The use of lactoferrin as a ligand for targeting the polyamidoamine-based gene delivery system to the brain

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Abstract

Development of an efficient gene vector is a key-limiting factor of brain gene therapy. In this study, lactoferrin (Lf), for the first time, was investigated as a brain-targeting ligand in the design of polyamidoamine (PAMAM)-based non-viral gene vector to the brain. Using polyethyleneglycol (PEG) as a spacer, PAMAM–PEG–Lf was successfully synthesized. This vector showed a concentration-dependent manner in the uptake in brain capillary endothelial cells (BCECs). The brain uptake of PAMAM–PEG–Lf was 2.2-fold compared to that of PAMAM–PEG–transferrin (Tf) in vivo. The transfection efficiency of PAMAM–PEG–Lf/DNA complex was higher than that of PAMAM–PEG–Tf/DNA complex in vitro and in vivo. The results of frozen sections showed the widespread expression of an exogenous gene in mouse brain via intravenous administration. With a PAMAM/DNA weight ratio of 10:1, the brain gene expression of the PAMAM–PEG–Lf/DNA complex was about 2.3-fold when compared to that of the PAMAM–PEG–Tf/DNA complex. These results provide evidence that PAMAM–PEG–Lf can be exploited as a potential non-viral gene vector targeting to the brain via noninvasive administration. Lf is a promising ligand for the design of gene delivery systems targeting to the brain.

Keywords: Brain targeting; Lactoferrin; Receptor-mediated gene delivery; Polyamidoamine

1. Introduction

Non-viral brain gene therapy poses two major problems: (1) how do the genes accumulate in the brain? (2) How do the genes cross the blood–brain barrier (BBB) efficiently? Intracerebral injection is the most frequently used method to locate exogenous genes in the brain [1,2]. However, this method is highly invasive and cannot deliver exogenous genes to all areas of the brain. One current candidate is the receptor-mediated targeting via intravenous injection of ligand-conjugated vector/gene complexes that target to receptors expressed on the luminal side of the BBB [3,4]. Based on the receptor-mediated mechanism, the gene vector technique enables the efficient transfer of exogenous genes across the BBB.

Till date, receptors discovered on the BBB mainly include transferrin (Tf) receptors, insulin receptors, epidermal growth factor receptors, insulin-like growth factor receptors, and so on [5,6]. The Tf receptors are of particular interest because their expression in capillaries is restricted to brain capillaries [7]. However, the Tf receptors are almost saturated under physiologic conditions because of high endogenous plasma Tf concentration [8]. Thus, Tf itself is limited as a brain drug transport ligand [9]. Another candidate, a mouse monoclonal antibody to the rat Tf receptor (OX26), has been studied recently and shown great potential in the delivery of therapeutic agents to the brain [4,10]. Nevertheless, Ji et al. [11] argued that there was only a slight amount of OX26 in the brain after intravenous administration, and the brain uptake of OX26 and Tf was not significantly different. Furthermore, commercially available OX26 antibodies are less prone to uptake into the brain tissue, unlike the one purified by the researchers themselves [12]. In our previous studies, lactoferrin (Lf) was suggested as a promising brain-targeting ligand [13].

Lf, a single-chain iron-binding glycoprotein, belongs to the Tf family. It is becoming increasingly evident that Lf is a multifunctional protein to which several physiological...
roles have been attributed [14]. The multiple biological activities of Lf are mediated by Lf receptors [15]. Several lines of evidence including the results of immuno-histochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) have indicated the presence of specific Lf receptors in the brain [16,17]. The Lf receptor-mediated transcytosis through the BBB has also been demonstrated [18]. Recently, the Lf receptors were characterized in our lab, exhibiting two classes of binding sites with high affinity (dissociation constant \( K_d = 6.8 \) nm) and low affinity \( K_d = 4815 \) nm) [13]. The plasma concentration of endogenous Lf is approximately 5 nm [16], much lower than \( K_d \) of Lf receptors in the BBB, showing the promising use of Lf as a ligand for facilitating drug delivery system into the brain. Moreover, an increased expression of Lf receptors has been reported in patients with Parkinson’s disease [19,20], which showed further potential of Lf for clinical application.

