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Further Evaluation of a Novel Nano-Scale Gene Vector for In Vivo Transfection of siRNA

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ABSTRACT

In this research, a lipid-cationic polymer (LCP) containing the side-chain branching of brassidic acid was synthesized using chemical methods. As a gene vector for small interfering ribonucleic acid (siRNA) transfection, the efficiency and biosafety of LCP were preliminarily evaluated to investigate its possible application on tumor gene therapy. The toxicity, side-effects, and biosafety of LCP were investigated in animals based on the results of in vitro experiments. The siRNA against cyclooxygenase-2 (COX-2) was transfected by LCP to interfere with the COX-2 expression in nude-transplanted tumors. Hematoxylin and eosin stains, immunohistochemistry, reverse transcription-polymerase chain reaction, and Western blot were performed to evaluate the efficiency of LCP for siRNA transfection. The animal toxicity experiment showed that a high concentration of LCP had a low toxic effect on animals and did not induce allergic or pyrogenic reactions. The results from the in vivo transfection indicated that LCP could efficiently transfect siRNA and silence the target gene expression. The LCP gene vector for siRNA transfection is highly efficient during in vivo transfection and had low toxicity. From all aspects of tumor gene therapy and basic research, LCP is valuable for scientific research and medical applications. J. Cell. Biochem. 112: 1329–1336, 2011.

KEY WORDS: LIPID-CATIONIC POLYMER; SIRNA; GENE VECTOR; TRANSFECTION

Small interfering ribonucleic acids (siRNAs) have been extensively used to silence gene expression. It plays great roles in the regulation of gene expression and cell activity. This provides a new way for gene therapy [Takahashi et al., 2007; Nguyen et al., 2008; Racz and Hamar, 2008; Bonoiu et al., 2009]. The most important clinical application of RNA interference (RNAi) is in gene therapy. Theoretically, RNAi can affect various diseases, but now is mainly applied in cancer therapy and research [Fountaine et al., 2005; Morrissey et al., 2005; Ashihara et al., 2010; Vazquez-Vega et al., 2010].

A key point in RNAi technology is whether siRNA can be efficiently transported into target cells and inhibits gene expression. Currently, gene vectors for RNAi are classified as two groups: viral vectors and non-viral vectors. Although the viral vector is highly efficient, its biosafety and complicated preparation limit its wide application [Verma and Somia, 1997; Marshall, 1999; Descamps and Benihoud, 2009]. Non-viral vector has been receiving much more attention for its high level of biosafety. For small molecule deoxyribonucleic acid (DNA) transfection we designed and synthesized a degradable nano-scale cationic polymer containing the side-chain branching of brassidic acid and named as lipid-cationic polymer (LCP) [Jing et al., 2010]. In our previous in vitro transfection, we explored the physical and chemical properties of LCP, as well as its transfection efficiency, cellular cytotoxicity, biocompatibility, and degradability. LCP had high transfection efficiency and specificity, low cell cytotoxicity, and better biocompatibility when used for in vitro transfection. Based on our previous results, present research investigated the factors influencing LCP transfection efficiency and in vivo toxicity. We used gastric cancer xenografts in nude mice as the model, and

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silenced the expression of cyclooxygenase-2 (COX-2) by using siRNA against the COX-2 gene. We aimed to provide additional scientific evidence for the in vivo transfection of siRNA using LCP vector, especially for tumor gene therapy.

MATERIALS AND METHODS

BIOMATERIALS

Plasmid pEGFPN1 was purchased from Clontech Company. HeLa-EGFP and TH1080-EGFP cell lines were from the American Type Culture Collection (ATCC). Fetal calf serum, Dulbecco's modified Eagle medium (DMEM), and Opti-MEM® were all from Gibco. All reagents used to synthesize LCP were analytically pure and provided by Xiamen Luyin Co. (China). Mice, guinea pigs, BALB/c nude mice, and rabbits were from the Experimental Animal Center of the Xiamen University School of Medicine. Trizol was the product of Invitrogen. Reagents for the reverse transcription-polymerase chain reaction (RT-PCR) kits were from Qiagen.

PREPARATION OF LCP VECTOR FOR siRNA

Two steps were involved in constructing LCP vectors for siRNA transfection: synthesizing LCP and preparing the working solution for the siRNA gene vector. LCP is a cationic polymer containing the side-chain branching of brassidic acid with a molecular mass of 50 kDa. The detailed build-up procedure for LCP can be found in published literature and patents in China [Yang, 2009; Jing et al., 2010]. According to the different requirements of in vivo and in vitro experiments, two working solutions for the gene vector were prepared. LCP working buffer I consisted of 77 mmol/L sodium chloride, 49% DMSO, and 1% glycerol, which was suitable for in vitro application. LCP working buffer II consisted of 27.5 mmol/L glucose and 1% glycerol, which was suitable for in vivo application. Each 50 mg LCP was dissolved in 10 ml buffer I and buffer II at 56°C. After filtration and sterilization, LCP-SRI and LCP-SRII (each 5 mg/ml) were prepared for in vitro and in vivo application, respectively.

