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ORIGINAL ARTICLE

Three-dimensional *ex vivo* imaging and analysis of intraportal islet transplants

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Summary

In clinical islet transplantation, because the long-term insulin-independence rate is still poor, a method for detailed analysis of the transplanted islets in the liver after transplantation is required. We have established a novel imaging technique suitable for analysis of transplanted islets in liver using an optical projection tomography (OPT) method. A three-dimensional tomographic image of the transplanted islets in liver was reconstructed. The number of islets transplanted and the number of transplanted islets observed using OPT showed good correlation. The OPT method was used to compare the numbers of transplanted islets in mouse syngeneic and allogeneic transplantation models. Blood glucose concentrations of streptozotocin (STZ)-induced diabetic mice transplanted with syngeneic islets remained normoglycemic and the number of transplanted islets was largely preserved 11 days after transplantation. In mice transplanted with allogeneic islets, hyperglycemia recurred from 7 days after transplantation and the number and the volume of transplanted islets was significantly reduced 11 days after transplantation. These results indicate that OPT imaging and analysis may be a useful tool to quantitatively and sterically evaluation of transplanted islets in liver at the cellular level.

Introduction

Islet transplantation is a promising therapeutic approach for patients with insulin-dependent diabetes mellitus to achieve insulin independence [1,2]. A remarkably high rate of freedom from insulin therapy is achieved in insulin-dependent type 1 diabetic patients after islet transplantation by the Edmonton Protocol [1]. However, it was reported that long-term maintenance of glucose homeostasis without the use of insulin is poor [3]. This decline may be attributed to progressive islet loss as well as to various reactions during and after islet transplantation, including mechanical injury, ischemia, and nonspecific inflammatory reactions [4].

Until now, the total functional volume of islets transplanted intraportally in liver could be monitored only indirectly by measurements such as blood glucose and serum c-peptide levels. Modalities including bioluminescence imaging (BLI) [5–7], magnetic resonance imaging (MRI) [8–11], and positron emission tomography (PET) [12–14] have been used, and transplanted islets were detected by MRI [11] and PET [13] in human. These methods are suitable for *in vivo* examination because they are noninvasive and can be repeated over time. However, islets cannot be evaluated at the cellular level by these methods because the resolution is too low. Conventional immunohistochemical method permit evaluation of beta-cell volume at the subcellular level, but

can only restricted, sliced areas of the sample can be observes. Recently, Hara *et al.* reported subcellular analysis of intact pancreas, but the method can analyze only thin neonatal samples [15]. To investigate engraftment of transplanted islets scattered in solid liver at the subcellular level, another method is required. We have demonstrated an optical projection tomography (OPT) technique for precisely, three-dimensionally evaluating transplanted islets at the cellular level in liver.

Optical projection tomography is a microscopic imaging technique for obtaining three-dimensional, reconstructed images of small biological samples [16]. The principle of OPT is that the light passes through the specimen labeled and cleared for a standard back-projection algorithm to generate a relatively high resolution tomographic image. A three-dimensional image of the specimen is reconstructed using the individual tomographic images. The advantage of OPT is the capability to investigate spatial distribution of such target molecules as RNA and protein without slicing of the target organs and at a higher resolution.

In this report, we show that the number and volume of intraportally transplanted islets in liver can be investigated using OPT analysis. In addition, comparing syngeneic and allogeneic rodent islet transplantation models, we demonstrate that the number and volume of transplanted islets is considerably more decreased in allogeneic islet transplantation than in syngeneic transplantation. Thus, *ex vivo* imaging of intraportal islet transplant using OPT may be a useful tool for evaluation and improvement of islet transplantation outcome.

Materials and methods

Animals

Male C57BL6 Cr Slc mice (Shimizu Laboratory Supplies Co. Ltd, Kyoto, Japan) aged 8–10 weeks were used as recipients and donors and male BALB/c mice (Shimizu Laboratory Supplies Co. Ltd) aged 8 weeks were used as recipients for allogeneic transplantation. All experiments were approved by the Kyoto University Animal Care Committee.

