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Delivery of two-step transcription amplification exendin-4 plasmid system with arginine-grafted bioresorbable polymer in type 2 diabetes animal model

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Abstract

Exendin-4, glucagon-like peptide 1 (GLP-1) receptor agonist, is an exocrine hormone, which has potent insulinotropic actions similar to GLP-1 such as stimulating insulin biosynthesis, facilitating glucose-concentration dependent insulin secretion, slowing gastric emptying, reducing food intake and stimulating β -cell proliferation. Exendin-4, also, has a longer half-life than GLP-1, due to its resistance to degradation by dipeptidyl peptidase IV (DPP-IV). In spite of its many advantages as a therapeutic agent for diabetes, its clinical application is still restricted. Thus, to improve the activity of exendin-4 *in vivo*, gene therapy system was developed as an alternative method. An exendin-4 expression system was constructed using the two-step transcription amplification (TSTA) system, which is composed of p β -Gal4-p65 and pUAS-SP-exendin-4 with combining the advantages of signal peptide (SP) in order to facilitate its secretion in ectopic cells or tissue. Arginine-grafted cyctaminebisacrylamide-diaminohexane polymer (ABP) was used as a gene carrier. Increased expression of exendin-4, glucose dependent insulin secretion in NIT-1 insulinoma cells, and high insulin expression in the presence of DPP-IV were evaluated *in vitro* after delivery of ABP/TSTA-SP-exendin-4. Blood glucose levels in diabetic mice were decreased dramatically from the third day for experimental period after single intravenous administration with ABP/TSTA-SP-exendin-4. The highest insulinotropic effect of exendin-4 was also observed in the ABP/TSTA/SP-exendin-4-treated mice groups, compared with the others groups from the 3rd day after injection. TSTA exendin-4 expression system with SP and ABP polymer has a potential gene therapy for the treatment of type 2 diabetes.

Keywords

Diabetes; exendin-4 polyplex; TSTA system; gene delivery

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Introduction

Glucagon-like peptide-1 (GLP-1) receptor agonist, exendin-4 was isolated from the parotid gland of the Gila monster lizard. Exendin-4 is an exocrine hormone, which has opposing effects against the features of type 2 diabetes [1]. Exendin-4 is also known to share with the action and function of GLP-1 as an incretin. Exendin-4 inhibits inappropriately high glucagon secretion, improves glycemic control, stimulates insulin secretion, protects against β -cell apoptosis via engagement of antiapoptotic pathway, promotes β -cell proliferation, promotes satiety, and slows gastric emptying [2-4]. Exendin-4 has also extrapancreatic effects: facilitates glucose transport in muscle and increases glucose transporter expression and glycogen synthesis in liver [5].

In addition, exendin-4 is well known to bind to the GLP-1 receptor, because it has a 53% amino acid sequence homology of GLP-1 [6]. The GLP-1 receptors are expressed in various tissues such as brain, liver, and cardiomyocytes [5]. Several studies demonstrated that exendin-4 improves heart function against cardiac remodeling after myocardial infarction and enhances central nervous system cell survival and function via neuronal degeneration [7, 8]. At recent, exendin-4 was used as a target moiety for *in vivo* pancreatic islet imaging [9].

Despite exendin-4 has many advantages with respect to reducing diabetes-related complication, it is still not allowed as therapeutic agents for the treatment of type 2 diabetes. Anti-exendin-4 antibodies and nausea as adverse effect are induced after administration of exendin-4 [10, 11] and its *in vivo* half-life is still short for sufficient treatment of type 2 diabetes, although exendin-4 has more resistance against the degradation by dipeptidyl peptidase-IV (DPP-IV) than GLP-1 [12]. Thus, exendin-4 has not only a short-term therapeutic effect, but is required once- or twice-daily injections or combination therapies with oral GLP-1 analogue drugs to maintain the normal glucose level in patients with type 2 diabetes [13].

Consequently, to overcome the barriers to clinical application, several strategies have been employed as alternative approaches by some modification methods to develop more stability and efficacy of exendin-4 such as modification of exendin-4 using various polymers. It was recently shown that exendin-4 conjugate with human serum albumin (HAS) via polyethylene glycol (PEG) lowered blood glucose levels and had greater circulating time in db/db mice [14]. Moreover, adenoviral vectors encoding exendin-4 linked to a leader sequences, palmitoyl acylation of exendin-4 in porous poly(lactic-co-glycolic acid) (PLGA) microspheres and amino acid modification of exendin-4 containing disulfide bond have been tested for the long-acting exendin-4 and extended therapeutic duration [15-17]. The results suggested that , exendin-4 is an attractive therapeutic material for the diabetes treatment.

Gene therapy provides an approach to maintain expression level of therapeutic gene for prolonged period by single injection. There are two possible ways to improve the exendin-4 gene therapy system. One is to develop a more efficient gene delivery carrier and the other is to develop a more efficient exendin-4 expression plasmid system.

In the present study, arginine-grafted cyctaminebisacrylamide-diaminohexane polymer (ABP) was used as a gene carrier. ABP has many disulfide bonds in its backbone, which are degradable in reducing condition of cytosol. With degradability in cytosol, ABP polymer was low toxic to cells. Furthermore, ABP had higher transfection efficiency than polyethylenimine (PEI) [18, 19].

A exendin-4 expression system was constructed using the two-step transcription amplification (TSTA) system. TSTA system is composed of p β -Gal4-p65 and pUAS-SP-exendin-4. The former plasmid expresses an artificial potent transfection factor, which in

turn binds to the promoter of the latter plasmid. This system amplifies gene expression compared with a single plasmid system. TSTA system was combined signal peptide (SP) to facilitate its secretion in ectopic cells or tissues [20].