In this study, a novel non-viral vector using Lf as the brain-targeting ligand was constructed. Polyamidoamine (PAMAM) dendrimer [generation=5, 5% w/w solution in methyl alcohol, containing 128 surface primary amino groups (MW 28,826)], human holo-Tf, Lf from bovine colostrum, Bolton and Hunter reagent, 2-iminothiolane hydrochloride (Traut’s reagent) and \( 125^I \)-labeled Bolton and Hunter reagent in benzene was carefully dried under a stream of nitrogen. PAMAM (10 mg) was dissolved in borate buffer (pH 8.5, 0.1 m), added to the \( 125^I \)-labeled Bolton and Hunter reagent (0.69 mCi) and reacted for 15 min on ice, mixing periodically. The \( 125^I \)-labeled PAMAM solution was carefully purified by dialysis against NaCl (1%) and stored at 4°C until use.

2.2. Iodination of PAMAM

Bolton and Hunter reagent (3 \( \mu \)g) was iodinated with \( 125^I \) (1.01 mCi) as described previously [25] with a 79.2% yield. \( 125^I \)-labeled Bolton and Hunter reagent in benzene was carefully dried under a stream of nitrogen. PAMAM (10 mg) was dissolved in borate buffer (pH 8.5, 0.1 m), added to the \( 125^I \)-labeled Bolton and Hunter reagent (0.69 mCi) and reacted for 15 min on ice, mixing periodically. The \( 125^I \)-labeled PAMAM solution was carefully purified by dialysis against NaCl (1%) and stored at 4°C until use.

2.3. Synthesis of PAMAM derivatives

A 1:2 conjugate of PAMAM and NHS-PEG4000-MAL was prepared through specific reaction between the primary amino groups on the surface of PAMAM and the NHS groups of the bifunctional PEG derivative. The reaction was performed in phosphate-buffered solution (PBS, pH 8.0) for 15 min at room temperature. The resulting conjugate, PAMAM-PEG, was purified by ultrafiltration using a 5-kDa molecular weight cutoff membrane to remove unreacted PEG and the buffer was exchanged into PBS (pH 7.0). At the same time, Tf was thiolated using Traut’s reagent according to Lu et al. [26] and Lf was modified with thiold groups using SADA [5]. Different thiolation methods were used due to different isoelectric points of Tf and Lf. The amount and stability of the thiol groups was determined with Ellmann’s reagent [27]. For both Tf and Lf, the mole ratio of thiol group to protein maintained around 1.3–1.5:1. Then PAMAM-PEG was reacted with thiolated proteins, 1:1 (mol/mol), in PBS (pH 7.0) for 24 h at room temperature. The MAL groups of PAMAM-PEG were specifically reacted with the thiol groups of thiolated proteins, yielding PAMAM-PEG–Tf and PAMAM-PEG–Lf. SDS-PAGE was used to demonstrate the almost full reaction of thiolated Lf. For the synthesis of BODIPY-labeled conjugates, PAMAM was first reacted with BODIPY in 100 mM NaHCO\(_3\) for 12 h at 4°C, purified and identified as described previously [28]. The corresponding conjugates, BODIPY-labeled PAMAM-PEG–Tf and BODIPY-labeled PAMAM-PEG–Lf were synthesized as described above. For the synthesis of \( 125^I \) labeled conjugates, \( 125^I \)-labeled PAMAM was used.

2.4. Characterization of PAMAM derivatives

The characteristics of PAMAM derivatives were analyzed by nuclear magnetic resonance (NMR) spectroscopy, PAMAM, PAMAM–PEG, and PAMAM–PEG–Lf was solubilized in D\(_2\)O and analyzed in a 400 MHz spectrometer (Varian, USA).

