Efficient serum-resistant lipopolyplexes targeted to the folate receptor

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**Abstract**

In this work, we have developed and evaluated a new targeted lipopolyplex (LPP), by combining poly-ethylenimine (PEI), 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP)/Chol liposomes, the plasmids pCMVLuc/pCMVIL-12, and the ligand folic acid (FA), able to transfect HeLa and B16-F10 cells in the presence of very high concentration of serum (60% FBS). These complexes (Fol-LPP) have a net positive surface charge. The combination of folic acid with lipopolyplexes also enhanced significantly the transfection activity of the therapeutic gene interleukin-12 (IL-12), without any significant cytotoxicity. The specificity of the folate receptor (FR)-mediated gene transfer was corroborated by employing a folate receptor-deficient cell line (HePG2). This formulation improved gene delivery showed by conventional lipoplexes or polyplexes resulting an efficient, simple, and nontoxic method for gene delivery of therapeutic genes in vitro and very probably in vivo.

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1. Introduction

One of the most important reasons why nonviral vectors have not been effective in cancer gene therapy is the low level of gene delivery and expression achieved by these systems. Transgene expression in vivo for therapeutic purposes will require methods that allow for an efficient gene transfer into cells. Although current vector technologies are being improved, the development of novel vector systems with improved targeting specificity, higher transduction efficiencies, and improved safety is necessary.

Two general strategies have been developed for the targeted delivery of drugs to folate receptor-positive tumor cells: by linkage to a monoclonal antibody against the receptor and by coupling to a high-affinity ligand, like folic acid (FA), which is a vitamin required for one carbon transfer reactions in several metabolic pathways. Because, folic acid is essential for the biosynthesis of nucleotide bases, the vitamin is consumed in elevated quantities by proliferating cells [1]. The attractiveness of folate has been further enhanced by its high binding affinity (Kd ≈ 10⁻¹⁰ M), low immunogenicity, ease of modification, small size (Mw 441.4), stability during storage, compatibility with a variety of organic and aqueous solvents, low cost, and ready availability [2].

Folate receptors (FRs) are frequently overexpressed on cancer cells, identifying the receptor as a potential target for a variety of ligand and antibody-directed cancer therapeutics [3]. These receptors are elevated in malignant tissues of the ovary, uterus [4], endometrium [5], brain, kidney [4], head and neck [4], and skin [6]. Folate binding to different nonviral systems (lipidic or polymeric) for in vitro and in vivo gene delivery has been studied by different authors [6–11].

In this way, targeting by folic acid has been studied and developed by several researches, showing the use of the ligand as a good targeting molecule for gene delivery in vitro and in vivo. Specifically, folate-targeted cationic liposomes have proven to be efficient vectors in the treatment for disseminated peritoneal tumors [12]. Folate-targeted gene transfer in vivo in the presence of PEI has also been documented in the case of glioma [9] and lung tumors. In our case, the designed LPP presented in this study are stable complexes, which have demonstrated to be efficient for targeting melanoma and cervix carcinoma cells in an improved manner compared to conventional lipoplexes or polyplexes. Therefore, we think that these systems would be pertinent for tumor cell targeting in vivo.

Lipopolyplexes (LPPs) (a ternary complex of cationic liposomes, cationic polymer, and DNA) represent a second generation of nonviral gene delivery vectors that can improve gene transfer compared to the first generation cationic-liposome–DNA complexes [13–15]. In general, these vectors are compact particles that exhibit superior colloidal stability, reduced cytotoxicity and elevated transfection efficiency compared to polypeplexes and lipoplexes. On the other side, in designing of lipid or polymer based-nonviral vectors the parameter “particle size” should be considered [16,17].

One of the major limitations in the application of nonviral vectors for gene therapy is the inhibition of their biological activity in the presence of serum [18,19]. Because of that, the design of new
nonviral vectors efficient in the presence of serum results of great interest.

It is interesting to note that the transfer of immunostimulatory genes is a potentially powerful therapeutic approach for treating cancer, considering that interleukin 12 (IL-12) is a very potent antitumoral cytokine [20]. Taking these observations into account and based on our previous work [21–23], the main objective of this study was the development and evaluation of targeted lipopolyplexes containing folic acid (Fol-LPP) able to transfect cancerous cells in the presence of high concentrations of serum.

