Anti-GAD antibody targeted non-viral gene delivery to islet beta cells

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Abstract

An islet cell targeting polymeric gene carrier was synthesized by conjugating anti-GAD Fab’ fragment to PEI via PEG linker (PEI-PEG-Fab’). The Fab’ fragment was prepared from a murine monoclonal antibody against glutamic acid decarboxylase (GAD), which has been identified as one of the major auto-antigens expressed in islet cells, and used as a targeting moiety for islet cell targeting. The electrophoretic migration of plasmid DNA (pCMVLuc)/PEI-PEG-Fab’ complexes in agarose gel was completely retarded above the N/P ratio of 2. The complexes demonstrated a size of 100–275 nm with an almost neutral surface charge. Confocal microscopy revealed that the PEI-PEG-Fab’ complexes showed much higher cellular binding and uptake efficiency compared to PEI-PEG complexes. The PEI-PEG-Fab’ showed about 10-fold higher transfection efficiency (relative luciferase activity) than PEI-PEG in GAD-expressing mouse insulinoma cells (MIN6), however the transfection efficiency of PEI-PEG-Fab’ reduced to that of PEI-PEG in GAD negative cells (293) and in the presence of competitive free Fab’. Considering the neutral surface charge of its complexes with DNA, and selectivity toward the islet cells expressing a specific antigen, the PEI-PEG-Fab’ conjugate could be thought as a potential candidate of the systemic gene therapy for the treatment of type I diabetes.

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1. Introduction

Type I diabetes results from the destruction of the insulin-producing beta-cells of the pancreatic islets. Since the destructive process is mainly mediated by immune response [1], immune intervention using gene therapy has been thought as one of the possible...
approaches to treat the autoimmune disease. Furthermore, the slow progressive nature of immune destruction makes it even feasible of prevention at a preclinical stage [2]. Non-viral gene delivery systems having specificity toward islet beta cells could be an attractive approach for a systemic treatment of type I diabetes. Polyethyleneglycol (PEG) has been popularly used in cationic polymer vectors to reduce cytotoxicity [3], and increase the stability of the polyelectrolyte complexes between the polycation and DNA [4]. Owing to the presence of flexible PEG, the pegylated carriers could minimize the non-specific binding of serum proteins in the bloodstream to the complexes resulting in prolonged in vivo circulation time [5,6]. However, with the same mechanism, the pegylation could hinder the binding of the complexes to cells, which could cause the reduction of cellular uptake resulting in lower transfection efficiency [7]. The incorporation of specific ligands to the gene carriers would offer additional advantages of targeting specific cells and enhancing cellular uptake by the receptor mediated endocytosis. The targeted gene delivery system is also expected not only to minimize the expression of trans-gene expression in non-targeted cells but also to enhance the therapeutic efficacy of a given genetic drug at the same plasma concentrations. Several targeting moieties, including folic acid [8,9], transferrin [10], RGD peptide [11,12], EGF [13], and sugar moieties [14,15] were employed in polymeric gene carriers to enhance target cell specificity and transfection efficiency.

Glutamic acid decarboxylase (GAD) is one of the major auto-antigens in type I diabetes since GAD specific autoantibody was identified in ca. 80% of newly diagnosed patients [16,17]. GAD is found as a doublet of proteins commonly referred to as GAD-65 and GAD-67. Whereas GAD-67 is soluble, GAD-65 is post-translationally altered via multi-step processing involving modification of lipid within the N-terminal domain, the result of which anchors the molecule within the membrane [18]. GAD is mainly expressed in gamma aminobutyric acid (GABA) containing neurons of the adult mammalian central nervous system, in which it catalyzes the conversion of glutamic acid to GABA. Besides its presence in the nervous system, GAD is observed only within pancreatic islet cells, epithelial cells of the fallopian tube, and spermatozoa of the testes. Although the exact function of GAD in tissues other than the nervous system has yet been revealed, GAD is thought to play an important role in the development of diabetes [18,19].

