Tumor-targeting magnetic lipoplex delivery of short hairpin RNA suppresses IGF-1R overexpression of lung adenocarcinoma A549 cells in vitro and in vivo

Chunmao Wang, Chao Ding, Minjian Kong, Aiqiang Dong*, Jianfang Qian, Daming Jiang, Zhonghua Shen

Department of Cardiothoracic Surgery, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, PR China

Abstract

Liposomal magnetofection potentiates gene transfection by applying a magnetic field to concentrate magnetic lipoplexes onto target cells. Magnetic lipoplexes are self-assembling ternary complexes of cationic lipids with plasmid DNA associated with superparamagnetic iron oxide nanoparticles (SPIONs). Type1 insulin-like growth factor receptor (IGF-1R), an important oncogene, is frequently overexpressed in lung cancer and mediates cancer cell proliferation and tumor growth. In this study, we evaluated the transfection efficiency (percentage of transfected cells) and therapeutic potential (potency of IGF-1R knockdown) of liposomal magnetofection of plasmids expressing GFP and shRNAs targeting IGF-1R (pGFPshIGF-1Rs) in A549 cells and in tumor-bearing mice as compared to lipofection using Lipofectamine 2000. Liposomal magnetofection provided a threefold improvement in transgene expression over lipofection and transfected up to 64.1% of A549 cells in vitro. In vitro, IGF-1R specific-shRNA transfected by lipofection inhibited IGF-1R protein by 56.1 ± 6% and by liposomal magnetofection by 85.1 ± 3%. In vivo delivery efficiency of the pGFPshIGF-1R plasmid into the tumor was significantly higher in the liposomal magnetofection group than in the lipofection group. In vivo IGF-1R specific-shRNA by lipofection inhibited IGF-1R protein by 43.8 ± 5.3%; that by liposomal magnetofection inhibited IGF-1R protein by 43.4 ± 5.7%, 56.3 ± 9.6%, and 72.2 ± 6.8%, at 24, 48, and 72 h, respectively, after pGFPshIGF-1R injection. Our findings indicate that liposomal magnetofection may be a promising method that allows the targeting of gene therapy to lung cancer.

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1. Introduction

Lung cancer is the leading cause of cancer death worldwide, accounting for 29% of cancer deaths in men and 26% in women [1]. Non-small cell lung cancer (NSCLC) comprises more than 80% of lung cancers [2]. Furthermore, approximately 75% of patients with NSCLC are at an advanced stage at diagnosis [3]. The median survival time of a patient with advanced or metastasis NSCLC is approximately 6–8 months [4] due to the inability to control this malignant disease, despite advances in chemotherapy and radiation and surgical therapies [5]. In recent decades, some novel treatment strategies such as target-based therapeutic approaches have been applied to improve the NSCLC treatment effect.

Insulin-like growth factor-1 receptor (IGF-1R) is an important tyrosine kinase receptor that plays an important role in cell metabolism, proliferation, differentiation, apoptosis, chemoresistance, and angiogenesis [6,7]. Recently, key publications and epidemiological studies have confirmed a correlation between the IGF signaling pathway and carcinogenesis, including cancer of the breast, prostate, colorectum, and lung. Further studies have also showed that abnormal activation of IGF-1R promotes malignant transformation leading to a poor prognosis for patients with lung cancer [6,8–10]. RNA interference molecules specific for IGF-1R have been shown to be a very effective method for abrogating the cellular effects of the IGF-1R mediated signaling pathway in NSCLC [11–13]. However, the problem is that the various nonviral vectors currently available do not carry shRNA or plasmid DNA into lung cancer cells with high transfection efficiency in vitro, and with an even lower efficiency in vivo, whereby targeting delivery of therapeutic genes to tumor sites is required [14,15]. Although viral vector systems can transfer into cancer cells with high efficiency, they do have some disadvantages such as limitations on plasmid insert size and safety including mutagenic effects and induction of inflammatory responses [16,19]. Luo and Saltzman identified slow vector accumulation and consequently low vector concentration at target tissues to be simple but strong barriers to effective gene transfection [17].

Magnetofection, a novel and efficient gene delivery method, is showing great promise in addressing the limitations discussed.
Magnetofection exploits the magnetic force exerted upon gene vectors associated with magnetic particles to draw the vectors toward, possibly even into, the target cells and tissues [18–21]. As well as enhancing gene transfection into cultured cells ex vivo, the approach can be used to achieve in vivo gene delivery into targeted tissues and organs in the body under the control of a magnetic field [21–23]. Liposomal magnetofection is a process in which nucleic acids are associated with magnetic nanoparticles in combination with cationic lipids to form magnetic lipoplexes. These complexes are then concentrated onto the surface of targeted cells under the influence of a magnetic field.