2.5. Cellular uptake of BODIPY-labeled dendrimers in BCECs

BCECs were seeded at a density of 8 \( \times \) 10\(^4\) cells/well in 6-well plates (Corning-Coaster, Tokyo, Japan), incubated for 72 h, and checked under the microscope for confluence and morphology. Wells with the similar cell density were chosen for the experiments (data not shown). Following this, BCECs were incubated with BODIPY-labeled PAMAM-PEG–Tf and BODIPY-labeled PAMAM-PEG–Lf in a PAMAM concentration range 0.035–1.750 \( \mu \)g for 60 min. The cells were washed three times with PBS (pH 7.4) and visualized under an IX2-RFACA fluorescent microscope (Olympus, Japan). For quantitative analysis, BCECs treated as described above were trypsinized and centrifuged at 1600 rpm for 8 min to obtain a cell pellet, which was subsequently resuspended in PBS (pH 7.4) and analyzed using a flow cytometer (FACSCalibur, BD, USA) equipped with an argon ion laser (488 nm) as the excitation source. The fluorescence of BODIPY was collected at 520 nm (FL1). For each sample, 10,000 events were supplied by the Department of Experimental Animals, Fudan University, and maintained under standard housing conditions. All animal experiments were carried out in accordance with the guidelines evaluated and approved by the ethics committee of Fudan University.
were collected and data were analyzed with CELLQuest software. BCECs cultured under the normal conditions served as the control. The living cells were defined by gating the major population of the cells and only the cells within this gate were analyzed. The mean fluorescence intensity of the cells was calculated using the histogram plot.

2.6. Biodistribution of 125I-labeled dendrimers in Balb/c mice

Balb/c mice were injected via the tail vein with 125I-labeled dendrimers (125I-PAMAM, 125I-PAMAM-PEG, 125I-PAMAM-PEG-Tf or 125I-PAMAM-PEG-Lf) at a dose of 40 μCi/mouse. At 2h after injection, a blood sample (0.2 mL) was collected. Then the mice were humanely killed and the principle organs (including brain, heart, liver, spleen, lung and kidney) were removed and weighed. The radioactivity of 125I in the blood and organ samples was assessed using a γ-counter and the results expressed as percentage of dose administered accumulating in each organ (% ID/g).

2.7. Preparation of dendrimer/DNA complexes

Dendrimers (PAMAM, PAMAM–PEG, PAMAM–PEG–Tf and PAMAM–PEG–Lf) were freshly prepared and diluted to appropriate concentrations in distilled water. DNA solution (100 μg DNA/mL 50 mM sodium sulfate solution) was added to obtained specified weight ratios (6:1 or 10:1, PAMAM to DNA, w/w) and immediately vortexed for 30 s at room temperature. Agarose gel electrophoresis was carried out to verify the complete complexation of dendrimers with DNA. Freshly prepared complexes were used in the following experiments.

2.8. Efficiency of gene expression in BCECs

BCECs were seeded in 24-well plates at a density of 2.5 × 10⁴ cells/well. Cultured for 48 h, the cells reached 70% confluence. The complexes containing pEGFP, with PAMAM to DNA at weight ratios of 6:1 and 10:1 were added to the cells in FCS-free medium and the mixture was incubated at 37°C for 1 h. After 48 h, fluorescence images of gene products, green fluorescent protein (GFP), were acquired and photographed under a fluorescence microscope. The quantitative efficiency of gene transfer was determined by luciferase activity using pGL2-Control Vector. Two days post-transfection, the activity of the gene product, luciferase, was quantified by Luciferase Assay System (Promega, Madison, WI, USA) following the manufacturer’s instructions. In brief, the cells were lysed in a sufficient volume of cell culture lysis buffer (Promega, Madison, WI, USA) following the manufacturer’s instructions. In brief, the cells were lysed in a sufficient volume of cell culture lysis buffer (Promega, Madison, WI, USA). The lysate was vortexed for 15 s, and then centrifuged at 12,000 g for 2 min at 4°C. The supernatant was assayed for luciferase activity and the emitted light was measured over 10 s using a chemiluminescence analyzer (BPCL, Peking, China) giving light units.

