P < .0001$; FPG [in millimoles per liter] = 0.011 × age + 4.6). Fig. 1B shows that HOMA-β has a negative correlation with age ($r = 0.24$, $P < .0001$), whereas there is no significant correlation between HOMA-IR and age ($r = 0.06$, not significant).

3.3. Correlation between HOMA-IR and BMI and serum TG levels

Fig. 2A, B shows that BMI and serum TG levels are associated with HOMA-IR ($r = 0.49$, $P < .0001$ and $r = 0.33$,