EFFECTS OF PROTON PUMP INHIBITOR ON THE TRANSFECTION EFFICIENCY OF LCP

LCP-SRI was used for in vitro experiments.

HeLa-EGFP and HT1080-EGFP cells were seeded in 96-well plates with an initial cell number of 1.5×10^4. A total of 100 μl culture medium was added into each well. Cells were maintained at 37°C with 5% CO_2 for 24 h. The cells were 40–50% confluent. For the cells in the control group, 7.5 μl LCP-SRI was dropped into 7.5 μl 20 pmol (1 nmol siRNA equals 13.3 μg) siRNA against EGFP. After incubating at room temperature for 15 min, the LCP-SRI/siRNA (anti-GFP) was prepared and then added into the cell culture medium. The gene expression was analyzed after the cells were cultured at 37°C with 5% CO_2 for 48 h. For the cells in the experimental group, 2 μM bafilomycin A1 diluted in DMEM medium was added into the LCP-SRI/siRNA (anti-GFP) complex; the mixture was then added into the culture medium to transfect the cells. Changes in the EGFP signals were observed using a fluorescence microscope (Olympus, Japan). FLX800 was used to quantify the fluorescence signal.

EVALUATION OF IN VIVO TOXICITY OF LCP GENE VECTOR

LCP-SRII was used for the in vivo experiment.

Acute toxicity test. Ten male mice, each with a body weight of 18–22 g, and aged 6-week old, were injected with 0.5 ml 5 mg/ml LCP-SRII through the tail vein. They were observed for untoward effects such as convulsions, quadriplegia, unsteady gait, piloerection, and respiratory depression or death for 48 h. Another five mice were injected with normal saline as the control.

Sub-acute and chronic toxicity test. The sub-acute and chronic toxicity test is similar to the acute toxicity test, except for the reagent dosage. Ten male mice, each with a body weight of 18–22 g, and aged 6-week old, were injected with 0.5 ml 0.5 mg/ml LCP-SRII through the tail vein. The mice were continuously observed for changes in body weight, food and water intakes, and other untoward effects or death for 8 weeks to determine sub-acute toxicity, and for 5 months to determine chronic toxicity. The observation of the viscera was facilitated by both naked eyes and histochemical sections. Another five mice were injected with normal saline as the control.

Determination of half-lethal dose. The half-lethal dose, also called LD_{50}, represented the dosage that caused death in half of the experimental animals. Ninety male mice, each with a body weight of 18–22 g, were divided into nine groups with 10 mice in each group. A total of 0.5 ml LCP-SRII with different concentrations (10, 15, 20, 25, 30, 35, 40, 45, and 50 mg/ml) was injected through the tail vein. LD_{50} was determined after being observed for 72 h, using death as the main indicator. Another 10 mice were injected with normal saline for the control.

Allergic test. Six guinea pigs, each with a body weight of 250–300 g, and aged 8-week old, were intraperitoneally injected with 0.5 ml 0.5 mg/ml LCP-SRII three times every other day, and then were divided into two groups. One group was injected with 1 ml 0.5 mg/ml LCP-SRII through the jugular vein 14 days from the first injection; the other group was injected the same way 21 days from the first injection. All guinea pigs were observed for allergic reaction, which is characterized by excitation, expiratory dyspnea, and death from suffocation. Another five guinea pigs injected with normal saline were the control.

Pyrogen test. Three rabbits, each with a body weight of 3 kg, were selected. Before injection, the average rectal temperature was calculated from three independent measurements. Then, 3 ml 5 mg/ml LCP-SRII was injected through the auricular vein. The rectal temperature was recorded at 1, 2, and 3 h after injection. Two rabbits were injected with normal saline for the control.

