Combination of GLP-1 Receptor Activation and Glucagon Blockage Promotes Pancreatic β-Cell Regeneration In Situ in Type 1 Diabetic Mice

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Pancreatic β-cell neogenesis in vivo holds great promise for cell replacement therapy in diabetic patients, and discovering the relevant clinical therapeutic strategies would push it forward to clinical application. Liraglutide, a widely used antidiabetic glucagon-like peptide-1 (GLP-1) analog, has displayed diverse β-cell-protective effects in type 2 diabetic animals. Glucagon receptor (GCGR) monoclonal antibody (mAb), a preclinical agent that blocks glucagon pathway, can promote recovery of functional β-cell mass in type 1 diabetic mice. Here, we conducted a 4-week treatment of the two drugs alone or in combination in type 1 diabetic mice. Although liraglutide neither lowered the blood glucose level nor increased the plasma insulin level, the immunostaining showed that liraglutide expanded β-cell mass through self-replication, differentiation from precursor cells, and transdifferentiation from pancreatic α cells to β cells. The pancreatic β-cell mass increased more significantly after GCGR mAb treatment, while the combination group did not further increase the pancreatic β-cell area. However, compared with the GCGR mAb group, the combined treatment reduced the plasma glucagon level and increased the proportion of β cells/α cells. Our study evaluated the effects of liraglutide, GCGR mAb monotherapy, or combined strategy in glucose control and islet β-cell regeneration and provided useful clues for the future clinical application in type 1 diabetes.

1. Introduction

Pancreatic β-cell dysfunction and cell mass loss is a pivotal pathogenesis in both type 1 diabetes (T1D) and type 2 diabetes (T2D). Therefore, it is highly necessary to preserve β-cell function and expand β-cell mass for diabetes treatment. Glucagon-like peptide-1 (GLP-1) based therapies, including GLP-1 receptor agonists and dipeptidyl peptidase-4 inhibitors, have several beneficial effects on pancreatic β cells, including upregulating insulin gene transcription and biosynthesis, potentiating glucose-stimulated insulin secretion, and promoting β-cell regeneration by promoting β-cell proliferation, inhibiting β-cell apoptosis, and inducing stem cells to differentiate into β cells [1]. Recent researches have proven that GLP-1 overexpression or GLP-1 receptor agonists promoted β-cell regeneration via α- to β-cell transdifferentiation [2–4]. Although GLP-1-based therapy shows various beneficial effects in T2D animals and humans, these drugs cannot be used alone for T1D treatment. Whether GLP-1-based therapy has similar effects on β-cell protection, especially for β-cell regeneration in T1D, needs to be determined, and which therapy should be combined with GLP-1 should be evaluated.

REMD 2.59, a fully competitive antagonistic glucagon receptor (GCGR) monoclonal antibody (mAb), has a strong hypoglycemic effect in T1D and T2D rodents and nonhuman primates [5, 6]. A randomized clinical trial showed that REMD 477, another GCGR mAb that has an affinity for the GCGR equivalent to that of REMD 2.59, improved glycemic control in patients with T1D without serious adverse effects [7]. Our previous findings suggested that treatment with the GCGR mAb increased the β-cell mass by promoting α- to β-cell conversion [8]. However, GCGR mAb substantially increased pancreatic α-cell mass and plasma glucagon levels.
Notably, GLP-1 receptor agonists have the ability of inhibiting glucagon secretion.

In this study, we investigated the possible effect of liraglutide, a commonly used GLP-1 receptor agonist, on β-cell regeneration in T1D mice, and evaluated the combined effect of liraglutide with GCGR mAb. Our study provides new clues for the clinical therapy to maintain glucose homeostasis and promote pancreatic β-cell neogenesis in T1D patients.

2. Materials and Methods

2.1. Animals and Intervention. All the animal experiments were conducted at the Peking University Health Science Center (Beijing, China) and approved by the Institutional Animal Care and Use Committee. Eight-week-old male C57BL/6j mice (Vital River Animal Center, Beijing, China) were injected intraperitoneally with 150 mg/kg streptozotocin (STZ; Sigma-Aldrich, Saint Louis, MO) in a citric acid buffer (0.1 mol/L, pH 4.2) to establish a T1D model. The diabetic condition was confirmed if fasting blood glucose was 11.1 mmol/L or the random blood glucose was 16.7 mmol/L at least twice. The diabetic animals were treated for four weeks by intraperitoneal administration of liraglutide (0.2 mg/kg, twice daily; Novo Nordish, Denmark) or REMD 2.59 (a human GCGR mAb, 5 mg/kg, once a week; REMD Biotherapeutics, Camarillo, CA, USA) or combined of two agents or saline (as control).