2.9. Distribution of gene expression qualitatively in mouse brain

The complexes containing pEGFP (10:1, PAMAM to DNA, w/w) were injected into the tail vein of Balb/c mice at a dose of 50 μg DNA/mouse. About 48 h later, the animals were humanely sacrificed. The brains were removed, fixed in 4% paraformaldehyde for 48 h, placed in 15% sucrose PBS solution until subsidence (about 6 h), and then in 30% sucrose until subsidence (about 24 h). Following this, the brains were frozen in OCT embedding medium (Sakura, USA) at −80°C. Frozen sections of 20 μm thickness were prepared with a cryotome Cryostat (Leica, CM 1900, Germany) and stained with 300 nM DAPI for 10 min at room temperature. After washing twice with PBS (pH 7.4), the sections were immediately examined under the fluorescence microscope.

2.10. Quantification of gene expression in Balb/c mice

The complexes containing pGL2-Control Vector (10:1, PAMAM to DNA, w/w) were injected into the tail vein of mice at a dose of 50 μg DNA/mouse. At 48 h after injection, the mice were humanely decapitated and the principle organs (including brain, heart, liver, spleen, lung and kidney) were extirpated. The organs were carefully washed with distilled water, and homogenized in 1 mL lysis reagent (Promega, Madison, WI, USA) using a JY92-II tissue homogenizer. The homogenate was centrifuged at 14,000 g for 20 min at 4°C. The luciferase activity and cellular proteins in the supernatant were quantified by Luciferase Assay System and Bio-Rad Protein Assay Kit, respectively. The results were expressed as light units/mg protein.

2.11. Statistical analysis

All of the quantitative measurements were collected in quadruplicate, and the experiments were repeated four times. The data are expressed as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed by one-way ANOVA followed by Bonferroni’s post-test using Stata software (version 7.0).

3. Results

3.1. Characterization of PAMAM derivatives

The solvent peak of D₂O was found at 4.65 ppm. The methylene protons of branching units of PAMAM have multiple peaks between 2.2 and 3.4 ppm (Fig. 1A), similar to the NMR spectrum of PAMAM (generation = 3) reported previously [29]. The NMR spectrum of PAMAM–PEG had multiple peaks of the repeat units in PEG at 3.5 ppm and a characteristic peak of the MAL group in PEG at 6.7 ppm (Fig. 1B), showing the conjugation of PEG to PAMAM. However, the MAL peak disappeared in the NMR spectrum of PAMAM–PEG–Lf, even as the repeat units of PEG still presented a sharp peak at 3.5 ppm (Fig. 1C), indicating that the MAL group had reacted with the thiol group of Lf. The NMR spectra demonstrated the successful synthesis of PAMAM–PEG–Lf. Furthermore, the integrated areas of NMR peaks were used to quantify the number of PEG chains per PAMAM with the assumption of 280 methylene protons per PEG and 2032 per PAMAM. As shown in Fig. 1B, PAMAM–PEG had a PEG/PAMAM proton ratio of 0.33, implying an average of 2.4 PEG chains per PAMAM.

3.2. Cellular uptake of BODIPY-labeled dendrimers in BCECs

The results of cellular uptake of BODIPY-labeled dendrimers are shown qualitatively using fluorescent images and quantitatively as a percentage of BODIPY-positive cells. BCECs treated with BODIPY-labeled PAMAM–PEG–Lf exhibited fluorescence intensity corresponding to the concentrations of BODIPY-labeled PAMAM–PEG–Lf (Fig. 2, Table 1). When the concentration of BODIPY-labeled PAMAM–PEG–Lf ranged from 0.035–1.750 μg/mL after a 60-min exposure, the percentage of...
BODIPY-positive cells increased from 60.65% to 89.00%, and mean fluorescence intensity increased from 38.97 to 121.88 (Table 1). The cellular uptake of BODIPY-labeled PAMAM–PEG–Tf also exhibited a concentration-dependent manner (Fig. 3). At a fixed concentration, BODIPY-labeled PAMAM–PEG–Lf was taken up by BCECs more efficiently than BODIPY-labeled PAMAM–PEG–Tf (Fig. 3).