IN VIVO TRANSFECTION USING LCP GENE VECTOR

COX-2 was highly expressed in gastric cancer tissues and was closely associated with the development and transfer of gastric cancer. We probed the effect of COX-2 on nude-transplanted gastric tumor using siRNA against COX-2 to silence the expression of COX-2. We also discussed the in vivo application of the LCP gene vector. The sequence of anti-COX-2 siRNA was as follows: target sequence: 5′-GCTGGGAAGCCTTCTCTAA-3′; sense sequence: 5′-GCUGGG-AAGCCUUCUCUAAdT dT-3′; anti-sense sequence: 3′-dTd TCGA-CCCUUCGGAAGAGAUU-5′; negative control: sense sequence:

5′-GUCGGAGGACACUUUCUCAdTdT-3′; anti-sense sequence: 3′-dTdTCAGCCUCCUGUGAAAGAGU-5′.

Model establishment of nude-transplanted tumor-siRNA. Human gastric cancer BGC-823 cells were routinely cultured and collected after being digested with trypsin. After centrifugation, the pellet was re-suspended in RPMI-1640 at a final cell density of 1×10^7/ml. Ten BALB/c, aged 5- to 6-week old, were each inoculated with 0.5 ml cells. When the diameter of the tumor was 5 mm, the nude mice were divided into two groups, with five mice in each group. In the experimental group, 0.1 ml 5 mg/ml LCP-SRII/siRNA complexes (anti-COX-2) were injected into the tumor body; in the control group, mice were injected with LCP-SRII/siRNA-negative complexes. All injections were performed every other day for a total of seven injections. After 14 days, the nude mice were euthanized by breaking their necks. The tumor tissues were separated, and tumor volume was determined by measuring the volume of water drained from the tumor tissues.

Pathology changes and volume of tumor. The sizes of the tumor tissues were measured, and hematoxylin and eosin (HE) staining was used to observe the tumor sections.

mRNA levels of COX-2. A total of 100 mg fresh tumor tissues were frozen in liquid nitrogen and ground with mortar and pestle to yield tissue powder. Each 100 mg of tissue or 10^7 cells of BGC-823 were digested with 1 ml Trizol reagent (Invitrogen). Total RNA was isolated from the cells and tissues according to the manual instruction. The RNA precipitate was then dissolved in 10–15 μl of RNAse-free water. A spectrophotometer was used to analyze the quantity and quality of the RNA precipitate.

A one-step RT-PCR procedure for COX-2 gene was performed using a kit from Qiagen. The resultant complementary DNA (cDNA) was then quantified by Bio-rad C1000 (Bio-rad). β-Actin was amplified as an internal control. The PCR primers used were as follows: (i) COX-2-F (F for forward) (5′-TCAAGTCCCTGAGCATC-TAC-3′) and COX-2-R (R for reverse) (5′-CATTCCTACCACCAG-CAACC-3′); (ii) β-actin-F (5′-GAAACTACCTTCAACTCCATC-3′) and β-actin-R (5′-CGAGGCCAGGATGGAGCCGCC-3′). The amplified products of COX-2 gene and β-actin were 488 and 219 bp, respectively. PCR was performed as follows: (i) 30 min at 50°C, 5 min at 94°C and (ii) 35 cycles, with 1 cycle consisting of 55 s at 94°C, 55 s at 54°C, and 55 s at 72°C. The amplified products were separated with 1% agarose gel for 30 min followed by EB. The gel was visualized and analyzed by UviPro (UVP).

Detection of COX-2 expression by IHC. Cancer tissue microarrays (TMAs) for the immunohistochemical analysis were constructed through the following methods. Sections 5 μm in size were taken from a tissue array block, affixed to 3-aminopropyl triethoxysilane-coated slides, and air-dried overnight at 37°C. After dewaxing and antigen retrieval, endogenous peroxidase was quenched with 3% hydrogen peroxide for 10 min. Immunohistochemistry (IHC) was performed on the two-step plus®poly-horseradish peroxidase (HRP) anti-mouse/rabbit IgG detection system (ZSGB-Bio Co., Ltd.). The monoclonal anti-COX-2 (1:1,000 dilution) was produced by our lab and the specificity was confirmed [Dan et al., 2010]. After overnight incubation, the sections were washed thoroughly with PBST (PBS, 0.05% Tween-20). The next steps were performed according to the manual of the two-step plus®poly-HRP anti-mouse/rabbit IgG detection system (ZSGB-Bio Co., Ltd.). The antigen–antibody complex was visualized with the diaminobenzidine (DAB) substrate.

Changes in COX-2 expression by Western blot. The fresh tumor tissues from mice before and after transfection were ground and solubilized in lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 1.5% Triton X-100, 65 mmol/L DTT, 40 mmol/LTris, 1 mmol/L PMSF, 3 mmol/L EDTA). They were then sonicated for 20 min at 0°C and centrifuged at 12,000*g* for 15 min at 4°C. Protein concentrations were determined using the Bradford assay. For Western blotting experiments, 20 μg cell lysates were loaded and separated on 12% polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA) according to standard protocol. The blots were blocked for 1 h at room temperature in 5% skim milk. The target proteins were probed with primary antibodies and HRP-labeled secondary antibodies. β-Actin was used as an indicator of equality of the lane loading. Antibody positive bands were visualized using ECL Western blot detection reagents (Pierce).