In the present study, we employed a GAD-recognizable monoclonal antibody fragment (Fab’) as a targeting ligand to achieve pancreatic islet specific gene delivery. PEG was used as a linkage between Fab’ and PEI to increase solubility and stability of the complexes in the presence of serum protein. With the PEI-PEG-Fab’ conjugate, the gene transfer efficiency and specificity toward GAD expressing cells (MIN 6) were evaluated.

2. Materials and methods

2.1. Materials

Polyethylenimine (branched PEI, 25,000 Da), 2-mercaptoethylamine, ethidium bromide (EtBr), and (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide) (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). A heterofunctional poly(ethylene glycol) (N-hydroxysuccinimide-PEG-vinylsulfone, NHS-PEG-VS, Mw 3400) and N-hydroxysuccinimide-derivatized poly(ethylene glycol) (mPEG-NHS, Mw 2000) were obtained from Nektar (Huntsville, AL). Artificial capillary bioreactor module for in vitro antibody production was purchased from Spectrum (Rancho Dominguez, CA). Cell culture materials, serum free medium (Hybridoma SFM), Dulbecco’s modified Eagle’s medium (DMEM), and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). All other chemicals were of analytical grade and used without further purification.

2.2. Antibody production

Murine anti-GAD monoclonal antibody was produced by in vitro capillary bioreactor culture of HB184 hybridoma cells (ATCC, Manassas, VA). The antibody was purified by using protein G column chromatography (Amersham Biosciences, Uppsala, Sweden). To prepare F(ab’)2, the antibody was digested by mixing with immobilized pepsin (Pierce, Rockford, IL) in 20 mM sodium acetate buffer (pH 4.5) at 37 °C for 8 h. The F(ab’)2 was then purified by
using protein A affinity chromatography system (Pierce, Rockford, IL) followed by dialysis (MWCO 50,000) against PBS (pH 7.4). Fab’ was prepared by reducing the F(ab’)2 with 2-mercaptoethylamine (6 mg/ml) in reduction buffer (100 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.2) for 90 min at 37 °C. The excess 2-mercaptoethylamine was removed by using Sephadex G-25 column chromatography (Amersham Biosciences, Uppsala, Sweden).

2.3. Synthesis of PEI-PEG-Fab’ conjugate

Five milligrams of PEI dissolved in 3 ml of reaction buffer (10 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), 150 mM NaCl, 10 mM EDTA, pH 7.2) was reacted with 2-fold molar excess of a hetero-functional PEG, NHS-PEG-VS at room temperature for 30 min. The reaction mixture was dialyzed in a buffer containing 10 mM HEPES, 150 mM NaCl, 10 mM EDTA, pH 7.2 for 5 h to remove unreacted NHS-PEG-VS (MWCO 8000, Spectum Laboratories Inc., Rancho Dominguez, CA). A fraction of the reaction product was dialyzed against deionized water and freeze-dried to determine the conjugation ratio of PEG/PEI by 1H-NMR. A freshly reduced Fab’ was added to the reaction containing the PEI-PEG-VS intermediate. The reaction stoichiometry of Fab’/PEI-PEG was 1:1. The reaction was carried out for 12 h at room temperature. The unconjugated PEI was removed by dialysis in PBS (MWCO 50,000). The concentration of PEI was determined by measuring the formation of cuprammonium complex between PEI and copper (II) using UV spectrophotometry at 630 nm [20,21].

2.4. Characterization of PEI-PEG-Fab’/DNA complexes

To characterize the formation of PEI-PEG-Fab’/DNA complexes, electrophoretic mobility shift assays were carried out. Varying amounts of PEI-PEG-Fab’ conjugate and 0.3 μg of plasmid DNA (pCMVLuc) were diluted separately in PBS. The diluted PEI-PEG-Fab’ and DNA solutions were combined in different N/P ratios, mixed with gentle vortexing, and left for 15 min at room temperature prior to analysis. Electrophoresis was performed in 0.8% agarose gel with a current of 100 V for 20 min in TAE buffer (40 mM Tris/HCl, 1% (v/v) acetic acid, 1 mM EDTA). The retardation of migration of the complexes was visualized by the ethidium bromide staining.