B6.Cg-Tg(Gcg-cre)1Herr/Mmc (cre expression in pancreatic α-cell lineage) and B6.Rosa26-LSL-Cas9-tdTomato/J (when crossed to a cre recombinase-expressing strain, red fluorescence protein (RFP) expression is observed in the cre-expressing tissues) mice were crossed to generate pancreatic α-cell lineage-tracing mice, namely, glucagon-RFP mice.

There were 6-8 mice in each group. The diabetic mice were fasted 8 h for the measurement of fasting blood glucose by the glucose oxidase method. Specific ELISA kits were used for detecting insulin (Millipore, Saint Charles, MO, USA) and glucagon (R&D System, Minneapolis, MN, USA) according to the manufacturer’s instructions.

2.2. Immunofluorescence Staining. Pancreases were fixed with 10% formalin at 4°C overnight and embedded in paraffin, and 5 μm thick sections were prepared. The sections were incubated with primary antibodies at 4°C overnight and secondary antibodies for 1 h at room temperature, followed by washing and staining with DAPI (1 μg/mL, Sigma-Aldrich). Images were captured under a confocal fluorescence microscope (Leica TCS SP8 (Leica Microsystems Inc., Wetzlar, Germany)). Areas of insulin- and glucagon-positive cells were analyzed using ImageJ software.

The following antibodies and dilutions were used: rabbit antiguacagon (1:800, Cell Signaling Technology, Beverly, MA), mouse antiguacagon (1:400, Sigma-Aldrich), mouse anti-insulin (1:800, Sigma-Aldrich), mouse antiproliferating cell nuclear antigen (PCNA; 1:400, Cell Signaling Technology), rabbit anticytokeratin 19 (CK19; 1:400, Abcam), Alexa Fluor 594-conjugated AffiniPure goat antitmouse IgG (H+L) (1:800, Jackson ImmunoResearch Laboratories, West Grove, PA), Alexa Fluor 488-conjugated AffiniPure goat antirabbit IgG (H+L) (1:800, Jackson ImmunoResearch Laboratories), and Alexa Fluor 647-conjugated AffiniPure goat anti-guinea pig IgG (H+L) (1:800, Jackson ImmunoResearch Laboratories).

2.3. Statistical Analysis. Data are expressed as the mean ± S.E.M. Difference between two groups was analyzed by ANOVA followed by the post hoc Tukey-Kramer test or Student’s t-test (two-tailed) when appropriate. A p value < 0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Liraglutide and GCGR mAb Treatments Improve Diabetic Phenotype in T1D Mice. Compared with the normal control group, the body weight of the T1D mice displayed a significant decrease while the blood glucose level showed obvious increment (Figures 1(a)–1(c)). Neither liraglutide nor GCGR mAb had any effects on the body weights (Figure 1(a)). Liraglutide alone did not decrease the random blood glucose (Figure 1(b)), while displaying a trend to lower fasting blood glucose (p = 0.17, Figure 1(c)). The GCGR mAb significantly reduced the random and fasting blood glucose levels (Figures 1(b) and 1(c)). However, the combination of GCGR mAb and liraglutide (mAb+Lira) did not decrease the blood glucose levels further compared to the GCGR mAb group (Figures 1(b) and 1(c)).

After STZ treatment, plasma insulin level was significantly lower and plasma glucagon level increased slightly than those of the normal control. Liraglutide did not affect the insulin level or the glucagon level (Figures 1(d) and 1(e)). The GCGR mAb treatment significantly upregulated the plasma insulin level and glucagon level when compared with vehicle treatment in T1D mice (Figures 1(d) and 1(e)). The mAb+Lira combination did not increase the plasma insulin level when compared with mAb treatment alone (Figure 1(d)). Notably, the glucagon level is significantly lower in the combination group, when compared with that in the GCGR mAb group (Figure 1(d)).