LCP OFF-TARGET EFFECT TEST

To investigate the extent of off-target effects, LCP was used to transfect siRNAs against EGFP and luciferase into HeLa-EGFP cells utilizing the method described in present study. The scrambled siRNA and LCP were used as the negative control and the mock control, respectively. Twenty-four hours after transfection, the fluorescence of EGFP in HeLa-EGFP cells was observed under fluorescence microscope and FLX800 fluoroanalyzer was used to quantify the fluorescence signal.

STATISTICAL ANALYSIS

All statistical analyses in this research were performed using SPSS 13.0. One-way analysis of variance (ANOVA) was used to determine the univariate differences between multiple groups, and *t*-test was performed to probe the differences between the two different groups. $P < 0.05$ was used as the significant difference.

RESULTS

EFFECTS OF THE PROTON PUMP INHIBITOR ON LCP TRANSFECTION EFFICIENCY

Fluorescence intensity in untransfected cells was set as 100% (relative fluorescence unit: RLU). It was negatively correlated with siRNA transfection efficiency; high transfection efficiency meant lower fluorescence intensity and higher inhibited degree of EGFP. The relative fluorescence intensity and inhibition rate were 27% and 73%, respectively, in HeLa-EGFP cells transfected with siRNA anti-EGFP mediated by LCP. With bafilomycin A1, the relative fluorescence intensity was 99% and the inhibition rate was only 1%, indicating that bafilomycin A1 could inhibit transfection activity. A similar result was acquired in HT1080-EGFP cells; the relative fluorescence intensity was 98% and the inhibition rate was only 2% with the presence of an inhibitor (Fig. 1).

EVALUATION OF IN VIVO TOXICITY OF LCP GENE VECTOR

Acute toxicity tests. After injecting with 0.5 ml 5 mg/ml LCP-SRII through the tail vein for 48 h, 10 mice did not show any adverse reaction such as convulsions, quadriplegia, unsteady gait, piloerection, and respiratory depression or death. There was no significant difference compared with the control group injected with normal saline.

Sub-acute and chronic toxicity tests. No adverse reaction was seen after injecting with 0.5 ml 5 mg/ml LCP-SRII through the tail vein and continuously observing for 8 weeks. Until the 5th month, the body weight and food and water intakes did not change, and no other side-effects or deaths were observed. No aberrant changes were found in the viscera by naked eyes. The sections of the liver and the kidney did not show cytohistological changes (data not shown), similar to the control group.

Determination of half-lethal dose. Ninety mice in nine groups were injected with different dosages of LCP-SRII through the caudal vein (highest concentration of 50 mg/ml and volume of 0.5 ml). After observing for 72 h, no mice died. All mice were still alive after being supplemented with another injection and being observed for another 72 h. The result is similar to the control group.

Allergic tests. After intraperitoneally injecting with 0.5 ml LCP-SRII three times every other day, guinea pigs were divided into two groups, which were supplemented with another intravenous injection of 1 ml LCP-SRII 14 and 21 days from the first injection, respectively. On the first day, the six guinea pigs in the two groups did not show allergic reactions, such as excitation, expiratory dyspnea, or death. The result is similar to the control group.

Pyrogen tests. Before injection, the average body temperature of the three rabbits was 38.9°C. At 1, 2, and 3 h after being injected with 2.5 ml LCP-SRII, the body temperature was 39.1, 39, and 39°C, respectively. The maximum temperature difference was only 0.2°C before and after injection, indicating that LCP-SRII was free of pyrogen. A similar result was acquired in the control group.

IN VIVO TRANSFECTION EFFICIENCY OF LCP GENE VECTOR

Preparation of nude-transplanted tumor. Ten BALB/c mice were inoculated with human gastric BGC-823 cells through inguinal subcutaneous injection. After 3 weeks, the tumor body formed with an average diameter of 5 ± 1 mm; the rate of tumor formation was 100%. All nude mice were randomly divided into two groups of five mice each. The experimental group was injected with LCP-SRII/siRNA (anti-COX-2) complex every other day, while the control group was injected with LCP-SRII/negative-siRNA. After 14 days, the tumor tissues were separated. The volume of tumors was determined by measuring the volume of water drained from the tumor tissues. The average tumor volume in the control and experimental groups were 545.12 ± 137.92 and $371.32 \pm 107.24\ mm^3$, respectively; there was a significant difference between the control and experiment groups ($P < 0.01$). The inhibition rate reached 44.8%. HE staining showed that the size of the nuclei of the tumor cells in the control group were not uniform and were deeply stained with less intercellular substances. Tumor cells were arranged in nests. The size of the tumor cells in the experimental group was relatively small and the ratio of nucleus to cytoplasm was smaller than that of the control group (Fig. 2).