Islet isolation and islet transplantation

Islets were isolated from mouse pancreas using collagenase digestion method [17]; 3–4 ml Hank's Balanced Salt Solution (HBSS) containing 0.5 mg/ml collagenase (Nitta Gelatin, Osaka, Japan) was infused through the common bile duct. The pancreas was dissected and digested at 37 °C for 21 min. Islets were separated from exocrine cells by centrifugation with Ficoll-Conray gradient solution for 10 min. Diabetes was rendered by a single intraperitoneal injection of streptozotocin (STZ) (Nacalai Tesque, Kyoto, Japan), 120 mg/kg body weight, freshly dissolved in 10 mM citrate buffer (pH 4.5). These mice were used as diabetic recipients if the blood glucose concentration was more than 20 mM on two consecutive days. Recipient mice were anesthetized by isoflurane (Forane; Abott, Chicago, IL, USA) during transplantation. Fresh islets in a volume of about 400 μl HBSS were injected into the portal vein and transplanted into the right hepatic lobe as previously reported [18]. For validation of the OPT method, 75, 150 or 300 islets were transplanted into the right hepatic lobe, which was dissected immediately after transplantation. For comparison of syngeneic and allogeneic transplantation, C57BL6 mice (H-2^b) were used as recipients; 300 islets isolated from C57BL6 mice or Balb/c mice (H-2^d) were transplanted, respectively. The blood glucose concentration was determined by glucose meter (Glucocard, Arkley, Japan).

Tissue preparation and immunostaining

Mice with transplanted islets were sacrificed by cervical dislocation. The transplanted right hepatic lobes were dissected clean and immediately immersed for fixation in 4% paraformaldehyde in PBS for 3 h at 4 °C. The fixed samples were washed in PBS and then transferred stepwise to 100% methanol (MeOH) and stored at −20 °C. The immunostaining was performed according to the previous report [19] as follows. The right hepatic lobe was immersed in 15% H_2O_2, 16.7% DMSO solution in MeOH for 24 h to bleach pigmented cells and to reduce auto fluorescence. The liver then was washed in MeOH, which was repeated five times and then kept at −80 °C for at least 1 h before return to room temperature. The organ was rehydrated by Tris Buffered Saline-TritonX (TBST) [0.15 M NaCl (Nacalai Tesque, Kyoto, Japan), 0.1 M Tris (hydroxymethy)aminomethane (Nacalai Tesque, Kyoto, Japan) pH 7.4, and 0.1% Triton X-100 (Nacalai Tesque, Kyoto, Japan)]. TBST containing 10% normal goat serum (Dako Corp., Glostrup, Denmark) and 0.01% sodium azide (Nacalai Tesque, Kyoto, Japan) was used as blocking solution for 24 h. The organ was incubated in insulin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in 5% DMSO containing blocking solution for 48 h. After washing, Alexa594 goat anti rabbit IgG (Invitrogen, Carlsbad, CA, USA) was used as secondary antibody for 48 h.

Optical projection tomography and image reconstruction

For the observations, the immunostained liver was embedded in 1% agarose gel (low melting point agarose; Sigma Aldrich, St. Louis, MO, USA) to fix the sample.

OPT was performed using an OPT scanner (OPT scanner 3001; Bioptonics, Scotland, UK) according to the manufacturer's instructions [16,19]. The specimens were maintained within the BABB (benzyl alochol/benzyl benzoate 1:2 ratio), rotated to a series of angular positions (0.9° apart) and images were captured at each orientation. High-resolutional tomographic images were reconstructed from raw images by NRecon software (SKYSCAN, Kontich, Belgium). The tomographic images obtained from OPT were reconstructed to three-dimensional form and analyzed by Avizo software (Visualization Science Group, Inc., Burlington, MA, USA). Three-dimensional images of islets and liver were obtained by isosurface treatment. Total volume of all islets was calculated by summation of the selected islets.

Statistical analysis

Data and graph were presented as medians (interquartile range) and statistical analysis was performed with Mann–Whitney's *U*-test. A value of $P < 0.05$ was considered significant.

Results

Observation of transplanted islets in liver by OPT

Transparency of the liver and immunostaining of transplanted islets without sectioning were achieved by the preparation protocols. Figure 1a is a raw OPT image of liver; the insulin-stained transplanted islets are seen as dots in the high magnification image (Fig. 1b, white arrows). One of the tomographic images obtained is shown in Fig. 1c. Vertically reconstructed images are shown in Fig. 1d and e and islets pointed out by arrow and arrowhead in Fig. 1c are located as in Fig. 1d and e, respectively. Some islets appear to be located at the terminal end of the portal vein (Fig. 1c and d) and other islets are located at the wall of the proximal branch of the portal vein (Fig. 1e). Figure 1f is the reconstructed target-specific image of an islet (arrowhead in Fig. 1e) and portal vein. Thus, a three-dimensional image as well as the size and location of transplanted islets in liver can be investigated (Fig. 1g and h and Supplementary movie).