In this study, *in vitro* effects of exendin-4 by TSTA expression system and ABP polymer were evaluated compared with 25 K PEI or one vector expression system. In addition, insulinotropic and glucoregulatory effects of this system were assessed with GLP-1 expression system in diabetic mice.

Materials and Methods

Cell lines and plasmid construction

The following cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA): A549, a non-small cell lung carcinoma cell line; NIT-1, insulinoma cell line established from NOD/Lt mice. A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, Grand Island, NY) or NIT-1 cells were maintained in DMEM/F12 (1:1) (Gibco-BRL) with 10% fetal bovine serum (FBS; Gibco-BRL) and penicillin/streptomycin (Gibco-BRL) at 37°C with 5% CO₂.

A plasmid containing exendin-4 expression cassette with secretion signal peptide (SP) was constructed similar with in a previously experiment [20, 21]. To create pUAS-SP-exendin-4 plasmid, the exendin-4 cDNA from p β -SP-exendin-4 at *KpnI* and *BglII* was inserted into the *KpnI* and *BglII* of pUSA-GLP-1 plasmid. The construction of the plasmids was confirmed by restriction enzyme studies and direct sequencing. The plasmids were amplified in *Escherichia coli* strain DH5 α , and purified by Qiagen plasmids Maxi Kits (Qiagen, Valencia, CA). The purity of the plasmids was measured by absorbance at 260 and 280 nm and the quantity was determined with absorbance at 260 nm. The plasmids were stored at – 20 °C until use. Scheme 1 shows the expression mechanism of exendin-4 in TSTA system.

Cationic reagents and materials

Hyperbranched poly(ethylenimine) (25 K PEI) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). The synthesis and characterization of the arginine-grafted bio-reducible poly(disulfide amine) (ABP) cationic polymer was performed and validated as previously described [18].

Preparation of plasmid DNA polyplexes with a cationic polymer

Total 1.5 μ g plasmid DNA was used for the formation of polyplexes with polymer. Polyplexes between each DNA and ABP cationic polymers at the described w:w ratios in Fig. 1 were formed by pre-diluting the ABP cationic components and the DNA components in an E-tube using serum-free DMEM. The DNA solution was added to the solution of ABP polymer, rapidly mixed by vortexing. The polyplexes were allowed to stand at room temperature for 25 ~ 30 min before application to cells in culture medium without serum.

Size and zeta potential measurements of DNA/polymer polyplexes

The average size and zeta potential of DNA/polymer polyplexes were examined with a Nano ZS (ZEN3600, Malvern Instruments) with a He-Ne ion laser beam (633 nm, fixed scattering angle of 90°) at 25°C. TSTA polyplexes with ABP polymer were prepared as described above. After the formation of polyplex at each ratio in HEPES buffer, polyplex solutions were added in filtered-water to a final volume of 500 μ L before measurement, and then the sizes and potential values of polyplexes were presented as the average values from three runs. DNA quantification assay using Quant-iT PicoGreen assay kit (Molecular Probes Inc., Willow Creek Rd, Eugene, OR) was used to measure the condensing ability of the polymers

and plasmids. Each polyplex was prepared in Hepes buffered saline (10 mM HEPES, 1 mM NaCl, pH 7.4) at various weight ratios (w/w). The level of fluorescence in each polyplex was measured by Qubit Fluorometer (Molecular probes Inc.)

MTT assay

The cytotoxicity of polyplexes was determined by measuring the conversion of the tetrazolium salt MTT to formazan. After 48 hrs of treatment with polyplexes, the medium was removed, and fresh medium containing 2 mg/mL MTT was added to each well. The cells were then incubated for 4 hrs at 37°C in the dark. After removing the MTT solution, the remaining crystals were solubilized with 1 mL dimethyl sulfoxide (DMSO). After 20 min of incubation, the absorbency was determined using a microplate reader at 540 nm. Cell viability was expressed as a percentage of control uninfected cells. Each experiment was repeated at least three times.

Measurement of secreted exendin-4 and GLP-1

A549 cells were seeded at about 60-70% confluence in 12-well plates (2×10^5 cells/well) one day before transfection with polyplexes at 37°C in a humidified 5% CO₂ incubator. At 24 hrs before transfection, the medium from each well was exchanged with fresh serum-free medium, and then cells were transfected with polyplexes. The cells were incubated with polyplexes for 6 hrs. Then the polyplexes solution was removed, and fresh 10% serum-containing medium was added. After 48 hrs, the level of exendin-4 and GLP-1 in the culture media was determined by exendin-4 Enzyme Immunoassay Kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA) and GLP-1 ELISA (Millipore, Billerica, MA) according to the manufacturer's protocol, respectively.

Expression level of insulin of exendin-4 or GLP-1 in NIT-1 insulinoma cells

Exendin-4 medium secreted in the culture media as above mentioned method was harvested and then harvested media was exchanged with medium of NIT-1 cells prepared in 12-well plates. At 5 hrs after change, insulin level secreted in supernatant was determined by Rat/mouse insulin ELISA (Millipore) according to the manufacturer's protocol. To assess the induction of insulin by ABP/TSTA-SP-exendin-4 or /TSTA-SP-GLP-1 polyplexes in the presence of DPP-IV enzyme, exendin-4 or GLP-1 obtained from A549 cells after polyplexes transfection was incubated with 50 or 300 ng (mg/mL) DPP-IV for 15 min at 37°C and then exendin-4 or GLP-1 incubated with DPP-IV was treated to NIT-1 cells. After 5 hrs, insulin ELISA was carried out. NIT-1 insulinoma cells were also used to examine the insulin levels induced by ABP/p β -SP-exendin-4, 25 K PEI/TSTA-SP-exendin-4, or ABP/TSTA-SP-exendin-4 in condition depending on glucose concentration. Exendin-4 produced from A549 cells was incubated to NIT-1 in fresh 10% serum-containing medium with 50 or 300 mg/dL glucose concentration. After 5 hrs, the insulin levels in the medium were measured by insulin ELISA.