2. Materials and methods

2.1. Materials

The cationic lipid 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Polyethylenimine (MW 25 kDa, branched) (bPEI 25) was acquired from Aldrich (Madrid, Spain) and linear PEI of 22 kDa (PEI22) was kindly supplied by Dr. Manfred Ogris. Folic acid dehydrate, HEPES, and D(+)-glucose were purchased from Sigma (Madrid, Spain). The plasmids, pCMVLuc (VR-1216) (Clontech, Palo Alto, CA, USA), and pCMV100-IL-12 (kindly provided by Dr. Chen Qian, University of Navarra) encoding luciferase and interleukin-12 (IL-12) [21–23], respectively, were used for carrying out the transfection experiments. Alamar blue dye was purchased from Accumed International Companies (Westlake, OH, USA).

2.2. Cell culture

HeLa (human cervix carcinoma), B16-F10 (mouse melanoma), and HepG2 (human hepatoblastoma) cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained at 37 °C under 5% CO2 in Dulbecco’s modified Eagle’s medium–high glucose (DMEM). Each cell culture medium was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml), and l-glutamine (4 mM) (Gibco BRL Life Technologies). Cells were passaged 1:10 by trypsinization once a week.

2.3. Preparation of plain and Fol-lipopolyplexes

Lipopolyplexes for in vitro experiments were prepared with plasmid DNA and b-PEI (branched of 25 kDa) or L-PEI (linear, of 22 kDa) at a N/P ratio of 4. The N/P ratio of nitrogen atoms of PEI to DNA phosphates describes the amount of polymer used for polyplex formation. Lipids were added to prepare complexes at a total lipid/DNA charge ratio (+/-) of 5/1. First, DOTAP/Chol (1:0.9 molar ratio) liposomes were prepared by drying a chloroform solution of the lipids by rotary evaporation under reduced pressure. The film was hydrated with a 10 mM HEPES, 10% (w/v) glucose buffer (pH 7.4). The polyplexes were formulated at a PEI/DNA (N/P) ratio of 4 and, after 15 min incubation, the preformed cationic liposomes were added to obtain complexes at a lipid/DNA charge ratio (+/-) of 5/1. The lipopolyplexes were extruded through polycarbonate membranes with 200 nm pore diameter using a Liposofast device (Avestin, Toronto, Canada) to obtain a uniform size distribution. Targeted lipopolyplexes were prepared by mixing plain (nontargeted) lipopolyplexes with a variable amount of folic acid (Fol-lipopolyplexes), following incubation for 15 min at room temperature. This methodology has been also applied by us in order to prepare lipoxes, polyplexes, or lipopolyplexes in previous studies [23–26].

2.4. Particle size and zeta potential measurements

The particle size of the complexes was measured by dynamic light scattering and the overall charge by zeta potential measurements, using a particle analyzer (Zeta Nano Series, Malvern Instruments, Spain). Samples of the prepared complexes were diluted in distilled water and were measured at least three times immediately after preparation of the complexes.

2.5. In vitro transfection activity

For transfection, cells were seeded in medium in 48-well culture plates (Iwaki Microplate, Japan) and incubated for 24 h at 37 °C in 5% CO2. After this, the medium was removed, and 0.2 ml of the complexes (containing 1 μg of plasmid) and 0.3 ml of FBS were added to each well. After a 4 h incubation in 60% FBS, the complexes were replaced, and the cells were further incubated for 48 h in medium containing 10% FBS. Cells were washed with phosphate-buffered saline (PBS) and lysed using 100 μl of Reporter Lysis Buffer (Promega, Madison, WI, USA) at room temperature for 10 min followed by two freeze–thaw cycles. The cell lysate was centrifuged for 2 min at 12,000 g to pellet debris. Twenty microliters of the supernatant were assayed for total luciferase activity, using the Luciferase Assay Reagent (Promega), according to the manufacturer's protocol. A luminometer (Sirius-2, Berthold Detection Systems, Innogenetics, Diagnóstico y Terapéutica, Barcelona, Spain) was used to measure luciferase activity. The protein content of the lysates was measured by the DC protein assay Reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. The data were expressed as pg of luciferase (based on a standard curve for luciferase activity) per mg of protein. In vitro IL-12 levels were determined by an ELISA kit for murine IL-12p70 (BD OptEIA ELISA sets, Pharmingen, San Diego, CA, USA), following the manufacturer’s instructions.

2.6. Cell viability

Cell viability was quantified by a modified Alamar Blue Assay. Briefly, 2 ml of 10% (v/v) Alamar blue dye in DME-HC supplemented with 10% (v/v) FBS medium was added to each well 48 h post-transfection. After 2.5 h of incubation at 37 °C, 200 μl of the supernatant was assayed by measuring the absorbance at 570 and 600 nm. Cell viability, expressed as a percentage of control cells (without treatment), was calculated according to the formula \[
\frac{A_{570}}{A_{600}}\times 100/(A_{570} \text{ control} - A_{600} \text{ control})
\] of treated cells \times 100/(A_{570} \text{ control} - A_{600} \text{ control}) of control cells.