Determination of messenger RNA (mRNA) level of COX-2 by RT-PCR. The amplified products from RT-PCR were separated by agarose gel electrophoresis. The mRNA level of COX-2 in tumor tissues transfected with LCP-SRII/siRNA (anti-COX-2) was markedly lower than that in negative-LCP-SRII/siRNA-transfected tumor tissues ($P < 0.01$). The inhibition rate reached 63.2%. The mRNA level of COX-2 in control BGC-823 cells was identical to that in

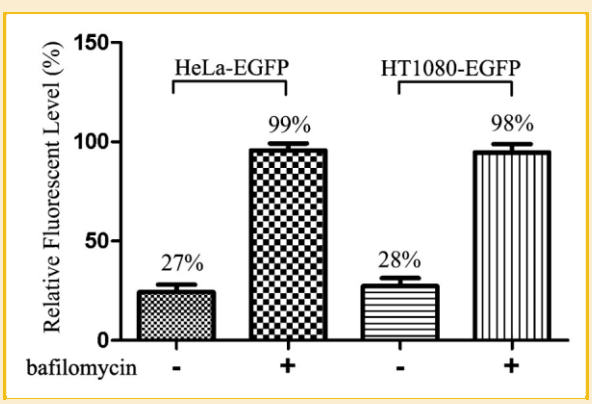

Fig. 1. Proton pump inhibitor's effect on LCP transfection.

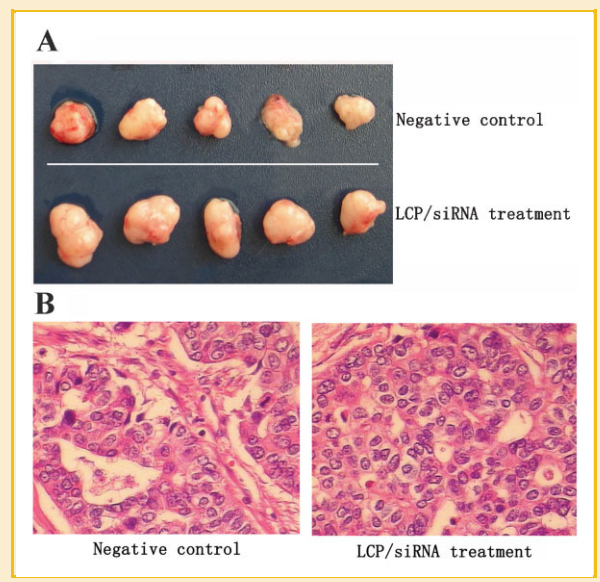

Fig. 2. Effects of transfection of LCP/siRNA (anti-COX-2) on the growth of nude-transplanted tumors. A: Transfection of siRNA against COX-2 mediated by LCP affected the growth of nude-transplanted tumors. The tumor volume in experimental group was reduced, and the inhibition rate reached 44.8%. B: HE staining showed nude-transplanted tumor sections transfected with LCP/siRNA (anti-COX-2) or LCP/negative-siRNA. The nuclei of the tumor cells in the control group were not of uniform size and were deeply stained with less intercellular substance. Tumor cells were arranged in nests. The size of the tumor cells in the experimental group was relatively small and the ratio of nucleus to cytoplasm was smaller than that of control group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

negative-LCP-SRII transfected cells. No significant difference was found between the control and experimental groups ($P > 0.05$). All results indicated that LCP could efficiently transport siRNA into the target cells and down-regulate the mRNA level of the COX-2 gene (Fig. 3).

Detection of COX-2 protein expression by IHC. The immunohistochemical results showed that the cytoplasm of the tumor cells in the control group was stained tawny and the nucleus was counterstained blue. These indicate that COX-2 was strongly expressed in tumor cells. In the experimental group transfected with LCP/siRNA (anti-COX-2) complex, the cytoplasm was slightly stained, and the nucleus was counterstained blue, indicating that the expression of COX-2 protein was silenced by the siRNA (Fig. 4).