3.3. Biodistribution of 125I-labeled dendrimers in Balb/c mice

The brain uptake of dendrimers 2 h after intravenous administration was as follows: 125I-PAMAM–PEG–Lf > 125I-PAMAM–PEG–Tf > 125I-PAMAM–PEG < 125I-PAMAM (Fig. 4A). Using 125I-PAMAM as a comparison, 125I-PAMAM–PEG–Lf increased the brain uptake by 4.6-fold when 125I-PAMAM–PEG–Tf uptake was 2.1-fold. The brain uptake of 125I-PAMAM–PEG–Lf was 2.2-fold of that of 125I-PAMAM–PEG–Tf. 125I-PAMAM–PEG–Tf showed highest accumulation in liver, spleen, lung, and kidney. The blood concentration of 125I-PAMAM–PEG–Lf was higher than the other dendrimer derivatives (Fig. 4B).

3.4. Efficiency of gene expression in BCECs

For the different complexes, PAMAM/DNA, PAMAM–PEG/DNA, PAMAM–PEG–Tf/DNA, and PAMAM–PEG–Lf/DNA, an increase in the weight ratio of PAMAM to DNA resulted in an increase in gene expression in BCECs. The modification of PEG did not change the gene expression in BCECs markedly. The conjugation of Lf increased the expression efficiency most significantly. Compared to PAMAM–PEG–Tf/DNA complex, the number of GFP-positive cells increased after the treatment with PAMAM–PEG–Lf/DNA complex at fixed PAMAM to DNA weight ratios (Fig. 5A–D). This was verified by the luciferase activity assay. With a PAMAM to DNA weight ratio at 6:1, the luciferase activity in BCECs treated with PAMAM–PEG–Lf/DNA complex was 7.55 ± 0.88 × 10^5 units/mg protein, which was about 3.6-fold of that with PAMAM/DNA complex (2.10 ± 0.11 × 10^5 units/mg protein) and PAMAM–PEG/DNA complex (2.21 ± 0.26 × 10^5 units/mg protein), and 1.9-fold of that with PAMAM–PEG–Tf/DNA complex (4.01 ± 0.83 × 10^5 units/mg protein). With a PAMAM to DNA weight ratio at 10:1, the luciferase activity in BCECs treated with PAMAM–PEG–Lf/DNA complex was 9.46 ± 0.89 × 10^5 units/mg protein, which was about 3.0-fold of that with PAMAM/DNA complex (3.19 ± 0.54 × 10^5 units/mg protein) and PAMAM–PEG/DNA complex (3.66 ± 0.32 × 10^5 units/mg protein), and 1.5-fold of that with PAMAM–PEG–Tf/DNA complex (6.49 ± 0.82 × 10^5 units/mg protein) (Fig. 5E).

3.5. Distribution of gene expression qualitatively in mouse brain

The GFP expressions in sections of the cortical layer, hippocampus, caudate putamen, substantia nigra, and fourth ventricle at 48 h after the intravenous injection of 50 μg/mouse of DNA complexed with PAMAM–PEG–Tf and PAMAM–PEG–Lf are shown in Fig. 6A–J. For both PAMAM–PEG–Tf/DNA and PAMAM–PEG–Lf/DNA complexes, the pEGFP-N2 gene expression was observed...
in all the five regions. The GFP expression of PAMAM–PEG–Lf/DNA complex was higher than PAMAM–PEG–Tf/DNA complex (Fig. 6A–J). There was almost no background fluorescence (data not shown).

3.6. Quantification of gene expression in Balb/c mice

To examine the transfection efficiencies of PAMAM/DNA, PAMAM–PEG/DNA, PAMAM–PEG–Tf/DNA, and PAMAM–PEG–Lf/DNA complexes in animals, the complexes with a PAMAM to DNA weight ratio at 10:1 were injected via the tail veins of Balb/c mice, and the expression of pGL2-Control Vector in principal organs was measured after 48 h (Fig. 6K and L). The brain luciferase expression of PAMAM–PEG–Lf/DNA complex was 7.20 ± 0.41 × 10^3 units/mg protein, which was about 5.2-fold of that with PAMAM/DNA complex (1.38 ± 0.39 × 10^3 units/mg protein) and PAMAM–PEG/
DNA complex (1.31 ± 0.18 × 10^3 units/mg protein), and 2.3-fold of that with PAMAM–PEG–Tf/DNA complex (3.12 ± 0.41 × 10^3 units/mg protein) (Fig. 6K). The gene expression of the PAMAM–PEG–Lf/DNA complex in the kidney was increased, (p < 0.05) compared to that of PAMAM/DNA, whereas the heart, liver, spleen, and lung level were not changed markedly (p > 0.05) (Fig. 6L). For all the complexes, the gene expression in the heart was the highest.