2.7. Statistical analysis

Results are reported as the mean values ± standard deviation. Statistical analysis was performed with SPSS 15.0 (SPSS®, Chicago, IL, USA). The different formulations were compared with ANOVA and Student’s t-test. Differences were considered statistically significant at p < 0.05.

3. Results

3.1. Characterization of lipopolyplexes

To characterize and evaluate the influence of folic acid on the particle size and the zeta potential of complexes, nontargeted and targeted lipopolyplexes were prepared with different amounts of the ligand. Table 1 shows the values of the particle size and the zeta potential of complexes at N/P ratio of 4, containing 10 μg/ml of pCMVLuc, in the absence or presence of increasing amounts of folic acid. In the case of lipopolyplexes prepared with branched
Table 1

<table>
<thead>
<tr>
<th>µg FA/µg DNA</th>
<th>LPP25 Size (nm)</th>
<th>PDI</th>
<th>ZP (mV)</th>
<th>LPP25 Size (nm)</th>
<th>PDI</th>
<th>ZP (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>122 ± 1</td>
<td>0.12</td>
<td>30 ± 3</td>
<td>121 ± 1</td>
<td>0.30</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>1</td>
<td>131 ± 1</td>
<td>0.15</td>
<td>25 ± 2</td>
<td>129 ± 1</td>
<td>0.26</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>5</td>
<td>125 ± 1</td>
<td>0.16</td>
<td>20 ± 1</td>
<td>316 ± 9</td>
<td>0.23</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>10</td>
<td>1951 ± 116</td>
<td>0.22</td>
<td>10 ± 1</td>
<td>2258 ± 485</td>
<td>0.37</td>
<td>18 ± 1</td>
</tr>
</tbody>
</table>

PEI of 25 kDa (LPP25), no significant differences were observed in the particle size are observed by adding 1 or 5 µg of folic acid (FA). However, a big increase is detected when 10 µg of FA is added. In lipopolyplexes containing linear PEI of 22 kDa (LPP22), the particle size increased slightly with 1 and 5 µg of folic acid (FA), but also a much bigger particle size is observed when 10 µg of FA is added. By comparing LPP25 with LPP22 complexes, it can be observed that no significant differences were obtained at low doses of FA (1 µg), however, at 5 and 10 µg FA/µg DNA, the size of LPP22 complexes resulted to be bigger than of LPP25.

The size (in nm) of controls has also be determined, being as follows: PEI-DNA complexes: 170 ± 5; plain-lipoplexes: 185 ± 1; FA-lipoplexes: 188 ± 1; FA-PP22 complexes: 180 ± 1.

The zeta potential of Fol-lipopolyplexes shows clearly positive values in all cases. By increasing the amount of the ligand, a decrease in the values of zeta potential was obtained in LPP25 and LPP22, as expected, given that folic acid is negatively charged. The surface charge of LPP22 complexes resulted to be slightly higher than of LPP25. Measurements were carried out in triplicate immediately after preparation of the complexes.

3.2. In vitro transfection activity in HeLa and B16-F10 cells by complexes carrying pCMVLuc

Plain (non-targeted) and Fol-lipopolyplexes were prepared at lipid/DNA charge ratio (+/-) 5/1 and contained 1 µg of pCMVLuc and different amounts of folic acid. They were added to HeLa and B16-F10 cells to evaluate their transfection efficiency. Fig. 1 shows that the combination of folic acid with lipopolyplexes prepared with linear PEI of 22 kDa (LPP22) enhanced significantly transfection activity in HeLa cells in the presence of 60% of serum, compared to nontargeted ones (p < 0.01). It was observed that transfection activity increased by increasing the amount of the ligand in the lipopolyplexes LPP22. A 7- and 2.8-fold increase transfection efficiency was observed in LPP22 complexes prepared with 10 µg fol/µg DNA, compared to lipopolyplexes with 1 and 5 µg of folic acid/µg DNA, respectively (p < 0.01). Fol-lipopolyplexes containing 10 µg Fol/µg DNA and prepared using PEI 22 kDa (LPP22) resulted in 27 times higher luciferase expression compared to the lipopolyplexes prepared without the ligand. These complexes were also more effective in transfecting HeLa cells than lipopolyplexes containing branched PEI of 25 kDa (LPP25) and also more effective than the control lipoplexes or polyplexes (p < 0.01). The size of LP, PP25, and PP22 controls was 185 ± 1, 162 ± 3, and 163 ± 2, respectively. No measurable luciferase expression was detected with the naked DNA.