Alteration of COX-2 protein expression determined by Western blot. The results of the Western blot showed that a deep-colored protein band was detected in the negative control group with a molecular weight of 72 kDa, which was identical to the molecular mass of COX-2. In the experimental group transfected with LCP/siRNA (anti-COX-2) complex, a light-colored protein band was detected at the identical position of 72 kDa. These results indicated that RNAi could significantly inhibit the expression of COX-2 protein in tumor cells (Fig. 5).

LCP OFF-TARGET EFFECT TEST

The results indicated that the controls—LCP/siRNA (anti-Luciferase), LCP/siRNA (negative control), and LCP (mock control)—did not affect the GFP protein expression in HeLa-EGFP cells, whereas the siRNA (anti-EGFP) transfected via LCP dramatically repressed the GFP expression. The quantitative analysis showed that the fluorescence signal declined to 25% (the fluorescence level in untreated HeLa-EGFP cells was set as 100%, Fig. 6). These results indicated that the fluorescent intensity in HeLa-EGFP cells was specifically descended by the siRNAs transfection against EGFP and LCP did not have off-target effect.

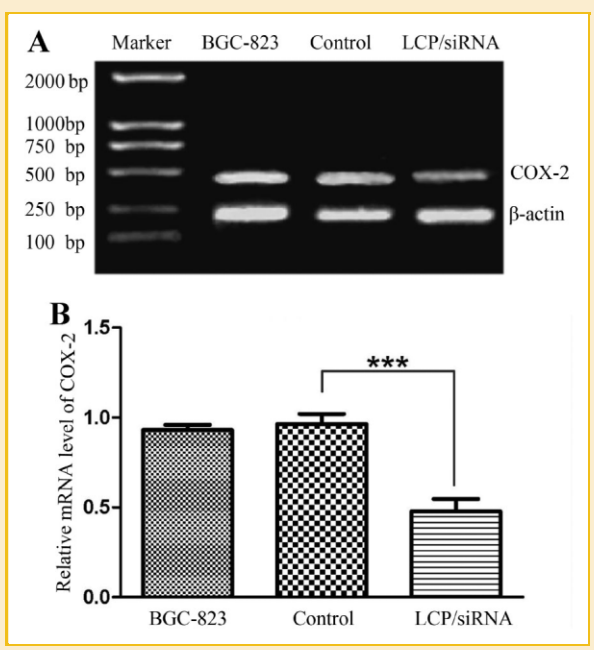

Fig. 3. A: 1% Agarose electrophoresis of RT-PCR of the gastric tumor cancer body. B: BGC-823 indicates no disposal BGC-823 cell, Control indicates LCP/siRNA (negative control) complex, LCP/siRNA indicates LCP/siRNA (anti-COX-2) complex.

DISCUSSION

The salt concentration in the working solution of gene vectors is important for transfection efficiency. In the buffer with salts, the positive charge on the surface of the cationic polymer gene vector/DNA complex was weakened. The repulsion among microparticles was reduced, which accumulated and formed particles with greater sizes [Goula et al., 1998; Wightman et al., 2001]. The accumulated complexes were rapidly deposited, which facilitated the interactions between the complexes and the cellular surface, improving transfection efficiency [Boussif et al., 1995; Goula et al., 1998; Ogris et al., 1998]. The sizes of complexes could be reduced by improving salt concentration, which would enhance transfection efficiency. The size of the cationic polymer gene vector/DNA complex was small in the salt-free solution (5% glucose), which made it more suitable for in vivo transfection. The transfection efficiency in the salt-free solution was 100 times higher than that in the solution with salts [Wightman et al., 2001]. Various hypotheses on how salt concentration affects transfection efficiency have been proposed, and the detailed mechanism is still elusive. Our trial experimental results confirmed this conclusion (data not shown). Therefore, we used salt-free buffer II for in vivo transfection and salt buffer I for in vitro transfection.

The LCP synthesized in this research used the cationic polymer as the basic skeleton, in which the side-chain branching of brassidic acid was cross-linked. Therefore, we postulated that LCP should possess some characteristics of the cationic polymer, and the LCP/siRNA complex should be similar to the cationic polymer/DNA complex, which was a proton pump. The process of releasing LCP/siRNA complexes from endosome to cytoplasm could also be interpreted by the proton sponge effect [Kichler et al., 2001]. To prove the proton pump characteristic of the LCP/siRNA complexes, we investigated the changes in the in vitro transfection efficiency of LCP/siRNA with the presence of a proton pump inhibitor. The inhibition rate of the target gene expression was 73% when cells were transfected with siRNA mediated by LCP without bafilomycin A1 (a proton inhibitor). With bafilomycin A1, the inhibition rate was only 1%, indicating that bafilomycin A1 could inhibit the transfection activity of LCP, which also proved our postulate.