4. Discussion

For the first time, Lf was exploited as a brain-targeting ligand for the rational design of DNA delivery systems. In this study, the Lf-conjugated gene vector exhibited enhanced brain-targeting capability and the Lf-conjugated vector/DNA complex showed higher expression of exogenous genes in the brain.

In this study, PEG served as a spacer between PAMAM and Lf. The successful synthesis of PAMAM–PEG–Lf was demonstrated by the results of NMR spectroscopy (Fig. 1). PEG has been extensively used for its ability to enhance the lifetime of liposomes in the bloodstream, via the so-called “steric stabilization” mechanism [30]. Plank et al. [31] also showed that coupling of PEG to polylysine/DNA complexes reduced complement system activation. Therefore, in this study, PEG3400-modified PAMAM was synthesized, having 1.6% of their amine functions derivatized. As PEG is expected to inhibit ionic interactions between positively charged macromolecules and negatively charged membranes, Lf was coupled to the remote end of the PEG chain, triggering the receptor-mediated mechanism to increase the accumulation of vectors in the brain. In addition, it has been shown that an increase in the generation of PAMAM resulted in an increase in not only the transfection efficiency but also the cytotoxicity [32]. The modification of low generation PAMAM with biocompatible PEG molecules would create a conjugate of PAMAM core with flexible PEG chains, which mimics the fractured high-generation dendrimer and produces high transfection efficiency [22].

The in vitro behavior of the gene vectors, PAMAM–PEG–Lf and PAMAM–PEG–Tf, were investigated in BCECs using BODIPY fluorescent dye. The cellular uptake of PAMAM–PEG–Lf was in a concentration-dependent manner in the experimental concentration range (Fig. 2, Table 1), not showing apparent competitive inhibition at higher concentration. And, at each concentration, the percentage of BODIPY-positive cells and mean fluorescence intensity of PAMAM–PEG–Lf were much higher than that of PAMAM–PEG–Tf (Fig. 3), suggesting that the conjugation of Lf makes the vector be taken up by BCECs more efficiently. Correspondingly, the Lf-modified delivery system showed the highest gene transfection and expression efficiency in BCECs in both the qualitative and quantitative evaluation (Fig. 5). This might partly attribute to the positive property of Lf itself, which facilitates the binding of the gene vector with the negatively charged biomembranes [33].

As shown in Fig. 4, PAMAM–PEG–Lf was measured in the brain. Then PAMAM–PEG–Lf was complexed with DNA, formulating the Lf-modified gene delivery system. The results of frozen sections showed that the products of exogenous genes were widely expressed throughout the brain, including neurons, as seen in the hippocampus (Fig. 6G), caudate putamen (Fig. 6H), etc. And the pEGFP-N2 gene was expressed in the hippocampus, substantia nigra, and 4th ventricle, but was almost non-existent in the cortical layer and caudate putamen, for PAMAM/DNA and PAMAM–PEG/DNA complexes (data not shown), which was in good consistence with previous studies [34]. These results suggest that PAMAM–PEG–Lf and its complex with DNA could transport into the brain and mediate efficient gene expression in the neuron cells. It has been reported that Lf could cross the BBB through receptor-mediated transcytotic mechanism [18]. To our knowledge, Tf receptors can mediate both the transcytosis through the BBB and the endocytosis of the vector/DNA complex into brain cells [3]. Therefore, it was assumed that the uptake of Lf-conjugated vector or vector/DNA complex by the brain was in a manner similar to the Tf receptor-mediated pathway. This means that the Lf-modified vector or vector/DNA complex might still possess the property of Lf and transverse both the BBB and the neuronal cell membranes through the Lf receptor-mediated pathway. In the future mechanism studies, we will further demonstrate the detailed cell types which express
exogenous genes. The fluorescent images of gene expression in different regions have clearly revealed the preferential localization of PAMAM–PEG–Lf in the brain when compared with other three controls (Fig. 4), further indicating the potent effect of Lf’s modification in the gene vector. Correspondingly, the quantitative assay showed that the brain luciferase expression of PAMAM–PEG–Lf/DNA complex was 2.3-fold of that of PAMAM–PEG–Tf/DNA complex (Fig. 6K). From the results described above, three possibilities for the enhanced brain accumulation of PAMAM–PEG–Lf or gene expression of the PAMAM–PEG–Lf/DNA complex can be proposed: (1) the binding between Lf and its receptors are not affected by the endogenous Lf, for the Lf receptors were not saturated under physiologic conditions due to the low plasma concentration of endogenous Lf[16], (2) Lf was positively charged itself, which can bind with the negative cellular membranes more easily than Tf, and (3) Lf transport across the BCEC monolayer was reported as unidirectional, from the apical to the basolateral side[18], which might result in higher accumulation of Lf-conjugated vector or vector/DNA complex in the neuron, compared to Tf-conjugated counterparts. In addition, the gene delivery system with Lf attachment might be more efficient under certain pathological conditions, such as Parkinson’s disease[19,20], as increased