In vivo anti-diabetic study

In study for *in vivo* anti-diabetic effects, male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME), 6 weeks of age, were used in same condition and management in our previous experiment [22]. Briefly, mice were fed with a high-fat and high-calorie diet (60 kcal% fat, D12492; Research Diet, New Brunswick, NJ) for 3 months. The animals were randomized as pre-glucose levels before experiment and then treated by intravenous single injection with PBS, DNA only, ABP/empty vector, ABP/one vector system, 25 K PEI/TSTA system, and ABP/TSTA system polyplexes of exendin-4 or GLP-1 with *w:w* ratio of 1:10 DNA/polymer. Blood samples were obtained from the retro-orbital vein after anesthesia at days 0, 3, 6, 9, 12 after injection to measure blood glucose, exendin-4, GLP-1, and insulin concentrations.

Extracted blood sample was added with DPP-IV inhibitor (1 mg/mL, Millipore). Blood glucose levels were measured by using a one-touch blood glucose meter (ACCU-CHEK sensor, Roche Diagnostics Corp., Indianapolis, IN). In addition, to evaluate the effects of each polyplex formulations on glucose tolerance, intraperitoneal glucose tolerance test (IPGTT) was done 3 days after injection to tolerate a standard glucose load. Mice (n = 5 each at 3 days after injection) were fasted overnight before the IPGTT. A preload blood sample was taken and a glucose load of 1 g/kg was administered via intraperitoneal injection. Subsequent blood samples were taken 0, 15, 30, 60, 90, and 120 min after injection and then were measured by one-touch blood glucose meter (Roche Diagnostics Corp.) to measure blood glucose levels. All animal studies were performed according to established NIH and University of Utah Animal Care Committee guidelines.

Biodistribution assessment of ABP/TSTA-SP-exendin4 polyplex in Balb/c mice

Mice were systemically injected once with each exendin-4 polyplexes. Heart, liver, lung, spleen, kidney, and pancreas were harvested at 48 hrs post administration, and then DNA was extracted from tissues using Maxwell 16 Tissue DNA Purification Kit (Promega, Madison, Wisconsin) according to the manufacture's instruction. The exendin-4 copy number in each tissue was measured by real time quantitative PCR (TaqMan PCR detection; Applied Biosystems, Foster City, CA). A fluorogenic probe (FAM-5'-CATTTACCAGTGACTTGTC-3'-NFQ) was designed to anneal to the target sequence between the sense primer (5'-ACGGCGGCATGGTGAA-3') and the antisense primer (5'-CTGCCTCCTCTCCATCTGTTT-3') in the region of the Ad gene encoding IX protein. Samples were amplified for 40 cycles in a StepOnePlus™ real-time PCR system (Applied Biosystems) with continuous fluorescence monitoring. All samples were analyzed in duplicate and data were processed with the StepOne™ v2.1 software package (Applied Biosystems).

Evaluation of liver toxicity in vivo

Balb/c mice were injected intravenously with each exendin-4 formulations. Serum levels of aspartate aminotransferase (AST) and alanine transaminase (ALT) were then measured at 2 days post-injection by ALT or AST enzyme assay kit (Bioo Scientific co., Austin, TX) according to the manufacture's protocol, respectively.

Statistical analysis

The data were expressed as mean $\pm$ standard error of the mean (SEM). Statistical comparisons were made using Stat View software (Abacus Concepts, Inc., Berkeley, CA) and the Mann-Whitney test (nonparametric method). Differences were considered statistically significant when *P* values were less than 0.05.

Results

Evaluation of exendin-4 expression of exendin-4 polyplexes according to p β -Gal4/p65 and pUAS-SP-exendin-4 weight ratios

TSTA system-mediated exendin-4 expression depends on the ratios between p β -Gal4/p65 and pUAS-SP-exendin-4 plasmids. To optimize exendin-4 expression efficiency according to the ratios between pUAS-SP-exendin-4 and p β -Gal4/p65, A549 cancer cells were incubated with the polyplexes at 3:1, 2:1, 1:1, 1:2, 1:3, and 1:4 p β -Gal4/p65/pUAS-SP-exendin-4 weight ratios with ABP polymer used as a gene carrier. pSI was added as an irrelevant plasmid to adjust the quantity of plasmid to 1.5 μ g. As show in Fig. 1A, the highest exendin-4 expression was obtained at a 1:1 p β -Gal4/p65/pUAS-SP-exendin-4 ratio compared with those in cells treated with polyplexes formed at the others ratios. Exendin-4

at this ratio was observed about 4-fold higher expression than that of pUAS-SP-exendin-4 without p β -Gal4/p65. pUAS-SP-exendin-4 without p β -Gal4/p65 showed the minimal exendin-4 expression. Interestingly, the exendin-4 expression of the polyplex from at a 1:3 ratio showed decreased tendency. These results indicate that exendin-4 can be expressed by TSTA system. Based on these results, we subsequently used a 1:1 TSTA system ratio as the optimal concentration for polyplex with ABP polymer for the following experiment.

Characterization of TSTA plasmids with ABP polymer weight ratio

To optimize the ratio between TSTA system and ABP polymer, picogreen assay was assessed after the formation of complex. Picogreen chelated on exposed DNA site after complex formation. Thus, low value of assay results means that DNA is condensed well by ABP polymer, indicating in the formation of compact nanoparticles. In Fig. 1B result, the value of DNA only used for polyplex formation normalized 100 % (560 ng/mL). However, the value after complex formation was lower depending on increase at ABP weight ratios. High optimized complexation was obtained at a 40 weight ratio.