Surface zeta potential measurements were carried out in PBS (pH 7.4) at 25 °C by using a zeta potential analyzer (Zeta Plus, Brookhaven Instrument Co., New York) equipped with a He–Ne laser at a wavelength of 632 nm. Effective hydrodynamic diameter was measured in the same condition as the surface charge measurement by dynamic light scattering using the same instrument. Every measurement was carried out in three serial measurements.

2.5. Cell culture and transfection

Mouse insulinoma cells (MIN6) and human kidney epithelial cells (293) were cultured in DMEM supplemented with 15% FBS and 10% FBS, respectively. Cells were maintained at 37 °C in a humidified 5% CO2 atmosphere. Before transfection, the cells were trypsinized and 10⁵ cells per well were plated in a six-well plate (35 mm diameter). The cells were then incubated 37 °C in a humidified 5% CO2 atmosphere for 24 h.

For transfection, 2 μg of the plasmid DNA and the desired amount of PEI-PEG-Fab’ were diluted separately in PBS and mixed. The mixture was allowed to form complexes for 15 min at room temperature. The cell culture medium was replaced with serum-free medium prior to the addition of the complexes. After 3 h incubation, the medium was removed and supplemented with fresh medium containing 15% (MIN6) or 10% (293) FBS. Luciferase gene expression was determined 24 h after transfection by using a commercial luciferase assay kit (Promega, Madison, WI). The luciferase activity was monitored for 15 s in a Lumat LB 9501 luminometer (Berthold, Wilbach, Germany). The transfection efficiency was expressed as relative light unit per mg of cell protein, of which concentration was measured by using Micro-BCA assay (Pierce, Rockford, IL).

2.6. In vitro toxicity assay

MIN6 cells were plated in a 96-well plate at a seeding density of 5000 cells per well in 0.2 ml of growth medium (DMEM) containing 15% FBS and maintained at 37 °C for 24 h. The growth medium was
Fig. 1. Synthetic scheme of PEI-PEG-Fab’ conjugate.
removed and the fresh growth medium containing DNA/polycation complexes was added at a desired N/P ratio. The metabolic activity of the cells was measured after an additional 48 h incubation. One hundred microliters of the fresh growth medium containing 50 μg MTT was added to each well and cells were incubated for 4 h. Lysis buffer (10% (w/v) SDS, 45% DMF, pH 4.7) was then added and incubated for 24 h. Absorbance was measured at 590 nm in a microplate reader (Bio-Rad, Model 550). Survival percentage was calculated as compared to mock-treated cells (100% survival).

3. Results and discussion

3.1. Synthesis of PEI-PEG-Fab’ conjugate

The synthetic scheme of PEI-PEG-Fab’ conjugate is shown in Fig. 1. To synthesize the PEI-PEG-Fab’ conjugate, a heterobifunctional PEG, NHS-PEG-VS, was used as a linker between Fab’ and PEI. An active NHS group of the NHS-PEG-VS was first reacted with the primary amine groups of PEI to form VS-PEI-PEG intermediate. The coupling efficiency was 1.37 PEG linkers per PEI as determined by 1H-NMR. The coupling efficiency was calculated by relating the relative peak area of –CH2CH2O– protons of PEG (δ 3.6 ppm) to the multiple peak areas of –CH2CH2NH2– protons of PEI (δ 2.6–3.2 ppm). The F(ab’)2 fragment was obtained by pepsin digestion of anti-GAD monoclonal antibody followed by protein A column chromatography. The F(ab’)2 was then cleaved to two Fab’ fragments by reduction with 2-mercaptoethylamine, which selectively reduces the disulfides in the hinge region that hold the heavy chains together [22]. The VS-PEI-PEG was then reacted with a sulfhydryl (–SH) group of the freshly reduced Fab’ fragment to obtain the