Transfection activity mediated by either plain or Fol-lipopolyplexes was also examined in B16-F10 cells as a function of the amount of folic acid added (1, 5, or 10 µg of FA/µg DNA), in the presence of 60% of serum. Fig. 2 shows that LPP22 complexes containing the ligand resulted in higher values of gene expression compared to plain-lipopolyplexes, showing maximal transfection activity with 10 µg FA/µg DNA (p < 0.01). At this point, a 2.5-fold increase was detected compared to nontargeted complexes. Moreover, higher values of gene expression were obtained respect to control lipoplexes (LP) or polyplexes (PP22) with 10 µg FA/µg DNA (p < 0.01). LPP22 lipopolyplexes showed higher gene expression than LPP25 complexes (p < 0.01). Transfection of cells by naked DNA did not show any transfection activity.

3.3. Viability studies

Cell viability following transfection was determined to evaluate whether plain and Fol-lipopolyplexes formulated with different amounts of folic acid were toxic to HeLa and B16-F10 cells. The Alamar Blue Assay showed a viability higher than 90% in all transfected wells, independently of the amount of the ligand, the type of PEI or the cell line used (Figs. 3 and 4). The relative cytotoxicity of...
the complexes was also determined by the total amount of extractable cellular proteins in the cell lysate per well and confirmed the results with the Alamar Blue Assay (data not shown).

3.4. Interleukin-12 gene delivery

To evaluate the capacity of our complexes to transfect cells carrying a therapeutic gene such as IL-12, plain, and Fol-lipopolyplexes were prepared in the presence of 1 μg of pCMVIL-12 and tested in HeLa cells. The levels of expressed IL-12 demonstrated high transfection activity of the targeted complexes compared to non-targeted ones (*p < 0.01). Fol-lipopolyplexes prepared with 10 μg folate/μg of DNA mediated higher IL-12 expression than lipopolyplexes prepared using lower amounts of the ligand, in accordance with the results obtained with the plasmid encoding luciferase (Fig. 1). Levels of expressed interleukin-12 obtained with Fol-lipopolyplexes, containing 10 μg folate/μg of DNA were 2.6-fold higher than those obtained with nontargeted complexes.

3.5. Specificity of targeting to the folic acid receptor

We also investigated whether the uptake of Fol-lipopolyplexes was mediated via interaction with the folate receptor. The experiment was performed using plain- and Fol-lipopolyplexes in a folate receptor-positive cell line as HeLa and in a folate deficient one as HepG2. Also, a competition experiment was performed in the presence of an excess (25 mg) of the ligand. In the Fig. 6, it can be observed that the results obtained with the plasmid encoding luciferase (Fig. 1). Levels of expressed interleukin-12 obtained with Fol-lipopolyplexes, containing 10 μg folate/μg of DNA were 2.6-fold higher than those obtained with nontargeted complexes.

4. Discussion

A crucial limiting factor in gene therapy is the low efficiency of gene transfer with the currently available vectors. In an attempt to solve this problem, we studied targeted complexes that can deliver genetic material into cancer cells. We examined the association of...
a ligand, such as folic acid, with lipopolyplexes containing DOTAP/Chol liposomes, PEI and pCMVLuc or pCMVIL-12. In these complexes, PEI acts as a DNA condensing and endosomal disruption agent because its positive charge at neutral pH and further positive charge generation as the pH decreases [27]. Since the folate receptor is highly over-expressed in a variety of cancer cells [2,4,5], gene and drug delivery via folate receptor-mediated endocytosis has been shown to be a powerful method for the specific delivery of drugs and genes to certain cell types of tissues [28–30]. Nevertheless, one of the major limitations of folate-targeted liposomal gene therapy lies in the low rate of vector escape from intracellular compartments following folate receptor-mediated endocytosis.

In a previous study, we explored the combined used of nontargeted lipoplexes and polyplexes (lipopolyplexes) for efficient gene delivery in vitro [21]. In this work, we have prepared and evaluated different Fol-lipopolyplexes containing growing amounts of folic acid, to evaluate their transfection efficiency in the presence of very high concentration of serum (60% FBS). We expected that the association of PEI with cationic liposomes and folic acid would increase luciferase expression, compared to plain nontargeted lipoplexes, polyplexes, and naked DNA. We also studied the influence of the type of PEI (branched or linear) in transfection activity, as well as the capacity of this vector to deliver a therapeutic gene, such as interleukin-12, into cells.