Evaluation of the effectiveness of OPT analysis of transplanted islets in liver

To correlate the number of islets transplanted and the number of islets detected by OPT, we resected and fixed livers immediately after transplantation of a range of numbers of islets. The three-dimensional reconstructed image shows that the number of spots indicating transplanted islets in the right hepatic lobes was increased in accord with the increased dosage (Fig. 2a–c); these numbers are well correlated ($r^2 = 0.9561$) (Fig. 2d). These findings indicate that OPT can be used for quantitative analysis of islets transplanted into liver.

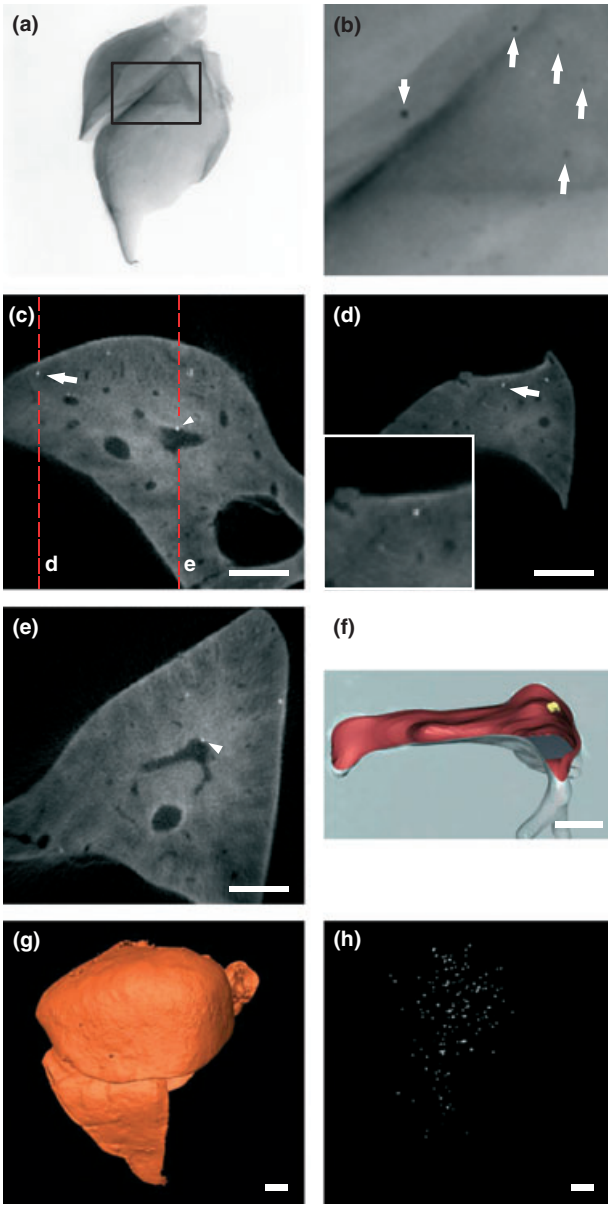

Figure 1 Optical projection tomography (OPT) images of liver containing intraportally transplanted islets. (a) Raw image of islet-transplanted right hepatic lobe, (b) high magnification image [in square of (a)], (c) representative slice image of the transplanted right hepatic lobe; (d and e) vertical slice image of islet with arrow in (c), arrowhead in (c), respectively. (f) Reconstructed three-dimensional image of islet [in (c) and (e), arrowhead] and portal branch. Three-dimensional image of (g) right hepatic lobe; (h) islets (white spots) in liver reconstructed from the same liver sample as (a). Scale bars indicate 1 mm in (c, d, e, g, and h) and 300 μm in (f).