![Fig. 2. Electrophoretic mobility shift assays of pDNA/PEI-PEG-Fab’ (A) and pDNA/PEI-PEG (B) complexes. The complexes were analyzed by 0.8% agarose gel.](image)

![Fig. 3. Hydrodynamic diameter (A) and surface zeta-potential (B) of pDNA/PEI-PEG-Fab’ and pDNA/PEI-PEG complexes at different N/P ratios. Every measurement was carried out three serial measurements. The data are given as the mean ± S.D.](image)
PEI-PEG-Fab’ conjugate. Unconjugated PEI was removed by dialysis. The amount of PEI was determined by Cu (II) chelating assay as previously described [20,21]. The molar conjugation ratio of Fab’ to PEI was 0.97 as determined by measuring at the absorbance of Fab’ at 280 nm with the background correction using PEI-PEG.

3.2. Characterization of PEI-PEG-Fab’/DNA complexes

Electrophoretic mobility shift assay (EMSA) was carried out to observe the PEI-PEG-Fab’ could stably form complexes by electrostatic interaction with plasmid DNA. The retardation of DNA band migration was occurred as the amount of the PEI-PEG-Fab’ increased, suggesting that the conjugate formed a tight complex with DNA by charge neutralization. The DNA band was completely retarded above the N/P ratio of 2 (Fig. 2). There were no significant differences in the retardation of DNA band between PEI-PEG-Fab’ and PEI-PEG.

The hydrodynamic diameter of the complexes was measured by a dynamic light scattering method. Both PEI-PEG-Fab’ and PEI-PEG showed smaller complex size at higher N/P ratios (Fig. 3A). However, much smaller complexes were formed from PEI-PEG compared to PEI-PEG-Fab’. The surface charges of the

Fig. 4. Cellular uptake of pDNA/PEI-PEG-Fab’ (A) and pDNA/PEI-PEG (B) complexes in MIN6 cells observed by confocal microscopy. Transfection complexes were prepared at N/P ratio of 10.
complexes formed from PEI-PEG-Fab’ were close to neutral over the range of N/P ratios, whereas those of the complexes from PEI-PEG increased up to 10 mV along with N/P ratio (Fig 3B). The results suggest that Fab’ would be located in surface layer of the complexes and, therefore, the presence of Fab’ in conjunction with the charge shielding effect of PEG would affect the physical characteristics of the complexes. These results are also consistent with previous observation with pegylated PEIs [23,24]. The surface localization of Fab’ is of prime importance for targeted gene delivery and the neutral surface charge would also be beneficial for in vivo application since it is known that the cationic surface charge of transfection complexes showed close relationship with the interaction of serum components [25].

3.3. Confocal microscopy

Uptake of plasmid DNA/PEI-PEG-Fab’ complexes by GAD expressing mouse insulinoma cells (MIN6 cells) was visualized under confocal microscope. For visualization, plasmid DNA was labeled by fluorescent dye, ethidium monoazide, which is known to intercalate DNA strand and form a stable covalent linkage by an exposure of UV radiation [26]. The complexes formed from PEI-PEG-Fab’ (Fig. 4A) showed much higher cellular uptake compared to those formed from PEI-PEG (Fig. 4B). This result suggests that the conjugation of Fab’ could greatly enhance the cellular uptake efficiency of the polymer/DNA polyelectrolyte complexes. The enhanced cellular uptake is possibly due to a higher degree of binding to MIN6 cells. The higher cellular binding of PEI-PEG-Fab’ is consistent with the previously published result with pegylated Fab’-PEI (PEI-PEG-Fab’) conjugate [7]. After 3 h incubation, although the majority of PEI-PEG-Fab’ complexes was localized in cytoplasmic region, possibly due to the antibody-mediate endocytosis, some of the complexes were still bound on plasma membrane as highly localized patches, which could then migrate to coated pit for endocytosis. In contrast, PEI-PEG complexes were mostly localized within cytoplasmic region. The patching phenomenon of PEI-PEG-Fab’ complexes was also found in previous report on anti-JL1 antibody-PLL conjugate, which was designed for targeting leukemia T cells [27].