LCP was a novel transfection reagent. In our previous research on the in vitro application of LCP, higher transfection efficiency occurred when it was used to transfect multiple cell lines, including SK-N-SH cell line and 2BS cell line, both of which were hard to be transfected (data not shown). On the condition of optimum transfection efficiency, cell livability was about 95%, indicating its lower cytotoxicity. Our results proved its good biocompatibility, degradability, and easy storage. Although the in vitro application of LCP was well developed, we ultimately aimed its in vivo application for gene therapy. Compared with in vitro gene transportation, we

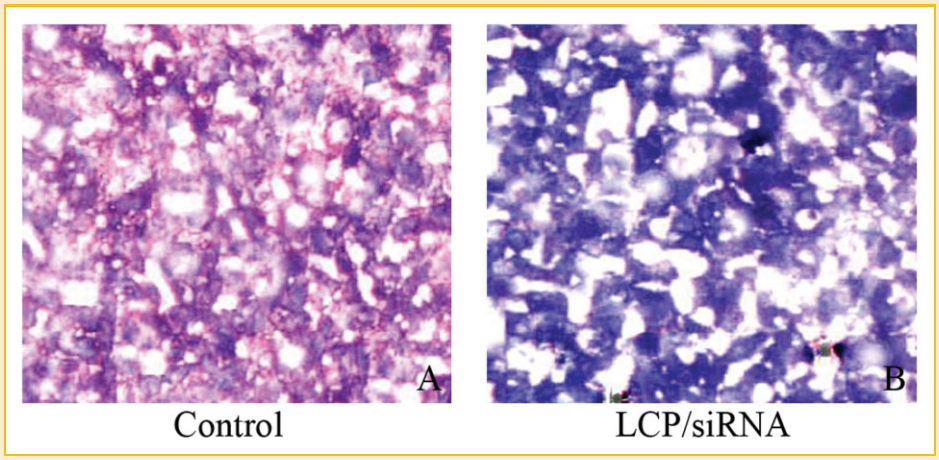

Fig. 4. Detection of COX-2 expression in gastric cancer tissues by IHC. A: The tawny cytoplasm in control group indicated that COX-2 was strongly expressed; (B) the slightly stained cytoplasm in LCP/siRNA-transfected cells indicated that COX-2 expression was significantly suppressed. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

faced a series of problems on the in vivo application of LCP [Jing et al., 2010]. For example, how would LCP cross anatomical and physiological barriers? How could LCP be prevented from interacting with body fluids and extracellular matrix (such as hyaluronic acid and heparin)? How could natural defense mechanisms, such as complement in blood serum, reticuloendothelial system (RES), and immune system, be overcome? How could LCP be prevented from binding with non-target cells [Kircheis et al., 2001]? These factors could affect the physicochemical characteristics of LCP and decrease its transfection efficiency. Therefore, this research focused on the in vivo application of LCP and its prospects on medical application.

Safety is the first consideration in performing clinical application or in vivo tests for transfection reagents. We evaluated the in vivo toxicity of LCP according to the requirements of national pharmacopeia. No animals injected with LCP showed acute, subacute, or chronic reactions. Allergy or pyrogen was also not detected. In the half-lethal dose tests, the highest concentration of the LCP gene vector used was 50 mg/ml, which is 10 times higher

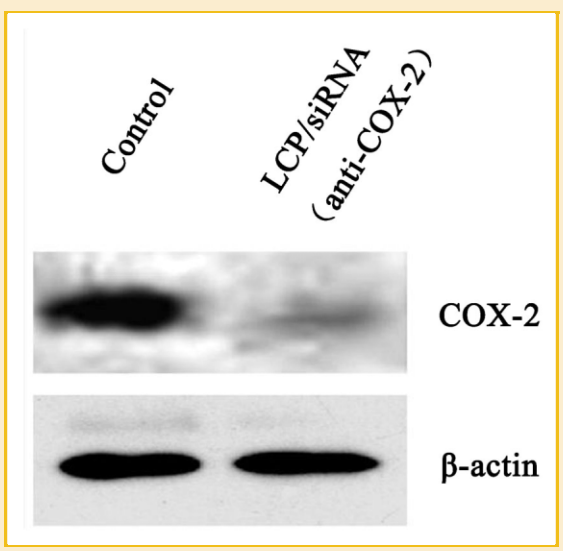

Fig. 5. Detection of COX-2 expression in gastric cancer tissues by Western blot.