3.2. Liraglutide and GCGR mAb Treatments Increase Pancreatic β-Cell Area of T1D Mice in Varying Degrees. Histological analysis of the pancreatic islets was carried out by using double-labeled immunofluorescence staining. STZ significantly decreased the entire islet area, with the level less than 1/3 of the normal control (0.364 ± 0.0096 vs. 0.113 ± 0.021 mm², Figures 2(a) and 2(b)). STZ strikingly decreased the β-cell area (0.00380 ± 0.00088 vs. 0.0748 ± 0.033 mm², Figure 2(c)) and increased the α-cell mass (0.0220 ± 0.00664 vs. 0.00993 ± 0.0034 mm², Figure 2(d)) compared with the normal control. Liraglutide treatment did not change the total islet area (Figure 2(b)), but liraglutide increased the β-cell area (0.00852 ± 0.0051 vs. 0.00380 ± 0.00088 mm², Figure 2(c)) and decreased the α-cell area (0.00993 ± 0.0034 vs. 0.0220 ± 0.0066 mm², Figure 2(d)), thus having a tendency to increase the β/α-cell area.
GCGR mAb treatment increased the total islet area (Figure 2(b)), with increased β-cell area (0.0348 ± 0.018 vs. 0.0038 ± 0.00088 mm², Figure 2(c)), α-cell area (0.100 ± 0.043 vs. 0.0220 ± 0.0066 mm², Figure 2(d)), and β/α-cell area proportion when compared with the vehicle T1D group (Figure 2(e)). Similar to the GCGR mAb treatment, the mAb+Lira combination also greatly increased the total islet area when compared with the vehicle T1D group (0.112 ± 0.039 vs. 0.0364 ± 0.0096, Figure 2(e)) but showed no difference with the mAb group. The mAb+Lira combination increased the α-cell area when compared with the vehicle T1D group (0.0634 ± 0.033 vs. 0.0220 ± 0.0066 mm², Figure 2(d)), while decreasing the α-cell area when compared with the mAb group. Therefore, the mAb+Lira combination increased the β/α-cell area proportion significantly (0.297 ± 0.0086 vs. 0.502 ± 0.074, Figure 2(e)).

3.3. Liraglutide and GCGR mAb Treatments Promote β-Cell Self-Replication in T1D Mice. As shown above, both liraglutide and GCGR mAb increased the β-cell area. Subsequently, we tried to determine the source of neogenic islet cells. We performed costaining of insulin and PCNA in pancreatic sections to detect β-cell proliferation. We found that the
proportions of PCNA+insulin+ cells (the proliferating β cells) were higher in the liraglutide group and the mAb+Lira group when compared to the vehicle group, while the proportion in the GCGR mAb group did not show differences with other groups (Figures 3(a) and 3(d)).

3.4. Liraglutide Induces Duct-Derived β-Cell Neogenesis in T1D Mice. Neogenesis from precursor cells is an important approach for the recovery of β-cell mass. Pancreatic precursor cells were often located near or within the adult pancreatic ducts. Our previous study has proven that GCGR mAb could induce pancreatic duct-derived α-cell neogenesis, rather than β-cell neogenesis, in T1D mice [8]. In this study, we costained insulin or glucagon with pancreatic duct marker CK19 to evaluate the cell neogenesis. Results showed that in the liraglutide group or the mAb+Lira group, glucagon-positive cells or insulin-positive cells could appear adjacent to CK19, suggestive of duct-derived α-cell or β-cell neogenesis (Figures 3(b) and 3(c)). However, the glucagon or insulin-positive cells that are located in the duct were rare, so we did not perform quantification further.

3.5. Liraglutide and GCGR mAb Treatments Induce α- to β-Cell Transdifferentiation in T1D Mice. To evaluate α- to β-cell transdifferentiation, we first performed glucagon and insulin double immunostaining. Compared with the STZ group, the proportion of glucagon+insulin+ cells was boosted by the liraglutide treatment (2.44 ± 0.43% vs. 1.83 ± 0.61%, p = 0.031) (Figures 4(a) and 4(c)). Next, we established pancreatic α-cell lineage-tracing (glucagon-RFP) mice. Results showed that the proportion of RFP+insulin+ cells in the liraglutide treatment group was significantly higher than that in the STZ group (3.68 ± 0.92% vs. 2.32 ± 0.71%, p < 0.01) (Figures 4(b) and 4(d)), which confirmed that some
newborn β cells were derived from the transdifferentiation of pancreatic α cells. GCGR mAb could promote α- to β-cell transdifferentiation, as indicated by the increased proportion of glucagon+insulin+ cells (3.31 ± 0.56% vs. 1.83 ± 0.61%, p < 0.01), which was consistent with our previous report [8]. Notably, the proportion of glucagon+insulin+ cells in the mAb+Lira combination group was even higher than that in the liraglutide group (3.44 ± 0.71% vs. 2.44 ± 0.43%, p = 0.038).