Fig. 5. The qualitative and quantitative gene expression in vitro. The fluorescence images of GFP expression in BCECs were taken 48 h post-transfection with PAMAM–PEG–Tf/DNA (A, C) and PAMAM–PEG–Lf/DNA (B, D) complexes, with a PAMAM to DNA weight ratio of 6:1 (A, B) and 10:1 (C, D), respectively. BCECs were incubated with different complexes for 1 h. Green: GFP. Original magnification: 200 \times\circ. (E) Transfection assay of PAMAM/DNA, PAMAM–PEG/DNA, PAMAM–PEG–Tf/DNA and PAMAM–PEG–Lf/DNA complexes in BCECs with 1 h incubation. Luciferase activity was measured 48 h post-transfection as described in Materials and Methods, and was expressed as light units per mg protein. Data represent the mean \pm S.E.M (n = 4). (**p < 0.01). Bar = 100 \mu m.
expression of Lf receptors on microvessels and neurons has been reported.

As seen in Fig. 4B, all the gene vectors were most densely accumulated in the kidney. This tropism is in good agreement with an earlier study where 111In- or 88Y-labeled PAMAM showed mainly renal accumulation, which was attributed to the strongly positive property of PAMAM by the researchers [35]. The clearance of Lf from liver [18] might result in the relatively high accumulation of PAMAM–PEG–Lf in the liver. However, the distribution of PAMAM–PEG–Lf in liver, spleen, lung, and kidney was much lower than that of PAMAM–PEG–Tf. This significantly increased the blood and brain concentrations of PAMAM–PEG–Lf, implying the prolonged lifetime in the bloodstream and the relatively higher accumulation of the Lf-conjugated gene vector in the brain. Interestingly, the gene expression of the vector/DNA complexes was primarily in the heart (Fig. 6L), whereas the vectors mainly accumulated in the kidney (Fig. 4B). The in vivo fate of vectors and the gene expression of vector/DNA complexes are affected by complicated factors. The detailed mechanisms of this discrepancy remain to be examined. Furthermore, the exogenous genes are also expressed in peripheral organs, so the selection of therapeutic genes must be cautious. Genes which have no negative effects in peripheral organs are preferred, such as the ones encoding neurotrophic factors.

In summary, the results suggested that Lf is a potential ligand in designing non-viral gene delivery system to brain. In the future studies, the potential of Lf-modified gene delivery system in treating brain disorders would be investigated in animal models.

5. Conclusion

The Lf functionalized PAMAM–PEG gene vector, PAMAM–PEG–Lf, was successfully achieved. This novel nanocarrier showed a promising targeting capability to the brain. In addition, it was shown that the functionalization of the gene vector with Lf was critical for facilitating their translocation into the brain tissue after intravenous administration. The data from this study suggest that Lf can be used as a potent ligand for the design of drug delivery systems targeting to the brain.
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