After polyplex generation of TSTA plasmids with dual DNA and the ABP polymer, average sizes and surface charge values of polyplexes were assessed by DLS and zeta potential analyzer (Table 1). 25 K PEI was used as a control cationic polymer. Polyplexes were formed in the following conditions: TSTA system at 1:1 ratio in total 1.5 μ g DNA and 1:40 ratio DNA to ABP polymer or 1:1 ratio DNA to 25 K PEI. As shown in Table 1, TSTA polyplexes formed with ABP or 25 K PEI were stably formed at below 100 nm in size, which would be proper size capable of inducing efficient cellular up-take [23], with minimal cytotoxicity (Supplementary Fig. 1). Also, zeta-potential values of polyplex with ABP polymer were measured a little higher than that of 25 K PEI polyplex due to strong positive charge of ABP surface [18], implying that TSTA system plasmids were compactly complexed with polymer. These results indicate that sizes and zeta potential values of TSTA-SP-exendin-4 or -GLP-1 polyplexes were caused by the binding of the positively-charged ABP polymer to negatively charged TSTA plasmids.

Exendin-4 expression and the ability of insulin induction by TATA system with ABP polymer

We next tested whether TSTA-SP-exendin-4 polyplex with ABP polymer can induce high exendin-4 expression in ectopic cells compared with those of one vector expression or 25 K PEI. The highest exendin-4 expression was examined in A549 cells treated with ABP/TSTA-SP-exendin-4 polyplex (Fig. 2A). The exendin-4 expression in the cells treated with 25 K PEI/TSTA-SP-exendin-4 was 1.4-fold lower than that of cells treated with ABP/TSTA-SP-exendin-4. Also, these patterns were observed in the case of 25 K PEI. Moreover, all TSTA expression systems with ABP or PEI induced higher exendin-4 expression than those of p β -SP-exendin-4. Taken together, these results suggest that TSTA system with the cationic polymer ABP can enhance the expression of exendin-4.

Exendin-4 can induce insulin secretion as an incretin effect [6]. Thus, to assess the ability of insulin induction of exendin-4 expressed by two kinds of expression systems or polymers, the media containing exendin-4 secreted from A549 cells was treated to NIT-1 insulinoma cells. Then, at 6 hrs after exendin-4 media treatment, insulin level in NIT-1 cell medium was measured by ELISA. As shown in Fig. 2B, the results showed that ABP polyplex with TSTA system had higher insulin expression than the others groups. The insulin expression by TSTA system with ABP was enhanced compared to that with PEI. Also, TSTA system induced more high insulin expression than one step expression system (p β -SP-exendin-4). These results showed similar pattern as shown by increased expression of exendin-4. These

results demonstrate that the enhanced exendin-4 expression by TSTA system is capable of stimulating insulin secretion in insulinoma cells.

Effect of DPP-IV on the insulin induction of exendin-4 and GLP-1 polyplex

Degradation by DPP-IV enzyme after injection of exendin-4 is also an important obstacle to overcome for successful exendin-4 delivery. We next verified whether exendin-4 expressed by TSTA expression system with ABP not only had more resistant to DPP-IV than GLP-1, but stimulated insulin secretion in the presence of DPP-IV. DPP-IV is well known as a ubiquitous enzyme, which is responsible for degradation of exendin-4 or GLP-1 [4, 24]. The secreted exendin-4 or GLP-1 was incubated with DPP-IV enzyme in a concentration dependent manner as shown in Fig. 3A. The insulin secretion by GLP-1 polyplex was significantly decreased, depending on the DPP-IV concentration. The result suggests that the biological activity of GLP-1 was significantly decreased by DPP-IV. In contrast, ABP/TSTA-SP-exendin-4 polyplex did not show a significant reduction of insulin secretion in the presence or absence of DPP-IV. Accordingly, these results suggest that the enhanced insulin secretion by ABP/TSTA-SP-exendin-4 polyplex might be due to the improved stability of exendin-4.

Effects of TSTA-SP-exendin-4 polyplex with ABP polymer on glucose concentration depending on insulin secretion

Exendin-4 is well known to induce insulin secretion in a glucose concentration dependent manner [25, 26]. Insulin secretion was measured in NIT-1 insulinoma cells, after treatment of the cells with the medium from the ABP/TSTA-SP-exendin-4 transfected A549 cells in order to confirm the biological activity of the produced exendin-4. Secreted exendin-4 obtained from A549 cells treated with ABP/TSTA-SP-exendin-4 was treated to NIT-1 insulinoma cells with two kinds of glucose concentration (50 and 300 mg/dL). At 6 hrs after exendin-4 media treatment, insulin level in NIT-1 cell medium was measured by ELISA.

As shown in Fig. 3B, insulin secretion under low glucose concentrations (50 mg/dL) was not induced in all the polyplexes transfected cells. However, a remarkable increment of insulin secretion occurred under high glucose concentrations (300 mg/dL). The highest insulin secretion was observed in cells treated with ABP/TSTA-SP-exendin-4 polyplex. Moreover, exendin-4 expressed by TSTA-SP-exendin-4 system significantly stimulated the secretion of insulin under high glucose condition, but not under low glucose condition. These results indicate that the expressed exendin-4 induced the insulin secretion in a glucose-dependant manner.

