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Oral delivery of an anti-diabetic peptide drug via conjugation and complexation with low molecular weight chitosan

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ABSTRACT

Despite the therapeutic potential of exendin-4 as a glucagon-like peptide-1 (GLP-1) mimetic for the treatment of type 2 diabetes, its utility has so far been limited because of the low level of patient compliance due to the requirement for frequent injections. In this study, an orally available exendin-4 was produced by conjugating it to low molecular weight chitosan (LMWC). Conjugation between the LMWC and cysteinylated exendin-4 was carried out using a cleavable linker system in order to maximize the availability of the active peptide. The LMWC-exendin-4 conjugate formed a nanoparticle structure with a mean particle size of 101 ± 41 nm through complexation between the positively charged LMWC backbone and the negatively charged exendin-4 of individual conjugate molecules. The biological activity of the LMWC-exendin-4 conjugate was evaluated in an INS-1 cell line. The LMWC-exendin-4 conjugate stimulated insulin secretion in a dose dependent manner as similar as that of native exendin-4. From the pharmacokinetic study after oral administration of the conjugate, a C_{max} value of 344 pg/mL and a T_{max} of 6 h were observed, and the bioavailability, relative to the subcutaneous counterpart, was found to be 6.4%. Furthermore, the absorbed exendin-4 demonstrated a significantly enhanced hypoglycemic effect. These results suggest that the LMWC-exendin-4 conjugate could be used as a potential oral anti-diabetic agent for the treatment of type 2 diabetes.

1. Introduction

Patients with type 2 diabetes have greatly impaired incretin-mediated insulin secretion, mainly owing to decreased secretion of glucagon-like peptide-1 (GLP-1) [1]. Exendin-4 is a GLP-1 mimetic peptide comprising 39 amino acids that has been used in the treatment of type 2 diabetes as it has a significantly enhanced half-life *in vivo* compared to endogenous GLP-1, and similar gluco-regulatory activity [2,3]. Even though exendin-4 has been widely used for the treatment of type 2 diabetes, patients are required to undergo frequent subcutaneous injections, resulting in poor patient compliance in addition to side effects such as infection at the sites of injection [4,5]. Since oral delivery is expected to result in drastically enhanced patient compliance, many attempts have been made to develop oral delivery systems for exendin-4 [6–8]. However, orally administered peptides encounter formidable barriers to absorption into the blood stream. These typically include physical barriers, such as viscous mucous layers and tight junctions of aligned enterocytes in the gastrointestinal (GI) track; chemical barriers, such as low stomach pH; and biological barriers, such as enzymatic degradation. Overcoming these problems is essential for improving the level of absorption of orally administered peptide-based drugs [9,10]. There have been a limited number of successful studies into the development of systems for oral exendin-4 delivery. It has been shown that site-specific covalent attachment of biotin to exendin-4 facilitated receptor-mediated intestinal absorption of the peptide, resulting in approximately 3.95% oral bioavailability (BA) [7]. Very recently, a relatively high oral BA of ~14.0% was attained using a nanoparticulate system formed by electrostatic complexation between negatively charged exendin-4 and positively charged chitosan, and subsequent coating with anionic poly(γ-glutamic acid) [8]. Unlike previous approaches, we have sought to overcome such oral delivery barriers using chemical conjugation between the therapeutic agent of interest and a mucoadhesive polymer. In the last few years, a large number of mucoadhesive materials have been investigated for oral delivery of peptides and proteins, including chitosan, methacrylate, and alginate, based polymers [11–16]. Rekha and Sharma reported that anionic/hydrophobic modification of chitosan (LSC) enhanced mucoadhesivity of the nanoparticles and orally administered FITC-insulin loaded LSC particles to diabetic rats showed enhanced insulin absorption and transported insulin across the enterocytes efficiently. We have shown that intestinal absorption of the therapeutic agent could be significantly enhanced by conjugating it to low molecular weight chitosan (LMWC) [17–19]. Such high absorption is attributed to unique features of LMWC, including its high mucoadhesiveness and its ability to open tight cell-cell junctions, facilitating paracellular transport of drugs [20–24]. Our previous results suggest that the conjugation

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of chemical or peptide-based drugs to LMWC may be an efficient strategy for enabling a high level of absorption into the blood stream [18].

In this regard, here we report the development of an oral delivery method for exendin-4 by conjugating it to LMWC. We describe the synthesis and characterization of the conjugate, its activity in the induction of *in vitro* cellular insulin secretion, and the pharmacokinetic parameters and glucoregulatory effect after oral administration in a diabetic mouse model.

2. Materials and methods

2.1. Materials

Custom synthesized cysteinylated exendin-4 (exendin-4-cys; *N′*-HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPSC-*C′*) was purchased from AnyGen Co. (Gwangju, South Korea). Low molecular weight chitosan (LMWC) was purchased from KITTOLIFE (Seoul, South Korea) and further purified by ultrafiltration before use. N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) immobilized bead for disulfide reduction were purchased from Pierce (Rockford, IL). All other solvents and reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Male C57BL/6 *db*/*db* mice (7–8 weeks old) were supplied by the Korean Research Institute of Bioscience and Biotechnology (Daejeon, South Korea) and maintained in pathogen-free conditions in the animal facility at Gwangju Institute of Science and Technology (GIST). The animal experiments were approved by the GIST Animal Care and Use Committee. The exendin-4 ELISA kit was purchased from Phoenix pharmaceuticals, Inc. (Burlingame, CA) and insulin ELISA kits were purchased from Mercodia (Uppsala, Sweden). The One-touch blood glucose meter (CodeFree) was purchased from SD Biosensor, Inc. (Suwon, South Korea). The glucose-sensitive pancreatic cell line (INS-1) was kindly donated by Prof. Kang Choon Lee (Sung Kyun Kwan University, Suwon, South Korea) and was cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Grand Island, NY), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Synthesis of the LMWC-PDP conjugate