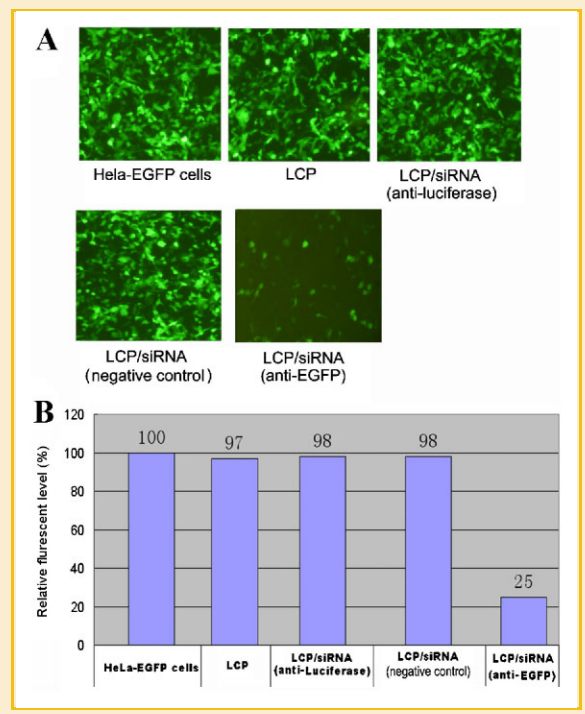

Fig. 6. Relative fluorescence level of HeLa-EGFP transfected by LCP. A: The fluorescence signal of HeLa-EGFP cells in different groups 24 h after transfection. B: Quantitative analysis of the fluorescence signal. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

than the normal working concentration of 5 mg/ml. Given the body weight of an adult and a mouse at 50 kg and 20 g, respectively, 0.5 ml 50 mg/ml LCP would not lead to death in mice. This means that the maximum tolerance dose in mice is equivalent to 2,500 times that in humans. The transfection reagent is safe for clinical application when the maximum tolerance dose in mice is over 100 times higher than that in humans according to body weight. Therefore, LCP is safe enough for in vivo application in animals.

To evaluate the in vivo transfection efficiency of LCP, we transported siRNA against COX-2 into the mice by LCP. Then, we detected the changes of COX-2 in the protein and mRNA levels. We investigated the effects of LCP on nude-transplanted gastric tumors. Finally, the efficiency of LCP-mediated target gene RNAi was determined. COX, also called prostaglandin peroxidase synthase, is an important restricted enzyme during PG synthesis. It could produce various prostaglandins by metabolizing arachidonic acid. It also affects physiological and pathological processes [Khunamornpong et al., 2009]. COX-2 is closely associated with the development of tumor and inflammation, and could be taken as the target to prevent and cure tumor and inflammation, which is of great clinical value [Antonio et al., 2010; Menter et al., 2010]. COX-2 could inhibit tumor proliferation and induce tumor cells into apoptosis, indicating its anti-tumor effects [Che et al., 2010; Huang et al., 2010]. We successfully established the nude-transplanted tumor model, and interfered against the COX-2 expression in nude-transplanted tumors. The tumor volume and the ratio of nucleus to cytoplasm in the experimental group were smaller than that in the control group. These indicate that the transfection of siRNA against COX-2 could efficiently suppress tumor growth. The results of the RT-PCR showed that the mRNA level of COX-2 in tumor tissues was markedly lower than that in BGC-823 cells with or without transfection of negative-LCP/siRNA ($P < 0.01$). The inhibition rate reached 63.2%. Results from IHC and Western blot proved that gene silence mediated by anti-COX-2 siRNA was efficient. All results indicated that the transfection of siRNA mediated by LCP could efficiently inhibit the expression of COX-2 and suppress tumor growth. Therefore, LCP showed higher transfection efficiency in transporting siRNA and could be used in tumor gene therapy.

Considering that the decrease in fluorescence might have been caused by the off-target effects of LCP, different control treatments such as LCP (mock control), LCP/siRNA (anti-luciferase), and LCP/siRNA (anti-EGFP), were set to eliminate this possibility. Cells in different groups were transfected under the identical conditions. Our results proved that the decline in fluorescence intensity in HeLa-EGFP cells was due to the transfection of siRNA against EGFP, and that LCP did not have off-target effect.

In summary, safety and high efficiency were the two key problems for in vivo transfection. This research and other published articles showed that LCP possesses high transfection efficiency and safety for in vivo and in vitro applications. Especially for in vivo tests, the transfection of siRNA mediated by LCP successfully silenced the expression of COX-2 in tumor tissues. This provided scientific evidence and aspects for the in vivo application of LCP gene vector, especially for application in tumor gene therapy.

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