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Development of fluorescence-conjugated islet-homing peptide using biopanning for targeted optical imaging of pancreatic islet

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ABSTRACT

Visualization of pancreatic islet can provide important information for diagnosis and monitoring of pancreatic islets. By using phage display technology, here we newly identified four candidate peptide sequences with islet-homing property. *In vitro* binding assay showed that LSALPRT peptide had stronger affinity with islet cells than other candidates. Then this islet-homing peptide (IHP) was chemically conjugated with TAMRA fluorophore for near infrared (NIR) imaging of islet cells. This TMARA-IHP was strongly bound to islets *in vitro*, but also to cryo-sectioned slice of pancreas tissue *ex vivo*. Collectively, it would be a considerable potential as therapeutics or diagnostics in future studies.

Introduction

Diabetes mellitus is a complicated, chronic disease resulting from an insufficient mass of functional beta cells. Beta cells are destroyed by the auto-reactive T lymphocyte in patients with type 1 diabetes mellitus (T1DM) [1]. Type 2 diabetes mellitus (T2DM) is related with beta-cells dysfunction and insulin resistance [2]. The reduction in beta cell mass is a common characteristic of both T1DM and T2DM, as a result of an increased beta cell apoptosis [3]. In current, there are several methods to assess beta cell mass, such as glucose tolerance testing, blood glucose levels, serum C-peptide levels, and hemoglobin A1c (HbA1c) levels. However, these indirect measurement of beta cell mass provide only end-point assessment of diabetes development. For these reasons, non-invasive, accurate imaging and quantification of beta cell mass *in vivo* are critically needed. Such methods may provide the potential for early detection of beta cell-related metabolic disease and for evaluation of islet graft recipients. In recent years, several potential islet imaging markers have been investigated for their suitability, *i.e.*, (1) cellular transporters such as glucokinase and glucose transporter 2 (GLUT2), (2) cellular receptors such as glucagon-like peptide 1 receptor (GLP-1R) and sulfonylurea receptor (SUR) [4–6]. However, none of these potential markers has allowed for successful islet imaging in the body. The challenges for islet imaging *in vivo* are the size of islet itself (average diameter = 100–150 μm) and their anatomical location in pancreas organ surrounded by the gastrointestinal system and the liver organ. Specially, endogenous beta cells are composed of only 2% of the total cells in pancreas organ, making their imaging even more challenging [7]. On the other hand, when pancreatic islets are implanted in diabetic patients, the relative volume of islet graft/host tissue is less than 0.2%. Therefore, a new strategy is highly required to clinically image the beta cell mass accurately.

One powerful strategy to overcome these limitations is the development of islet-specific peptides that is designed by using phage display. This phage display was first developed for display of peptides on filamentous bacteriophage [8]. This technology was further improved for display of proteins for therapeutic protein engineering [9,10]. Basically, the encoded peptides are displayed on the surface of the phage as a fusion product with one of the phage coating materials. This is processed by the introduction of defined exogenous peptide sequences in the genome of the phage capsid protein. Phage that binds to a target is eluted and then simply amplified by growing in bacteria (Fig. 1). This method is so called “biopanning”. It is repeated at least three times to find

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specific peptides that can strongly bind best to the target molecule with high affinity. The advantage of phage display technique is the large diversity of various peptides that can be screened (up to 10^{10}). In addition, after preparation of a phage library, phage display technique is relatively simple, rapid and cheap.

Several literatures reported phage display-based peptide sequences having interaction with pancreas tissue [11–13]. However, these 7-mer amino acid sequences such as CRGRRST, CKAAKNK, CRSRKG, CVSNPRWKC and CHVLWSTRC were just targeted to blood vessels or vascular endothelium within pancreas, meaning that the sequences do not directly targeted to pancreatic islets cells in pancreas organ [11,13]. In addition, although LSGTPERSGQAVKVKLKAIP sequence was specifically identified for targeting pancreatic islets, it was just evaluated with the peptide sequence-displayed phage but not directly with the peptide sequence only. That was not clinically acceptable because the peptide sequence-displayed phage microbe should be injected into the body for targeting pancreatic islets.

In this study, therefore, we first investigated new peptides that strongly bind to islet cells and evaluated their binding affinity at the peptide level. And then new peptide sequence with high affinity to pancreatic islets was chemically conjugated with near-infrared (NIR) dye for optical targeting imaging *in vivo*.

Experimental

Experimental animals

Male Sprague-Dawley (SD) rats (Daehan-Bio link, Chungcheongbuk-do, Republic of Korea) aged 7/8 weeks were used for all of the experiments in this study. Animals were housed in ventilated cages in a temperature-controlled room under specific pathogen-free (SPF) conditions. All animal experiments procedures were approved by the Institutional Animal Care and Use Committee in Hanyang University (HY-IACUC-13-030A).

Pancreatic islet isolation and culture

Pancreatic islets were isolated from SD rats as previously described [14]. In brief, Collagenase-P (1 mg/mL) (Roche, Basel, Switzerland) was injected into pancreas to digest extracellular matrix around islets. The pancreas was harvested and incubated at 37 °C for 15 min. After washing by Medium 199 (Sigma, St. Louis, MO, USA), islets were isolated by discontinuous density gradient centrifugation using Ficoll™ Histopaque and handpicked for further purification [15]. Isolated islets were cultured in RPMI-1640 (Invitrogen Inc., Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Sigma) and 1% antibiotics at 37 °C in 5% CO_2 before biopanning.