3.4. Transfection studies

In order to investigate the targeting property of PEI-PEG-Fab’ toward islet beta cells, transfection experiments were performed with GAD-expressing MIN6 cells and GAD-negative 293 cells. PEI-PEG-Fab’/pCMVLuc complexes showed about 10-fold higher transfection efficiency than PEI-PEG control in MIN6 cells (Fig. 5A). In 293 cells, however, there was no significant difference in transfection efficiency between PEI-PEG-Fab’ and PEI-PEG over the selected N/P ratio range (Fig. 5B). The results suggest that PEI-PEG-Fab’ possess the specificity toward GAD-antigen presenting cells. Expression of GAD antigen in MIN6 cells was reported in previous pub-
lication [28]. To verify that enhanced cellular uptake mediated by specific interaction between GAD antigen and Fab’ was responsible for the enhanced transfection efficiency, competition experiment was carried out in the presence of free Fab’ (100 μg/ml) during transfection with PEI-PEG-Fab’/DNA complexes. The transfection efficiency of PEI-PEG-Fab’/DNA complexes was significantly reduced to show similar level to that of PEI-PEG complexes (Fig. 6). This suggests that the free Fab’ preoccupy the available antigen on plasma membrane, which results in the reduction of the specific cellular uptake of the PEI-PEG-Fab’/DNA complexes. Taken all together, the PEI-PEG-Fab’ can significantly enhance the transfection in GAD-expressing islet cell line due to the increased cellular uptake of the PEI-PEG-Fab’/DNA complexes, which is mediated by specific antigen-antibody (Fab’) interaction.

3.5. Cytotoxicity

The influence of PEI-PEG-Fab’/DNA complexes on cell viability was monitored by using MTT assays. The PEI-PEG-Fab’/DNA complexes showed a slightly lower cytotoxicity compared to PEI-PEG complexes in a selected N/P ratio range (2.5–32.5), although the difference was marginal (data not shown).

4. Conclusions

In this study, anti-GAD Fab’ was introduced to cationic gene carrier, PEI, as a targeting moiety for islet cells. The transfection experiments showed clear selectiveness toward the cells (MIN6 expressing a specific antigen, GAD. Owing to the specific targeting property of it, PEI-PEG-Fab’ conjugate could successfully make up for the inherent problem of pegylated PEI (PEI-PEG), the reduced transfection efficiency. The surface charge of the complexes between the conjugate and plasmid DNA was almost neutral, suggesting that the complexes may exhibit less non-specific interactions with the serum components than complexes with highly positively charged surface. These results showed the possibility of using the PEI-PEG-Fab’ as a candidate for the targeted delivery of therapeutic DNAs to pancreatic islet cells through systemic administration. This islet-targeted delivery system would be especially useful, being applied in the blockade of the beta-cell specific expression of major auto-antigens, such as glutamic acid decarboxylase (GAD), protein tyrosine phosphatase-like molecule (IA-2), or islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP), by delivering a plasmid encoding an antisense RNA toward the specific auto-antigen to the pancreatic islet cells. Undesirable side effect of GAD-recognizable Fab’ in other GAD expressing tissues, including the GABA neurons of the central nervous system, epithelial cells of the fallopian tube and spermatozoa of the testes, could be minimized by employing islet-specific promoters, such as rat insulin promoter, to control the expression of the desired therapeutic genes. In conjunction with the islet-targeted gene carrier (PEI-PEG-Fab’), the study on the prevention of type I diabetes using a plasmid encoding antisense RNA, which could block the expression of the major antigens, in non-obese diabetic (NOD) mice is in progress.

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References