4. Discussion

Our results demonstrated that the GLP-1 receptor agonist liraglutide increased pancreatic β-cell mass in T1D mice through self-replication, differentiation from precursor cells, and transdifferentiation of pancreatic α to β cells. Although combination of liraglutide and GCGR mAb did not demonstrate remarkable synergistic effects on the glucose level and the β-cell area, the stimulating effects of GCGR mAb on the α-cell area and glucagon secretion were alleviated. Interestingly, transdifferentiation of pancreatic α to β cells was also boosted in the combination group. The combination strategy of GLP-1 receptor activation with glucagon blockage may be beneficial in the T1D context, with good glucose control, β-cell regeneration, and not-very-high glucagon levels.

The current therapy for T1D is limited, and it is highly needed to evaluate the new nontraditional therapy for glucose control and maybe even for β-cell regeneration in T1D. GLP-1 receptor agonists have various beneficial effects on pancreatic β cells, including promoting β-cell regeneration [9, 10]. However, most of the conclusions were obtained in T2D models. Our present study showed a GLP-1 receptor agonist liraglutide increased the pancreatic β-cell area in STZ-induced T1D mice. Moreover, we found that liraglutide not only promoted the proliferation of existing pancreatic β cells, inducing cells in the duct lining to transform into pancreatic islet cells, but also boosted α cells to transdifferentiate into insulin-positive cells. In this way, we confirmed that all above sources participated in the liraglutide-induced β-cell renewal. However, liraglutide could not decrease blood glucose in T1D mice. The GLP-1-based therapy cannot be used alone for T1D treatment, and the combination with other drugs is needed.

Our previous study, together with others, has proven that GCGR blockage could decrease blood glucose and
improve the phenotype of T1D mice. Strikingly, GCGR mAb increased the number of pancreatic β cells and upregulated circulating insulin levels by inducing α- to β-cell transdifferentiation in T1D mice [8, 11]. However, GCGR mAb substantially increased pancreatic α-cell mass, which brings a safety concern on the α-cell tumor [12].

Notably, GLP-1 receptor agonists have the ability of inhibiting glucagon secretion and inducing α- to β-cell transdifferentiation. In this study, we tried to evaluate the synergistic effect of GCGR mAb and liraglutide in T1D mice. Although the combination did not show obvious advantages in decreasing blood glucose or increasing β-cell mass, the plasma glucagon level in the combination group decreased significantly and the α-cell area showed a downward trend. The total pancreatic β-cell area did not elevate further after treatment of liraglutide in combination with GCGR mAb; therefore, we inferred that the mechanism of the two drugs in promoting β-cell regeneration might be similar.

However, there were some limitations in our research. First, we did not explore the underlying molecular mechanisms. Second, precursor-specific lineage-tracing mice were needed for verification of stem cell-derived β cells.

In summary, our present study evaluated the synergic effect of GLP-1 receptor activation and GCGR antagonism in T1D mice. Although we did not find better glucose control and β-cell regeneration, we discovered that a combination of liraglutide with GCGR mAb could promote α- to β-cell transdifferentiation, thus attenuating the GCGR mAb-induced α-cell hyperplasia and hyperglucagonemia. Our research may provide useful clues for the clinical therapy of T1D.

**Figure 4:** Pancreatic histological analysis of α- to β-cell transdifferentiation in T1D mice treated with liraglutide, GCGR mAb, or both for four weeks. (a) Representative images of insulin and glucagon colocalization. (b) Representative images of insulin and tracing marker, red fluorescence protein (RFP), colocalization in pancreatic α-cell-tracing T1D mice. (c) Quantification of the glucagon ‘insulin’ cells. (d) Quantification of the RFP ‘insulin’ cells. Data are expressed as the mean ± SEM. n = 6 – 9 sections/mouse multiplied by 6 mice/group. Statistical analysis was conducted by ANOVA or Student’s t-test when appropriate. *p < 0.05 vs. STZ group; †p < 0.05 vs. liraglutide group. Magnified views of box regions are shown in the upper right panels.
Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

None of the authors of this paper has any financial or personal relationships with other people or organizations that could influence (bias) the research results. No conflicts of interest exist for the authors of this study.

Authors’ Contributions

L.G. and R.W. designed the research; L.G., T.W., and X.C. performed the research; J.Y., K.Y., and R.W. analyzed the data; L.G. and D.W. wrote the manuscript; and J.Y., R.W., and T.H. revised the paper.

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