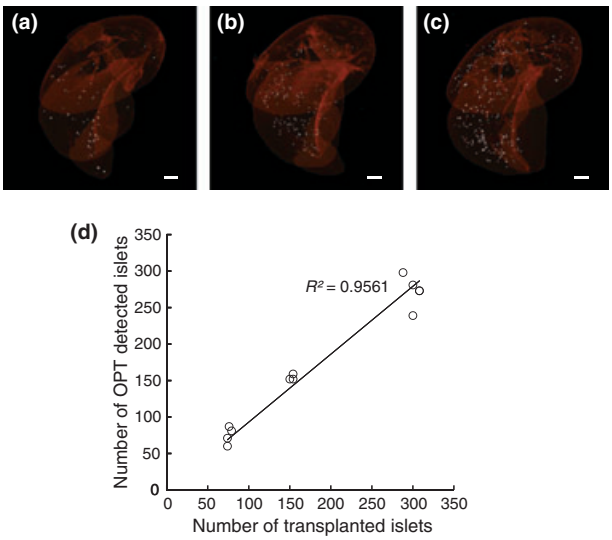

Figure 2 Comparison of the number of islets transplanted with that obtained by optical projection tomography (OPT) analysis. Representative OPT image of recipient liver transplanted with (a) 75, (b) 150, and (c) 300 islets, respectively. (d) Correlation of the number of islets transplanted and OPT-detected islets. Scale bar indicates 1 mm.

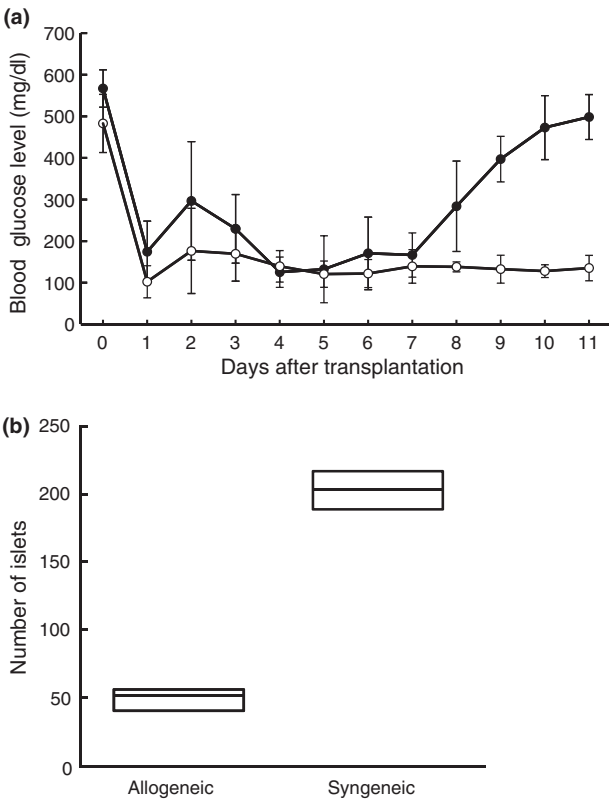

Figure 3 Glycemic level and number of islets of streptozotocin (STZ)-induced diabetic mice after syngeneic and allogeneic islet transplantation. (a) Random blood glucose level of recipients (open circles: syngeneic transplantation, filled circles: allogeneic transplantation). (b) Number of transplanted islets in syngeneic and allogeneic transplantation 11 days after transplantation.

Optical projection tomography analysis of islet grafts under syngeneic and allogeneic conditions

To evaluate the time course of transplanted islets in syngeneic and allogeneic conditions, we analyzed the number and volume of islets intraportally transplanted in liver of STZ-induced diabetic mice. Blood glucose concentrations under both syngeneic and allogeneic conditions were normoglycemic until a week after transplantation. However, blood glucose concentrations under allogeneic conditions thereafter became hyperglycemic, while those under syngeneic conditions remained normoglycemic (Fig. 3a). The islet-containing livers were resected on day 11 for analysis using OPT method. The number of islets in the syngeneic condition was dramatically greater than that in the allogeneic condition [52 (IQR 16.5) vs. 203 (28.5), respectively, $P < 0.05$] (Fig. 3b).

In OPT-detected islets classified by size, the number in each category was significantly greater in syngeneic than in allogeneic conditions and showed a similar histogram pattern (Fig. 4a). Total volume of islets in syngeneic condition was dramatically greater than that in allogeneic condition [8.6 (2.7) vs. 35.3 (10.1) ($\mu m^3 \times 10^6$)], respectively, ($P < 0.05$) (Fig. 4b).

Discussion

In this study, we demonstrate that islets transplanted intraportally in liver can be analyzed at the cellular level using OPT method, which permits three-dimensional analysis of the distribution of the islets in the liver. Comparing syngeneic and allogeneic islet transplantation models, we show by OPT that the volume of transplanted islets differs significantly at the cellular level.