The potential of using SPIONs to mediate gene delivery to a variety of cell lines and primary human cells has been elegantly demonstrated [24–26], but the feasibility of liposomal magnetofection for gene transfer to A549 cells has never been systematically assessed. We addressed this issue by applying plasmid DNA conjugated with SPIONs in combination with Lipofectamine 2000 (Lp2000, Invitrogen) to transfer in vitro cultured A549 cells, and thus perform targeting liposomal magnetofection in vivo.

2. Materials and methods

2.1. Cell line and culture

A549 cells, which belong to a NSCLC cell line with overexpression of IGF-1R, were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). A549 cells were grown in RPMI-1640 (Invitrogen-GIBCO) supplemented with 10% fetal bovine serum (FBS, Invitrogen-GIBCO), glutamate (10 mM/L Invitrogen-GIBCO) and antibiotics (100 U/ml streptomycin and 100 U/ml penicillin, Genom, China) at 37 °C in a humidified atmosphere containing 5% CO2.

2.2. Animals

Male BALB/cAnNCrj-nu mice (4 weeks old) were obtained from Shanghai Slac Laboratory Animal Co., Ltd. (Shanghai, China). The mice were maintained in the SPF environment and handled according to Zhejiang University’s institutional guidelines established for animal care and use.

2.3. Construction of plasmid expressing GFP and shRNA targeting IGF-1R (pGFPhIGF-1R)

In this experiment, the complementary sequences from the 5’ to 3’ of ds-oligos encoding shRNAs and a negative control (not to target any known vertebrate gene) were as follows:

IGF1R-shRNA1:TCGTGAAACGGTAGAGTACAGATTTGCGCAGTACTCTCTATCACCCTTTTTGAIGF1R-shRNA2:TCGTGAAAAGTGCCGCTCCTTGGAGGTTTTGGCAGTCTCAGTACGCAGCTCAGCCTGCTACCTTTNegative-control-shRNA:TCGTGAATGTACTCGGCGCTGGAGACGTTTTGGCAGTCTCAGTACATTT.

2.4. Transfection in vitro

The day prior to transfection, 4 × 10^5 cells per well were incubated in 2 mL of RPMI-1640 growth medium without antibiotics in a 6-well plate to allow the cells to become more than 90% confluent by the time of transfection.

For lipofection in a 6-well plate, we applied Lp2000 to deliver pGFPhIGF-1Rs into A549 cells according to the manufacturer’s protocol (Invitrogen).

For liposomal magnetofection in a 6-well plate, the transfection mixtures contained 5 μL of Lp2000 diluted in antibiotics and serum-free RPMI-1640 to a volume of 500 μL. A complex of 2 μg combiMAG (aqueous dispersion of SPIONs coated with a monolayer polyethyleneimines (PEIs, Chemicell) and 2 μg of pGFPhIGF-1Rs were diluted in antibiotics and serum-free RPMI-1640 to 500 μL, added to the diluted Lp2000 solution for a total volume of 1 mL, and mixed by pipetting. For the control, 500 μL of antibiotics and serum-free RPMI-1640 dilution of 2 μg of combiMAG were added instead of diluted pGFPhIGF-1R solution. Mixtures were incubated for 15 min at room temperature. Prior to liposomal magnetofection, the medium was removed and cells washed gently twice with phosphate-buffered saline (PBS). Transfection mixtures were added directly to the cells. The cells were placed in a culture plate upon Nd–Fe–B permanent magnets and incubated at 37 °C in a 5% CO2 incubator for 15 min or as indicated. The magnets were removed and the cells were continually incubated. Medium containing the transfection complex was replaced with the RPMI-1640 medium supplemented with 10% fetal bovine serum 4 h after removing the magnets. Cells were harvested 24 h after transfection to evaluate transfection efficiency using fluorescence microscopy and flow cytometry. The cells were analyzed 48 h after transfection by RT-PCR and Western blotting.

Mock transfected cells were applied to provide a more severe control than using untreated cells to account for possible nonspecific effects of the transfection reagents or the transfection methods.