In vivo effects of ABP/TSTA-SP-exendin-4 and -GLP-1 polyplexes on blood glucoregulation

In vivo efficacy of ABP/TSTA-SP-exendin-4 and -GLP-1 polyplexes were confirmed in diet-induced obese (DIO) diabetic mice. The diabetic mice were intravenously injected one time with exendin-4 and GLP-1 polyplexes with TSTA or one vector expression system and ABP or 25 K PEI. Among the exendin-4 polyplexes groups, the TSTA-SP-exendin-4 with ABP group showed better glycemic control compared with the other groups during the entire experiment period (Fig. 4A). In particular, the serum glucose level of ABP/TSTA-SP-exendin-4 was reduced to 116.8 ± 12.8 mg/dL, compared with 146.8 ± 14.5 mg/dL for ABP/p β -SP-exendin-4 or 132.3 ± 13 mg/dL for 25 K PEI/TSTA-SP-exendin-4 at the third day after the injection. At this time, glucose concentration of ABP/TSTA-SP-exendin-4 reached to normal physiological range. In the case of GLP-1-treated groups, lower glucose level showed in mice treated with TSTA-SP-GLP-1 with ABP (147.7 ± 14.4 mg/dL) (Fig. 4B). The glucose levels of ABP/p β -GLP-1 or 25 K PEI/TSTA-SP-GLP-1 were measured 168.5 ± 23.6 mg/dL or 153.4 ± 15.3 mg/dL, respectively. The treatment with either DNA only or

empty vector polyplex of both groups did not enhance insulin secretion after single-same dose injection during the same period.

In addition, blood glucose level in mice treated with ABP/TSTA-SP-exendin-4 polyplex was more efficient in regulation of the serum glucose level than that of ABP/TSTA-SP-GLP1 polyplex during the total period (Fig. 4C). Based upon the results, we concluded that exendin-4 delivered by TSTA system and ABP polymer induced better glucose control and long-lasting effect than GLP-1, indicating that ABP/TSTA-SP-exendin-4 polyplex induces improved glucose homeostasis in mice with diabetes.

Moreover, the enhanced incretin effects of exendin-4 may be caused by the higher stability of exendin-4, compared with GLP-1 *in vivo*. Typically, GLP-1 half-life is known to be less than 2 min *in vivo* because it has alanine residue that can be cleaved by DPP-IV at position 8 in N-terminal. On the other hand, exendin-4 has longer plasma stability due to the lack of the DPP-IV site. In our results, exendin-4 induced an increased insulin secretion than that of GLP-1 in the presence of DPP-IV (Fig. 3A).

Exendin-4 and GLP-1 expression level in TSTA-SP-exendin-4 or -GLP-1 polyplex-treated diabetic mice

The enhanced glucoregulatory effects shown in Fig. 4 may be also be caused by the increased exendin-4 or GLP-1 expression of each polyplex with ABP polymer. Thus, to evaluate the exendin-4 or GLP-1 expression in mice treated with ABP/TSTA-SP-exendin-4 or ABP/TSTA-SP-GLP-1 polyplexes, the serum levels of exendin-4 or GLP-1 were measured by exendin-4 or GLP-1 ELISA after the injection.

As shown in Fig. 5, the exendin-4 or GLP-1 expression increased in mice treated with TSTA-SP-exendin-4 or TSTA-SP-GLP-1 polyplex with ABP polymer. Serum exendin-4 levels in the mice treated with ABP/TSTA-SP-exendin-4 polyplex were 1.4- and 1.3-fold higher than those in the mice treated with ABP/p β -SP-exendin-4 or 25 K PEI/TSTA-SP-exendin-4, respectively. In the case of the GLP-1 groups, ABP/TSTA-SP-GLP-1 showed 1.4- or 1.2-fold higher GLP-1 expression than those of ABP/p β -GLP-1 or 25 K PEI/TSTA-SP-GLP-1. The control groups such as DNA only or ABP/empty vector polyplex showed insignificant minimal changes. Significant increase in the concentration of exendin-4 or GLP-1 was assessed in mice treated with ABP/TSTA-SP-exendin-4 or -GLP-1 compared with one vector expression system (p β -SP-exendin-4 or p β -GLP-1) or 25 K PEI polyplex from the 3rd day after injection during the experimental period and then gradually decreased. After all, these data suggest that the elevated exendin-4 or GLP-1 serum levels depended on the expression system and carrier, leading to high gene expression by TSTA system and ABP polymer. Also, these data delineate that TSTA system and ABP polymer serve to increase both exendin-4 and GLP-1 expression.

Insulinotropic effects of ABP/TSTA-SP-exendin-4 or -GLP-1 in diabetic mice

The anti-hyperglycemic effects of incretin hormones are mediated by the enhanced insulin secretion in a glucose dependent manner [27, 28]. We next verified whether exendin-4 or GLP-1 expressed by TSTA system with ABP polymer had insulin secretion-inducing ability as incretin action against diabetes. Fig. 6 showed increased insulin levels in mice treated with TSTA-SP-exendin-4 or TSTA-SP-GLP-1 with ABP or 25 K PEI polymer. The secreted insulin levels were raised dramatically to 627.3 ± 95.5 pmol/L for ABP/TSTA-SP-exendin-4 and 409.5 ± 98 pmol/L for ABP/TSTA-SP-GLP-1 compared with those of DNA only (120 ± 66.5 pmol/L for exendin-4 or 115.8 ± 92.3 pmol/L for GLP-1) at 3rd day after intravenous injection and then returned to basal levels (Fig. 6A and 6B). The insulin levels when used ABP polymer as a carrier was observed 2.1 or 1.7-fold higher in mice treated with ABP/

TSTA-SP-exendin-4 or -GLP-1 compared with those of 25 K PEI. Additionally, about 2-fold increased level of insulin was found in ABP/TSTA-SP-exendin-4 system in comparison with ABP/TSTA-SP-GLP1 at the 3rd day after polyplex administration. Then, insulin concentration steadily decreased to the 12th day of the study. These results indicate that exendin-4 expressed by TSTA system with ABP can induce the insulin secretion more efficiently than those of GLP-1.

Consequently, relative to the results shown in Fig. 5 when the blood exendin-4 or GLP-1 concentration was reduced near to basal level, the disappearance of the insulinotropic effect was observed. Taken together, these data imply that the expression of increased incretin hormones leads to glucoregulatory effects and this causes enhanced insulin secretion.