Biopanning

A phage library 1×10^{12} plaque forming units (pfu) mL^{-1} of Ph. D.™-7 phage display peptide library kit was used (New England Biolabs, Ipswich, MA, USA). Islet cells (1000 islet equivalent, IEQ) were plated into each well of a 6-well culture dish and incubated at 37 °C for 24 h in a CO_2 incubator (5% CO_2). The diluted M13 bacteriophage (1×10^{12} pfu mL^{-1}) using serum-free RPMI containing 0.1 wt-% bovine serum albumin (BSA) were added and reacted on a 3D shaker (Twist Shaker TW3, FINEPCR, Republic of Korea) for 1 h at room temperature. The culture medium containing unbound phages was discarded. Then, 1 mL of serum-free RPMI media was added into the wells to remove weak bound phages. After four intensive washes using 1 mL of PBS solution, the cell-bound phages were eluted by incubation with 400 μL of 0.1 wt-% BSA in 0.1 M HCl (pH 2.2) for 10 min at room temperature on the 3D shaker. The eluted product was immediately neutralized by the addition of 72 μL of 1 M Tris buffer (pH 10.0) at pH 7.0 to minimize damage of phage. The aliquot of eluted phage was centrifuged at 1800 rpm for 3 min at room temperature. The absorbance of the supernatant was read at 269 and 320 nm of spectrophotometer (ND-1000, Thermo Scientific, Waltham, MA, USA), and converted to pfu.

Enzyme-linked immunosorbent assay (ELISA)

On a 96 well culture plate, islet cells (300 IEQ) were placed and incubated in a CO_2 incubator at 37 °C for 24 h. M13 phages (wild type-, NERALTL-, RILITIP-, LSALPRT-, and MTSLSFS-displayed phages) were diluted to 2.0×10^{10} pfu mL^{-1} with 0.05% BSA in PBS. Each phages were added on each wells and incubated at 37 °C for 1 h. Unbound phase were discarded, and the cells were washed with 200 μL of 0.05% BSA in PBS. The Anti-M13 phage (FITC) antibody (MyBioSource, San Diego, CA, USA) was diluted 100-folds with 0.05% BSA in PBS. After the prepared 100 μL of secondary antibody was added into the wells and incubated on a 3D shaker at 100 rpm for 1 h, unbound antibodies were discarded by 200 μL of 0.05% Tween 20 in PBS. Then, the cell-phage-antibody mixture was washed three times with 200 μL of 0.05% Tween 20 in PBS by centrifugation at 1800 rpm for 3 min. Their fluorescence intensities were measured using a plate reader at excitation and emission wavelengths of 480 nm and 520 nm, respectively (1420 Multi-label Counter, PerkinElmer, Waltham, MA, USA). Fluorescence images were obtained using a fluorescence microscope (LEICA DMI3000B, Leica, Wetzlar, Germany).

In vitro *binding assay of TAMRA-labeled IHP to pancreatic islets*

LSALPRT peptide, a strong candidate of islet-homing peptides, was customer-synthesized by Anygen company (Gwangju, Republic of Korea). Then TAMRA (5-Carboxytetramethylrhodamine) was conjugated with the LSALPRT peptide sequence *via* amide linkage for optical targeting imaging of pancreatic islets.

For confirmation of specific binding of the LSALPRT peptide sequence, islet cells (100 IEQ) were placed on a 6 well culture plate at 37 °C. Then, TAMRA-labeled LSALPRT peptide (TAMRA-IHP; 10 μg/mL) was added on each wells and incubated for 1 h. Islets were washed three times to remove unbound TAMRA-IHP. Fluorescence images were obtained using a fluorescence microscope (Eclipse TE2000-S, Nikon, Tokyo, Japan). To quantitatively analyze binding affinity of TAMRA-IHP to islets, we further performed flow cytometry analysis. To do that, islets were dispersed into single cells using the trypsinization method. Islets were incubated with digestive trypsin for 6 min at 37 °C and dissociated with pipetting. The islet single cells were filtered using a 40-μm cell strainer filter to remove un-dissociated islets. Then the amount of islet cells bound with TAMRA-IHP was analyzed by flow cytometry (FACSCalibur™; BD Biosciences, Franklin Lakes, NJ, USA). Untreated islets were used as a background.

Ex vivo *binding of TAMRA-IHP to pancreatic islets in pancreas organ*

Pancreas organ was harvested from SD rat and embedded in tissue freezing medium (OCT compound; Sakura Finetek Japan, Tokyo, Japan). It was frozen and stored at −80 °C until sectioning. Immunohistochemical (IHC) staining was performed on 10-μm cryosections. For intracellular infiltration, the cryosections were treated with 0.1% Tween 20. Then cryosections were blocked for 30 min with a 20% goat serum albumin buffer to prevent non-specific binding of immunoglobulin. The prepared cryosections were incubated with a primary mouse monoclonal antibody against insulin (1:200 dilution; Abcam Cambridge, MA, USA) at

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