2.5. Analysis of transfection efficiency by flow cytometry and fluorescence microscopy

Briefly, A549 cells in the well plates were washed with PBS and trypsinized for 5 min to detach the cells from the well plates. The cell suspension was diluted to 500 μL using PBS. Expression of GFP was analyzed using a FACScalibur dual laser flow cytometer (Becton–Dickinson). For each cell sample, 10,000 events were collected using the high-speed mode (200–300 cells/s). Transfection efficiency was reported as the percentage of GFP-positive vital cells minus the mock transfected control cells.

Cells were also visually examined for the presence of green fluorescence using an Olympus IX71 fluorescence microscope.

2.6. RT-PCR

Total cellular RNA was extracted with TRizol reagent (Invitrogen) according to the manufacturer’s protocols. RT-PCR was performed as previously described [11]. The PCR product (5 μL) was electrophoresed on 2% agarose, stained with ethidium bromide, visualized by UV absorption and analyzed using Quantity One Software.

2.7. Western blot analysis in vitro and in vivo

Western blot analysis was performed as previously described [11]. Briefly, 100 μg of total cellular proteins from A549 cells (30 μg for β-actin) and 200 μg of total tissue proteins from human lung cancer subcutaneous xenografts of mice (100 μg for β-actin) were separated on 10% SDS–polyacrylamide gels, and then analyzed using anti-IGF-1R (Lab Vision) and anti-β-actin (Santa Cruz Biotechnology) antibodies.

2.8. Liposomal magnetofection in vivo and targeted gene therapy in vivo

Mice were inoculated subcutaneously with 5 × 10^8 A549 cells in 100 μL of serum-free RPMI-1640 medium into the right flanks. Tumor volumes were calculated using the formula V (mm^3) = ab^2/2, where ‘a’ is the largest dimension and ‘b’ is the perpendicular diameter. When the subcutaneous tumors had grown to the size of
about 400 mm³, the nude mice were randomly divided into three groups (n = 4 per group): control group, lipofection group, and liposomal magnetofection group. The control group received 200 µL PBS per mouse, the lipofection group received a pGFPshIGF-1R (50 µg/mouse):Lp2000 (125 µL/mouse) complex, and the liposomal magnetofection group received a pGFPshIGF-1R (50 µg/mouse):combiMAG (50 µg/mouse):Lp2000 (125 µL/mouse) complex under the influence of a magnetic field (400 mT). These complexes (200 µL) were injected into the mice via the tail vein. In the liposomal magnetofection group, prior to the injection, an Nd–Fe–B magnet (400 mT) was held onto the subcutaneous tumor surface throughout the infusion of 1 min and for an additional 14 min following the injection. The magnetic field was then removed. At 24 h postinjection, the mice in the lipofection and liposomal magnetofection groups were killed by cervical dislocation and the tumors were quickly removed and analyzed as a snap-frozen section.

Following the injection. The magnetic field was then removed. At 24 h postinjection, the mice in the lipofection and liposomal magnetofection groups were killed by cervical dislocation and the tumors were quickly removed and analyzed as a snap-frozen section (5 µm) using a freezing microtome (Leica CM1900). The GFP expressing cells in the tumor tissue sections were visualized using a fluorescence microscope at 100× original magnification. Mice in the control group and two treatment groups were killed and the tumors were quickly removed for Western blot analyses at 24, 48, or 72 h postinjection.

2.9. Statistical analysis

All quantitative data are presented means ± SD from at least three parallel experiments. The statistical significance of differences was determined by Student’s two-tailed t-test in two groups and one-way ANOVA in multiple groups. *p < 0.05 was considered statistically significantly. All data were analyzed with SPSS 17.0 software.

3. Results

3.1. Screening for IGF-1R-specific shRNA and GFP expressing plasmid (pGFPshIGF-1R)

To achieve the maximal interference effect, the shRNA: IGF-1R-specific shRNA-1 and shRNA-2 and GFP expressing plasmids (pGFPshIGF-1R-1s and pGFPshIGF-1R-2s), and the negative control shRNA expressing plasmids (pshneg-controls) were delivered into A549 cells by liposomal magnetofection as described in Section 2. RT-PCR analysis was performed to evaluate the interference effects of IGF-1R shRNA on IGF-1R expression at the level of transcription in A549 cells. The results showed that IGF-1R mRNA levels in A549 cells 48 h after transfection with the pGFPshIGF-1R-1 and pGFPshIGF-1R-2 were 38.2 ± 0.5% and 23.3 ± 1.0%, respectively, compared to the pshneg-control. Compared to the pGFPshIGF-1R-1 group, the pGFPshIGF-1R-2 group showed a more significant inhibition for IGF-1R mRNA expression (up to 76.7 ± 1.0% of the pshneg-control group; Fig. 1). These results indicated that the inhibition effect of shIGF-1R-2 was more potent than that of shIGF-1R-1 at the level of transcription in A549 cells.