In addition, long-lasting effect of exendin-4 is likely to be caused by an increase expression and secretion of exendin-4 by the transfection efficiency with ABP polymer (Fig. 2). Our group previously reported ABP polymer can be increased the transfection efficiency of therapeutic DNA or viral vector in various cell lines [18, 19, 29]. Actually, increased exendin-4 expression in several tissues was confirmed by biodistribution study of ABP/TSTA-SP-exendin-4 polyplex (Supplementary Fig. 2). In addition, we evaluated that severe liver toxicity (ALT and AST levels) associated with exendin-4 formulations was not induced by ABP/TSTA-SP-exendin-4 polyplex (Supplementary Fig. 3). After all, these data also support our results in respect to improved insulinotropic effect due to high exendin-4 expression by increased transduction efficiency of TSTA-SP-exendin-4 in ectopic cells about the potential utilization of the ABP.

Glucose tolerance test of exendin-4 and GLP-1 polyplexes upon the single dose injection

A glucose tolerance test was conducted after intravenous single administration of ABP/TSTA-SP-exendin-4 or /TSTA-SP-GLP-1. First, the mice were intravenously injected with each polyplex. Next day after injection, the mice were fasted overnight before the intraperitoneal glucose tolerance test (IPGTT). In IPGTT to verify the improvement of glucose tolerance, the mice treated with ABP/TSTA-SP-exendin-4 showed better glycemic control, compared with that of ABP/TSTA-SP-GLP-1 (Fig. 7). Especially, the ABP/TSTA-SP-exendin-4-treated mice had significantly lower glucose levels to 120 min after the injection. These results suggest that exendin-4 produced by TSTA system and ABP polymer has higher glucoregulatory effects in diabetic mice, leading to improved glucose homeostasis.

Conclusion

It is already known that exendin-4 has a biological effect against diabetes. Although it has many advantages, their clinical applications have been restricted, due to requirement of daily administration of exendin-4 by short half-life and no long-lasting actions.

If exendin-4 can be effectively delivered and be served sustained action, it would allow enough exendin-4 to maintain in the body for treatment of type 2 diabetes. In this study, we constructed a novel exendin-4 vector using TSTA system, which was consisted of dual plasmids and delivered the vector with ABP. *In vitro* data of ABP/TSTA-SP-exendin-4 polyplex showed the increased exendin-4 expression, higher resistance against DPP-IV, and the ability of glucose concentration depending on insulin induction. We also assessed the therapeutic potential of TSTA-SP-exendin-4 polyplex with ABP polymer via intravenous single administration in the DIO diabetic mice. The results confirmed that ABP-TSTA-SP-exendin-4 expressed high exendin-4, glucose homeostasis, the induction of increased insulin secretion and prolonged therapeutic effect in the diabetic mice.

Our *in vitro* and *in vivo* promising data shown in this study will be provided as the inspiring device of methods for development of more advanced exendin-4 based on therapeutic gene expression system with polymer. Taken together, ABP-TSTA-SP-exendin-4 system may have high potential for the treatment of type 2 diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- [1]. Kim SW. Polymeric gene delivery for diabetic treatment. *Diabetes Metab J*. 2011; 35:317–326. [PubMed: 21977450]
- [2]. Li Y, Hansotia T, Yusta B, Ris F, Halban PA, Drucker DJ. Glucagon-like peptide-1 receptor signaling modulates beta cell apoptosis. *J Biol Chem*. 2003; 278:471–478. [PubMed: 12409292]
- [3]. Brubaker PL. Minireview: update on incretin biology: focus on glucagon-like peptide-1. *Endocrinology*. 2010; 151:1984–1989. [PubMed: 20305008]
- [4]. Ahren B. The future of incretin-based therapy: novel avenues--novel targets. *Diabetes Obes Metab*. 2011; 13(Suppl 1):158–166. [PubMed: 21824270]
- [5]. Verspohl EJ. Novel therapeutics for type 2 diabetes: incretin hormone mimetics (glucagon-like peptide-1 receptor agonists) and dipeptidyl peptidase-4 inhibitors. *Pharmacol Ther*. 2009; 124:113–138. [PubMed: 19545590]
- [6]. Nielsen LL, Young AA, Parkes DG. Pharmacology of exenatide (synthetic exendin-4): a potential therapeutic for improved glycemic control of type 2 diabetes. *Regul Pept*. 2004; 117:77–88. [PubMed: 14700743]
- [7]. Bertilsson G, Patrone C, Zachrisson O, Andersson A, Dannaeus K, Heidrich J, Kortessmaa J, Mercer A, Nielsen E, Ronnholm H, Wikstrom L. Peptide hormone exendin-4 stimulates subventricular zone neurogenesis in the adult rodent brain and induces recovery in an animal model of Parkinson's disease. *J Neurosci Res*. 2008; 86:326–338. [PubMed: 17803225]
- [8]. Martin B, Golden E, Carlson OD, Pistell P, Zhou J, Kim W, Frank BP, Thomas S, Chadwick WA, Greig NH, Bates GP, Sathasivam K, Bernier M, Maudsley S, Mattson MP, Egan JM. Exendin-4 improves glycemic control, ameliorates brain and pancreatic pathologies, and extends survival in a mouse model of Huntington's disease. *Diabetes*. 2009; 58:318–328. [PubMed: 18984744]
- [9]. Wu Z, Todorov I, Li L, Bading JR, Li Z, Nair I, Ishiyama K, Colcher D, Conti PE, Fraser SE, Shively JE, Kandeel F. In vivo imaging of transplanted islets with ⁶⁴Cu-DO3A-VS-Cys40-Exendin-4 by targeting GLP-1 receptor. *Bioconjug Chem*. 2011; 22:1587–1594. [PubMed: 21692471]
- [10]. Ranganath LR. Incretins: pathophysiological and therapeutic implications of glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1. *J Clin Pathol*. 2008; 61:401–409. [PubMed: 18375745]
- [11]. Mikhail N. Incretin mimetics and dipeptidyl peptidase 4 inhibitors in clinical trials for the treatment of type 2 diabetes. *Expert Opin Investig Drugs*. 2008; 17:845–853.
- [12]. Schnabel CA, Wintle M, Kolterman O. Metabolic effects of the incretin mimetic exenatide in the treatment of type 2 diabetes. *Vasc Health Risk Manag*. 2006; 2:69–77. [PubMed: 17319471]
- [13]. Szayna M, Doyle ME, Betkey JA, Holloway HW, Spencer RG, Greig NH, Egan JM. Exendin-4 decelerates food intake, weight gain, and fat deposition in Zucker rats. *Endocrinology*. 2000; 141:1936–1941. [PubMed: 10830274]