The size of the complexes is an important parameter for efficient transfection of genes. The extent of nanoparticle uptake in vivo is variable and also dependent on the size, charge, rigidity, and other physicochemical properties of the particles. The systems developed in the present study were in the nanorange (excepting LPP with 10 μg FA/μg DNA (Table 1). The polydispersity index was lower than 0.3 in all the formulations. Zeta potential measurements showed that high concentrations of folic acid in the lipopolyplexes were associated with a decrease in the overall charge of the Fol-lipopolyplexes. This is due to the presence of α and γ carboxyl groups in the structure of the ligand. The targeted lipopolyplexes evaluated in cells (containing 1, 5 or 10 μg FA/μg DNA) had net positive zeta potentials, which facilitates the interaction with the negative cell surface.

In vitro transfection experiments were carried out in HeLa and B16-F10 cells in the presence of 60% of serum (Figs. 1 and 2). In both cell lines, the transfection efficiency increased by increasing the amount of folic acid in LPP22 lipopolyplexes. Complexes formulated with linear PEI22 were more effective in transfecting HeLa and B16-F10 cancer cells compared to lipopolyplexes formulated with bPEI25 (LPP25). In both cell lines, LPP22 complexes formulated with 10 μg FA/μg DNA improved transfection activity obtained by conventional lipoplexes (LP) or polyplexes (PP22). It is known that branched and linear PEI/DNA complexes differ in their ability to transfect cells. The greater efficiency of linear PEI may be due to an inherent kinetic instability [31]. Also, an increase in the buffering capacity, leading to a better and rapid lysosomal escape, resulting from the higher proton sponge capacity of the polymer can contribute to this effect. Moreover, linear PEI presents a more result from the higher proton sponge capacity of the polymer buffering capacity, leading to a better and rapid lysosomal escape, as explained by the higher concentration of folic acid in the lipopolyplexes associated with a decrease in the overall charge of the Fol-lipopolyplexes. This is due to the presence of α and γ carboxyl groups in the structure of the ligand. The targeted lipopolyplexes evaluated in cells (containing 1, 5 or 10 μg FA/μg DNA) had net positive zeta potentials, which facilitates the interaction with the negative cell surface.

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It is also important to note, that the anionic compounds present in serum often inhibit transfection activity [18,19] and represents a serious limitation for the use of nonviral vectors in vivo. Although other authors have shown that lipoplexes or polyplexes improved gene transfection in vitro even in the presence of folic acid [33,34], most of the experiments were performed in the absence or presence of 10% FBS, a condition that is far from that found in vivo. In this study, all the experiments were performed in the presence of 60% FBS to reflect in vivo conditions. Transfection studies by plain and Fol-lipopolyplexes LPP22 containing the IL-12 (Fig. 5) gene, a cytokine with potent antitumor activity [21], demonstrated that this vector is able to deliver therapeutic genes, and that the levels of gene expression obtained with Fol-lipopolyplexes prepared with 5 and 10 μg FA/μg DNA were higher than that with the untargeted complexes. No significant cytocytotoxicity was observed independently of the type of PEI and amount of folic acid used in the formulations (Figs. 3 and 4). Similar viability was found for the cell lines tested, and no significant differences in cellular toxicity were observed in the assay between plain and Fol-lipopolyplexes. This observation indicates that the difference in transfection activity between these sets of complexes cannot be explained by different toxicities of the formulations since cell viability was similar in all cases.

The specificity of FR-mediated gene transfer was illustrated by using the cell line HepG2, deficient in folate receptors [35,36], (Fig. 6), where a decrease in the level of transfection by increasing the amount of FA is obtained, given that the LPP22 complexes became less positive and consequently the interaction with the cell membrane is lower. When an excess of free FA was added to HeLa cells previous to the addition of targeted LPP, a decrease in gene expression is detected, confirming a receptor-mediated endocytosis mechanism. The results presented support the hypothesis that the targeted lipopolyplexes are recognized by folic acid receptors on the cell surface, which in turn facilitates receptor-mediated endocytosis. Although the bigger size of LPP with 10 μgFA/μgDNA could contribute to a higher transfection efficiency of complexes due to sedimentation, this fact cannot be the reason for the increase of transfection in the presence of the ligand, given that in HepG2 cells, where no receptors are present, the complex with 10 μgFA/μgDNA showed the lowest transfection activity. Based on these results, these systems seem to be a good alternative for gene delivery in vivo.

5. Conclusions

In this work, it has been developed by the first time a folate-lipopolyplex, efficient in targeting HeLa and B16-F10 cancer cells in a very high concentration of serum (60% FBS). These complexes are also able to transfect the therapeutic gene IL-12 and are capable of improving gene expression by conventional lipoplexes or polyplexes. This formulation provides a simple, nontoxic, efficient, and inexpensive method for gene delivery.

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References