3.2. Investigation of liposomal magnetofection efficiency in cultured A549 cells

To obtain the highest transfection efficiency and low cytotoxicity, we optimized transfection conditions in 24-well plates by varying the cell density per well and the concentrations and proportions of pGFPshIGF-1R, Lp2000, and combiMAG, respectively. Initially, we applied lipofection to determine the most effective ratio of pGFPshIGF-1R and Lp2000. The most effective transfection ratio of pGFPshIGF-1R and combiMAG was also determined for liposomal magnetofection.

When the cell confluence was greater than 90%, the ratio of the pGFPshIGF-1R and Lp2000 was 1:2.5 (w/v), and the ratio of the pGFPshIGF-1R and combiMAG was 1:1 (w/w), this produced the highest transfection efficiency and lowest cytotoxicity for both lipofection and liposomal magnetofection (data not shown). Increasing the pGFPshIGF-1R concentration increased the delivery efficiency, but also increased cytotoxicity from the transfection vector, especially Lp2000.

Fig. 2D shows that the lipofection efficiency of pGFPshIGF-1R was only 23.3 ± 3.5% compared to the control (mock transfected cells), while the liposomal magnetofection efficiency of pGFPshIGF-1R indicated by Fig. 3E was 59.7 ± 4.4% compared to the control (mock transfected cells) after 15 min exposure of cells to the magnetic field, approaching a threefold higher efficiency compared to lipofection.

3.3. Liposomal magnetofection allowed site-specific gene delivery in vivo

To investigate whether liposomal magnetofection could be a feasible strategy for directing therapeutic genes to one specific target site after intravenous injection in vivo, we injected tumor-bearing mice with the pGFPshIGF-1R:combiMAG:Lp2000 complex via the tail vein in the direction of the magnetic field. As a control, the pGFPshIGF-1R:Lp2000 complex also was intravenously injected into the tumor-bearing mice. High expression of GFP was observed in the subcutaneous tumors using liposomal magnetofection (Fig. 3B). However, only scattered GFP expression was found in tumors with lipofection (Fig. 3A), suggesting that liposomal magnetofection caused specific targeting and uptake of plasmid DNA into specific target sites.

3.4. Quantification of IGF-1R protein knockdown by Western blot in vitro and in vivo

3.4.1. IGF-1R protein knockdown in vitro

To further confirm the effect of IGF-1R gene silencing by delivering specific shRNA into lung cancer cells using liposomal
magnetofection with a magnetic vector, we compared the effect of gene silencing using lipofection with Lp2000. A549 cells were transfected using liposomal magnetofection and lipofection. pGFPshIGF-1R delivered by liposomal magnetofection was shown to be effective in downregulating the IGF-1R gene by 85.1 ± 3% compared to the control (untreated cells) (Fig. 4A). Fig. 4A also shows that pGFPshIGF-1R delivered by lipofection effectively silenced IGF-1R gene expression by 56.1 ± 6% compared to the control (untreated cells). Moreover, pGFPshIGF-1R delivered by liposomal magnetofection with magnetic vectors (combiMAG and Lp2000) was more potent than pGFPshIGF-1R delivered by lipofection with Lp2000 in the inhibition of lung cancer targeted gene expression.

3.4.2. IGF-1R protein knockdown in vivo

To elucidate whether the plasmid expressing GFP and shRNA specific for IGF-1R (pGFPshIGF-1R) is silencing IGF-1R expression in the tumor-bearing mice, we intravenously injected pGFPshIGF-1R into the mice via lipofection and liposomal magnetofection. Moreover, to obtain the differences of GFP and shRNA expression in the subcutaneous tumors over time, we killed the mice at 24, 48, and 72 h after injection, removed the A549 subcutaneous tumors, and performed an analysis by Western blotting. Fig. 4B shows that shRNAs delivered by both lipofection and liposomal magnetofection effectively inhibited IGF-1R expression of A549 tumors. Comparing these two processes, shRNAs delivered by liposomal magnetofection (**p < 0.01) silenced IGF-1R expression more significantly than shRNA delivered by lipofection (*p < 0.05). The difference within the lipofection group was not significant (p > 0.05), while the difference within liposomal magnetofection group was significant (**p < 0.05). The silencing efficiency of shRNAs delivered by liposomal magnetofection reached 43.4 ± 5.7%, 56.3 ± 9.6%, and 72.2 ± 6.8% by lipofection and reached an average of 43.8 ± 5.3% at 24, 48, and 72 h, respectively, after pGFPshIGF-1R injection.