- [14]. Kim I, Kim TH, Ma K, Lee ES, Kim D, Oh KT, Lee DH, Lee KC, Youn YS. Synthesis and evaluation of human serum albumin-modified exendin-4 conjugate via heterobifunctional polyethylene glycol linkage with protracted hypoglycemic efficacy. *Bioconj Chem*. 2010; 21:1513–1519. [PubMed: 20715855]
- [15]. Lee YS, Shin S, Shigihara T, Hahm E, Liu MJ, Han J, Yoon JW, Jun HS. Glucagon-like peptide-1 gene therapy in obese diabetic mice results in long-term cure of diabetes by improving insulin sensitivity and reducing hepatic gluconeogenesis. *Diabetes*. 2007; 56:1671–1679. [PubMed: 17369525]
- [16]. Kim H, Park H, Lee J, Kim TH, Lee ES, Oh KT, Lee KC, Youn YS. Highly porous large poly(lactic-co-glycolic acid) microspheres adsorbed with palmityl-acylated exendin-4 as a long-acting inhalation system for treating diabetes. *Biomaterials*. 2011; 32:1685–1693. [PubMed: 21126761]
- [17]. Li Y, Zheng X, Tang L, Xu W, Gong M. GLP-1 analogs containing disulfide bond exhibited prolonged half-life in vivo than GLP-1. *Peptides*. 2011; 32:1303–1312. [PubMed: 21515323]
- [18]. Kim TI, Ou M, Lee M, Kim SW. Arginine-grafted bioreducible poly(disulfide amine) for gene delivery systems. *Biomaterials*. 2009; 30:658–664. [PubMed: 19007981]
- [19]. Kim PH, Kim TI, Yockman JW, Kim SW, Yun CO. The effect of surface modification of adenovirus with an arginine-grafted bioreducible polymer on transduction efficiency and immunogenicity in cancer gene therapy. *Biomaterials*. 2010; 31:1865–1874. [PubMed: 19962189]
- [20]. Kim TI, Lee M, Kim SW. Efficient GLP-1 gene delivery using two-step transcription amplification plasmid system with a secretion signal peptide and arginine-grafted bioreducible polymer. *J Control Release*. 2012; 157:243–248. [PubMed: 21945681]
- [21]. Ah Kim H, Lee S, Park JH, Lee S, Lee BW, Ihm SH, Kim TI, Kim SW, Ko KS, Lee M. Enhanced protection of Ins-1 beta cells from apoptosis under hypoxia by delivery of DNA encoding secretion signal peptide-linked exendin-4. *J Drug Target*. 2009; 17:242–248. [PubMed: 19558363]
- [22]. Choi S, Oh S, Lee M, Kim SW. Glucagon-like peptide-1 plasmid construction and delivery for the treatment of type 2 diabetes. *Mol Ther*. 2005; 12:885–891. [PubMed: 16039908]
- [23]. Wagner E, Ogris M, Zauner W. Polylysine-based transfection systems utilizing receptor-mediated delivery. *Adv Drug Deliv Rev*. 1998; 30:97–113. [PubMed: 10837605]
- [24]. Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet*. 2006; 368:1696–1705. [PubMed: 17098089]
- [25]. Egan JM, Clocquet AR, Elahi D. The insulinotropic effect of acute exendin-4 administered to humans: comparison of nondiabetic state to type 2 diabetes. *J Clin Endocrinol Metab*. 2002; 87:1282–1290. [PubMed: 11889200]
- [26]. Parkes DG, Pittner R, Jodka C, Smith P, Young A. Insulinotropic actions of exendin-4 and glucagon-like peptide-1 in vivo and in vitro. *Metabolism*. 2001; 50:583–589. [PubMed: 11319721]
- [27]. Drucker DJ. Enhancing incretin action for the treatment of type 2 diabetes. *Diabetes Care*. 2003; 26:2929–2940. [PubMed: 14514604]
- [28]. Davidson JA, Parente EB, Gross JL. Incretin mimetics and dipeptidyl peptidase-4 inhibitors: innovative treatment therapies for type 2 diabetes. *Arq Bras Endocrinol Metabol*. 2008; 52:1039–1049. [PubMed: 18820816]
- [29]. Kim PH, Kim J, Kim TI, Nam HY, Yockman JW, Kim M, Kim SW, Yun CO. Bioreducible polymer-conjugated oncolytic adenovirus for hepatoma-specific therapy via systemic administration. *Biomaterials*. 2011; 32:9328–9342. [PubMed: 21924767]

Fig. 1. Weight ratios for optimized exendin-4 expression. (A) Exendin-4 expression depending on the pβ-Gal4/p65/pUAS-SP-exendin-4. The polyplexes with plasmid and ABP polymer (1:10 weight ratio) were prepared at various pβ-Gal4/p65/pUAS-SP-exendin-4 ratios from 3:1 to 1:4 in dual plasmids. The highest exendin-4 expression was induced at 1:1 pβ-Gal4/p65/pUAS-SP-exendin-4 weight ratio. Each experiment was done in triplicates and was statistically analyzed by student t test. **P* < 0.05 and ****P* < 0.01 versus pUAS-SP-exendin-4. (B) Condensed polyplexation depending on ABP polymer ratios. Polyplexes formed at various ABP polymer ratios with TSTA system of 1:1 pβ-Gal4/p65/pUAS-SP-exendin-4 ratio was assessed complexation by pico-green assay. ABP for polyplexation with plasmid was decided at 1:40 weight ratio.