A solution of SPDP (30 mg) dissolved in anhydrous DMF (5.6 mL, 50 mM) was added to a solution of LMWC (168 mg, average M_w = 9 kDa) dissolved in 2.8 mL of sodium tetraborate buffer (10 mM, pH 9). The mixture was stirred in an oil bath at 37 °C for 5 h. Distilled water (33.6 mL) was then poured into the reaction mixture and unreacted SPDP was subsequently extracted using ethyl acetate (42 mL, 5 times). The aqueous layer was collected and dialyzed against distilled water using a dialysis membrane tube with a MWCO of 5 kDa (Spectrum Laboratories, Rancho Dominguez, CA). After freeze drying for 2 days, LMWC-PDP (120 mg) was obtained as light yellow powder in 69% yield and further characterized using ^{1}H NMR (400 MHz, Jeol, Tokyo, Japan) and UV spectrophotometry (Varian, Palo Alto, CA).

2.3. Synthesis of the LMWC-exendin-4 conjugate

Exendin-4-cys (5 mg, 0.12 mmol) dissolved in distilled water (1 mL) was treated with a 10-fold molar excess of TCEP gel as a reducing agent at ambient temperature for 30 min. The excess TCEP gel was then removed before further reaction. The pre-treated exendin-4-cys was added to a solution of LMWC-PDP (48.8 mg) in PBS-EDTA buffer (pH 7.4) and reacted for 1 h at ambient temperature. Following the reaction, distilled water (8 mL) was poured into the reaction mixture, the pyridine-2-thione leaving group was extracted using ethyl acetate (10 mL, 5 times), and the solvent was evaporated using a centrifugal vacuum system (Hanil, Seoul, Korea). The resulting viscous material was purified using reverse phase high performance liquid chromatography (RP-HPLC; Shimadzu, Kyoto, Japan) on a C 18 column (150 mm × 4.6 mm Symmetry, Waters, Milford, MA) with a mobile phase consisting of 0.1% trifluoroacetic acid (TFA) in water and acetonitrile. The mobile phase was run with a linear gradient from 5 to 65% for 30 min at a flow rate of 1 mL/min and the detection wavelength was 230 nm.

2.4. Characterization of the LMWC-exendin-4 conjugate nanoparticles

The particle size and zeta potential of LMWC-exendin-4 were measured before and after freeze drying using electrophoretic light scattering apparatus (ELS 8000, Otsuka Electronics, Japan). The morphology was examined using transmission electron microscopy (TEM) using a TECNAI F 20 electron microscope (Philips Electronic Instruments Corp., Mahwah, NJ).

2.5. Proteolytic stability test

The LMWC-exendin-4 conjugate or exendin-4-cys (200 μg/mL each) was mixed with an equal volume of trypsin solution (Life Technologies, Grand Island, NY) at 37 °C, and further incubated at 37 °C for predetermined times. The reactions were then stopped by adding 200 μL of 1% TFA in H_2O. An aliquot of the reaction solution was taken and centrifuged at 1500 *g* for 5 min. The residual amount of each exendin-4 species in the supernatant was analyzed using RP-HPLC.

2.6. In vitro biological activity test

The biological activity of the LMWC-exendin-4 conjugate was determined through the measurement of insulin secretion from the glucose sensitive pancreatic-cell line (INS-1) after treatment with the compound. The cells were maintained in complete RPMI 1640 medium (11.1 mM glucose) containing 10% (v/v) FBS, 50 μM 2-mercaptoethanol, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin and incubated at 37 °C under 5% CO_2 atmosphere. *In vitro* biological activity was evaluated by incubating the INS-1 cells in 500 μL of Krebs-Ringer-HEPES (KRH) buffer (11.1 mM glucose) containing exendin-4 or the LMWC-exendin-4 conjugate (1–10 nM) for 1 h or 6 h. Levels of insulin released were measured using an insulin ELISA kit.

2.7. In vivo pharmacokinetics

The pharmacokinetic profile of exendin-4-cys and the LMWC-exendin-4 conjugate was assessed as follows. Either exendin-4-cys (50 μg/kg, s.c.) or LMWC-exendin-4 (400 μg/kg, p.o.) was administered to C57BL/6 mice (n = 5). Blood samples were withdrawn from the retro-orbital sinus and centrifuged (700 *g* at 4 °C for 20 min). The supernatant was filtered by using SEP-Column (RK-SEPCOL-1, phoenix pharmaceuticals Inc., Burlingame, CA) to remove plasma proteins. The filtered plasma was treated with TCEP bead for 1 h to cleave the disulfide linkage between LMWC and exendin-4-cys and centrifuged (700 *g* at 4 °C for 20 min). The concentration of exendin-4-cys in the collected supernatant was measured using an exendin-4 ELISA kit and calculated based on a standard curve for exendin-4-cys. The pharmacokinetic parameters were estimated using a WinNolin software package. The relative BA of LMWC-exendin-4 conjugate after oral administration was calculated using the following formula: $[(AUC_{oral} \times Dose_{sc}) / (AUC_{sc} \times Dose_{oral})] \times 100$ [25].

2.8. Intraperitoneal glucose tolerance test (IPGTT)

The *in vivo* anti-diabetic (hypoglycemic) activity of LMWC-exendin-4 conjugate was measured in *db*/*db* mice after oral drug administration (n = 5) by IPGTT [26]. The average body weight and blood glucose levels were the same for every group. Briefly, mice fasted for 18 h were orally

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