4. Discussion

The feasibility of magnetic fields to enhance SPION-mediated transfection has been described by Plank's group and other
researchers covering several cell types, including primary cells [24–26]. In vivo magnetofection has also been reported by Krotz et al., Scherer et al., and Xenariou et al. [25,27,28]. Our results further confirmed that liposomal magnetofection can effectively deliver plasmid DNA into cancer cells in culture and tumor tissue in tumor-bearing mice. Our results showed that the liposomal magnetofection efficiency of pGFPshIGF-1R was 59.7 ± 4.4% in NSCLC A549 cells (Fig. 2E); in contrast, lipofection efficiency with Lp2000 was only 23.3 ± 3.5% (Fig. 2D). This indicated that liposomal magnetofection with magnetic vectors was superior to lipofection with Lp2000 with respect to therapeutic gene transfection efficiency in lung cancer cells. During liposomal magnetofection, the magnetic force produces a translational force on the particles acting in the direction of the applied static magnetic fields, causing rapid sedimentation of magnetic lipoplexes over the cell surface. As a result, magnetofection enhances transfection by the application of static magnetic fields raising the local concentration of particles at the cell surface, thereby promoting uptake rather than activating cellular uptake mechanisms [27,29]. Furthermore, the mechanism of the cellular uptake of transfected genes and SPIONs has been elucidated by transmission electron microscopy and is the same as other transfection methods; i.e., it is a clathrin-dependent endocytosis process [26,29].

Fig. 3 shows that liposomal magnetofection contributed to targeting the delivery of cancer therapeutic genes attached to magnetic vectors in vivo, indicating that the targeting of tumors with magnetic vectors under the influence of an external magnetic field increased the site specificity, and thus selectivity of the therapy, contributing to the reduction of side effects and therapy costs. Similar results were reported by Krotz et al. and Scherer et al. [25,27]. Furthermore, IGF-1R-specific shRNA of liposomal magnetofection into A549 cells silenced expression of the cancer gene IGF-1R with high suppression efficacy of 85.1 ± 3% (Fig. 4A), while IGF-1R-specific shRNA of lipofection with Lp2000 silenced the expression of IGF-1R with a lower suppression efficacy of 56.1 ± 8% (Fig. 4A). This variability may be caused by different amounts of IGF-1R-specific shRNA delivery into A549 cells by liposomal magnetofection and lipofection. Fig. 4B shows shRNA delivered by liposomal magnetofection and lipofection in vivo both significantly downregulated the targeted gene expression 24, 48, or 72 h after pGFPshIGF-1R injection. Moreover, in the liposomal magnetofection group, the inhibition effect of shRNA to IGF-1R gene became obvious when the time of pGFPshIGF-1R in the body was extended, but this was not significant in the lipofection group. In contrast, shRNA delivered by liposomal magnetofection significantly silenced the targeted gene expression compared to lipofection in vivo whether 24, 48, or 72 h after pGFPshIGF-1R injection. These results provided very important kinetic parameters about magnetofection in vivo to allow further study of therapeutic gene delivery and targeted gene therapy in vivo utilizing magnetofection.

However, the liposomal magnetofection efficiency of plasmid DNA in A549 cells was not ideal: 55.3–64.1% level. One should be able to increase transfection efficiency through optimization of the structure of the magnetic nanoparticles, application of oscillating magnetic fields, and utilizing other transfection methods. For optimizing the structure of magnetic nanoparticles, we may reduce the diameter of the magnetic nanoparticles, change the matrix composition encapsulating the magnetic particles [30,31], or introduce an oscillating magnetic field. McBain et al. first demonstrated that applying the Nd–Fe–B oscillating magnetic array system dramatically increased magnetic nanoparticle-mediated transfection efficacy in a human lung epithelial cell line compared to the use of static magnetic fields [32]. Similar conclusions were reported by Pickard and Chari, who used oscillating magnetic field systems to enhance magnetic nanoparticle-mediated transfer efficacy of rodent astrocytes using commercially available particles conjugated with plasmids encoding a reporter protein [33].

In conclusion, we successfully applied commercially available superparamagnetic iron-oxide nanoparticles in combination with Lipofectamine 2000-mediated gene delivery under the influence of an external static magnetic field in a NSCLC cell line (A549) in vitro and further achieved targeting transfection in nude mice, which provided a novel method of targeting therapeutic gene delivery for gene therapy of lung cancer.

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References