Fig. 2.

In vitro exendin-4 expression in ABP/TSTA-SP-exendin-4 polyplex-treated A549 cells (A) and the induction ability of insulin secretion in NIT-1 insulinoma cells (B). * $P < 0.05$ versus 25 K PEI/TSTA-SP-exendin-4.

Fig. 3.

The evaluation of insulin induction ability of ABP/TSTA-SP-exendin-4 polyplex in the presence of DPP-IV degradation enzyme (A) Exendin-4 or GLP-1 in media secreted from A549 cells treated with ABP/TSTA-SP-exendin-4 or ABP/TSTA-SP-GLP-1 polyplex was incubated with 50 (+) or 300 (++) ng DPP-IV contents (1 mg/mL) for 15 min at 37°C and then this medium was treated to NIT-1 cells. At 5 hrs after treatment, insulin levels released from cells were measured by insulin ELISA. ** $P < 0.02$ for comparison of ABP/TSTA-SP-GLP-1 in the absence of DPP-IV versus ABP/TSTA-SP-GLP-1 in the presence of DPP-IV. (B) The induction of glucose concentration depending on insulin secretion of exendin-4 polyplex formulations. Each secreted exendin-4 was treated to NIT-1 cells in condition with 50 or 300 mg/dL glucose concentration. After 5 hrs treatment, insulin levels released from cells were measured by insulin ELISA. * $P < 0.05$ versus ABP/pβ-SP-exendin-4 and ** $P < 0.02$ versus 25 K PEI/TSTA-SP-exendin-4 in co-culture with 300 mg/dL glucose. Each data point represents the mean and standard errors of triplicate infected cultures.

Fig. 4.

Assessment of improved anti-hyperglycemic effects of ABP/TSTA-SP-exendin-4 polyplex. Mice were intravenously injected with DNA only (60 μ g/mouse), ABP/empty vector, ABP/p β -SP-exendin-4, 25 K PEI/TSTA-SP-exendin-4, or ABP/TSTA-SP-exendin-4 (A), for GLP-1 polyplexes (B), and for comparison of ABP/TSTA-SP-exendin-4 and -GLP-1 (C). The blood glucose levels were measured by ACCU-CCHEK glucose meter at each time point. Data represent means $\pm$ SE. * P < 0.05 for comparison of ABP/TSTA-SP-exendin-4 against ABP/p β -SP-exendin-4 and * P < 0.05, ** P < 0.02, and *** P < 0.01 for comparison of ABP/p β -GLP-1, 25 K PEI/TSTA-SP-GLP-1, and ABP/TSTA-SP-GLP-1 against DNA only, respectively. ** P < 0.02 versus ABP/TSTA-SP-GLP-1.

Fig. 5.

The exendin-4 (A) and GLP-1 (B) expression levels in mice treated with each polyplexes via intravenous single administration. The serum level of exendin-4 or GLP-1 was assessed by each ELISA ever 3 days until the end on study after injection. Data represent means $\pm$ SE.

** $P < 0.02$ versus ABP/pβ-SP-exendin-4. * $P < 0.05$ and *** $P < 0.01$ versus ABP/pβ-GLP-1.

Fig. 6. Increased insulintropic effects of ABP/TSTA-SP-exendin-4 after systemic administration. (A) Insulin levels in mice treated with exendin-4 polyplexes. DNA only, 25 K PEI/TSTA-SP-exendin-4, or ABP/TSTA-SP-exendin-4 polyplex was intravenously injected to DIO mice, and then blood samples were extracted at several time points. The insulin levels in blood were measured by insulin ELISA. * $P < 0.05$ and *** $P < 0.01$ versus 25 K PEI/TSTA-SP-exendin-4. (B) Insulin levels of GLP-1 polyplex formulation-treated mice. * $P < 0.05$ versus 25 K PEI/TSTA-SP-GLP-1. (C) Comparison of insulin induction ability between ABP/TSTA-SP-exendin-4 and -GLP-1. * $P < 0.05$ versus ABP/TSTA-SP-GLP-1.

Fig. 7.

Glucose tolerance of ABP/TSTA-SP-exendin-4 upon intravenous administration in diabetic mice. ABP/TSTA-SPexendin-4 or ABP/TSTA-SP-GLP-1 polyplex was intravenously injected before 3 days for IPGTT and then mice were fasted for overnight. Glucose was loaded via intraperitoneal administration (1 g/kg). Blood glucose levels were measured at each time points. Data represent means $\pm$ SE. $n = 5$ for each experimental condition. * $P < 0.05$ for comparison of NC groups versus ABP/TSTA-SP-exendin-4-treated groups.

Scheme 1.

The expression mechanisms of exendin-4 or GLP-1 in TSTA system consisted of p β -Gal4/p65 and pUAS-SP-exendin-4 or pUAS-SP-GLP-1.

Table 1

Average size and zetapotential of TSTA-SP-exendin-4 or -GLP-1 polyplex with ABP or 25 K PEI polymer.

	PEI25k(WR=1)		ABP (WR-40)	
	TSTA-SP-exendin4	TSTA-SP-GLP-1	TSTA-SP-exendin4	TSTA-SP-GLP-1
Size (nm)	71.34 $\pm$ 5.29	77.17 $\pm$ 0.76	80.78 $\pm$ 1.81	87.5 $\pm$ 6.87
Zeta potential (mV)	29.07 $\pm$ 1.68	27.53 $\pm$ 3.45	32.17 $\pm$ 2.55	31.3 $\pm$ 1.82