One of main problems in clinical islet transplantation, poor long-term achievement of insulin independence, is primarily attributed to graft loss caused by various stressors upon transplantation [4]. When islets are injected intraportally, each of them is thought to locate at the respective branched end of the portal vein in liver. In modalities such as BLI, MRI, and PET, only PET allows quantification of graft volume, but the resolution is still too low for detailed analysis of the transplanted islets. On the other hand, while the resolution of conventional immunohistochemistry is high, only restricted slices of the engrafted organ can be analyzed using this method.

Optical projection tomography, a newly developed method, is reported to permit analysis of a sample at resolution as high as 5 μm. Recently, Alanentalo *et al.* performed detailed analysis of NOD mice during progression

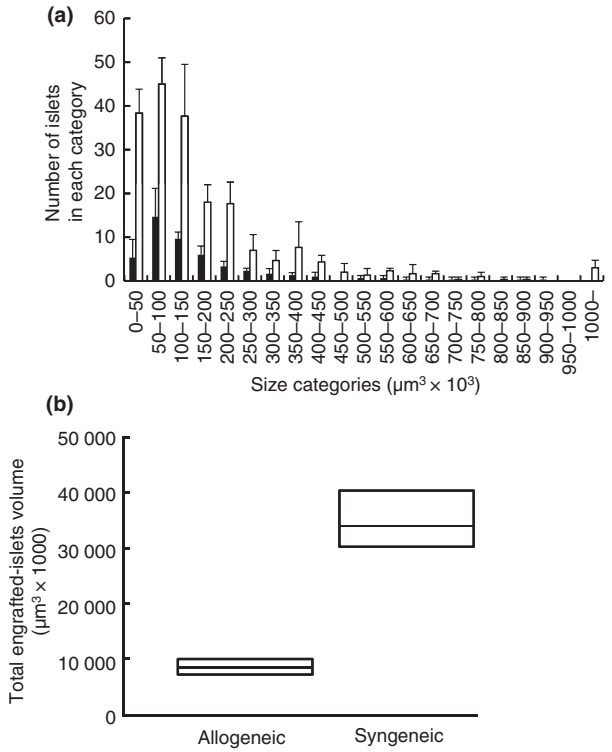

Figure 4 Quantitative analysis of transplanted islets in syngeneic and allogeneic model. (a) Size distribution of transplanted islets. (b) Total volume of transplanted islets in liver obtained using optical projection tomography (OPT) analysis.

of type 1 diabetes and showed that a reduction in volume of native islets in pancreas could be detected and quantified using the OPT method [20]. We have used OPT method for the first time in the intraportal islet transplantation model and confirm the efficacy of this method of islet imaging (Figs 1 and 2).

However, there are several limitations in use of the OPT method. It can be performed only *ex vivo*, and noninvasive, repeated observation is not possible. In this context, PET and MRI are suitable for *in vivo*, repeated monitoring of transplanted islets. In addition, the maximum sample size for analysis using OPT is about 2 cm. The OPT method also is not clinically applicable as it would require a large liver biopsy.

The OPT method is useful for evaluating small organs of small animals such as rodents, as in our present study. Indeed, using rodent islet transplantation models, the OPT method clearly shows quantitative difference of grafts in liver in syngeneic and allogeneic conditions. In this investigation, the OPT method revealed that 83% of the transplanted islets were lost in the allogeneic condition while about 70% were preserved in the syngeneic condition. Moreover, calculated beta-cell volume in the allogeneic condition was significantly reduced to 24.2% of that in the syngeneic condition (Figs 3b and 4b). This remarkable graft loss by allogeneic immune reaction seems not to be related to the size of the islet graft, as there was no difference in size distribution histogram between the two conditions (Fig. 4a). The OPT method also clearly shows the sites where islets adhere and are engrafted in the portal vein. Further investigation is required to determine the effect of islet location on islet engraftment.

In conclusion, we have constructed three-dimensional images of transplanted islets in liver using an OPT method that permits detailed analysis of transplanted islets in liver. This method should be useful for islet transplantation study.

Authorship

HF: designed the study, performed the study, collected the data, analyzed the data, and wrote the paper. KT: designed the study, performed the study, and wrote the paper. TO, SU, and NM: designed the study. XL, EM, and XZ: performed the study. NI: designed the study, wrote the paper.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Clip S1. Three dimensional image of islets transplanted in liver – Transplanted islets were scattered in liver. This image was reconstructed by software ''Avizo''.

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