RESEARCH ARTICLE

Cervical Cancer Gene Therapy by Gene Loaded PEG-PLA Nanomedicine

Bo Liu[1], Shu-Mei Han[1], Xiao-Yong Tang[1], Li Han[1], Chang-Zhong Li[2]*

Abstract

Background and Aims: Advances in the treatment of cervical cancer over the last decade have predominantly involved the development of genes directed at molecular targets. Gene therapy is recognized to be a novel method for the treatment of cervical cancer. Genes can be administered into target cells via nanocarriers. This study aimed to develop systemically administrable nano-vectors. Floate (Fa) containing gene loaded nanoparticles (NPs) could target HeLa human cervical cancer cells through combination with receptors on the cells to increase the nuclear uptake of genetic materials. Methods: Fa was linked onto Poly (ethylene glycol)-b-poly (D, L-lactide) (PEG-PLA) to form Fa-PEG-PLA, and the resulting material was used to load plasmids of enhanced green fluorescence protein (pEGFP) to obtain gene loaded nanoparticles (Fa-NPs/DNA). Physical-chemical characteristics, *in vitro* release and cytotoxicity of Fa-NPs/DNA were evaluated. The *in vitro* transfection efficiency of Fa-NPs/DNA was evaluated in HeLa cells and human umbilical vein endothelial cells (HUVEC). PEG-PLA without Fa was used to load pEGFP from NPs/DNA as a control. Results: Fa-NPs/DNA has a particle size of 183 nm and a gene loading quantity of 92%. After 72h of transfection, Fa-NPs/DNA displayed over 20% higher transfection efficiency than NPs/DNA and 40% higher than naked DNA in HeLa cells. However, in HUVECs, no significant difference appeared between Fa-NPs/DNA and NPs/DNA. Conclusions: Fa-PEG-PLA NPs could function as excellent materials for gene loading. This nano-approach could be used as tumor cell targeted medicine for the treatment of cervical cancer.

Keywords: Gene therapy - cervical cancer - folate coating - targeted nanomedicine

Asian Pac J Cancer Prev, **15 (12)**, 4915-4918

Introduction

Cervical cancer is one of the most common gynecological malignancies; it is a significant cause of morbidity and mortality among women worldwide, and its prevalence has increased during the last decades, especially in Asia countries (Zhou et al., 2012; Luu et al., 2013; Antic et al., 2014; Filipi et al., 2014; Karadag et al., 2014; Tungsrithong et al., 2014). Due to non-existent or inadequate screening, disease is normally detected at late stage. Improved treatment options for this type of malignancy are highly needed (Takai et al., 2011).

Gene therapy is recognized to be a novel method for the treatment of various disorders. Gene therapy strategies involve gene manipulation on broad biological processes responsible for the spreading of diseases. Cancer, monogenic diseases, vascular and infectious diseases are the main targets of gene therapy (Podolska et al., 2012). Recent technological advances in high-throughput genomics and the application of integrative approaches have greatly accelerated gene discovery, facilitating the identification of molecular targets (Narayan and Murty, 2010). New expression systems, viral and bacterial vectors for gene delivery, were widely used for cervical cancer treatment (Gersch et al., 2012).

As an alternative to the viral vectors, a variety of non-viral vectors have been developed for gene delivery (Vachani et al., 2011). Several general strategies have been developed to achieve this end, including liposomes (Chen et al., 2010; Buyens et al., 2011), polymers (Elzoghby 2013; Lee et al., 2014), and molecular conjugates (Elfinger et al., 2007; Geiger et al., 2010). As a research focus in recent years, polyethylene glycol-polylactic acid (PEG-PLA) block copolymer and its end-group derivative nanoparticles can enhance the drug loading of hydrophobic drugs, reduce the burst effect, avoid being engulfed by phagocytes, increase the circulation time of drugs in blood, and improve bioavailability (Xiao et al, 2010). There are also some reports on using PEG-PLA nanocarriers in vivo, showed its excellent property and safety for drug/gene delivery (Schädlich et al, 2011; Xia et al, 2012; Shi et al, 2013).

By adding end groups onto the PEG-PLA block copolymers, various nanocarriers may be prepared in order to increase the therapeutic effect of genes, such as long-circulating nanoparticles, immunonanoparticles, thermosensitive nanoparticles, and pH-sensitive nanoparticles (Xiao et al, 2010). Due to their core shell

*[1]Department of Internal Medicine Oncology, Shandong Tumor Hospital and Institute, [2]Department of Gynecology, Shandong Provincial Hospital Affiliated to Shandong University, Ji'nan, China *For correspondence: 15168888909@163.com*

structure, the surface of PEG-PLA block copolymer nanoparticles is often modifed by folic acid, peptide, lectin, albumin, and so on (Gao et al., 2006; Yu et al., 2010; Wang et al., 2013). Compared with PEG-PLA block copolymer nanoparticles with passive targeting, these modifed nanoparticles can actively target special locations for enhancing gene transfection efficiency and decreasing cytotoxicity.

In the present study, Fa was linked onto PEG-PLA to form Fa-PEG-PLA, the resulting material was used to load pEGFP to get Fa-NPs/DNA. The physical-chemical characteristics, in vitro release and cytotoxicity of Fa-NPs/DNA were evaluated. The in vitro transfection efficiency of Fa-NPs/DNA was evaluated on HeLa cells and HUVEC. PEG-PLA without Fa was used to load pEGFP to from NPs/DNA, applied as control.

Materials and Methods

Materials

NH2-PEG-PLA was purchased from Ji'nan Daigang Biology Co. Ltd. (Ji'nan, Shandong, China). pEGFP was obtained from Zhejiang University (Hangzhou, Zhejiang, China). Folic acid, and Cell Counting Kit-8 (CCK-8) were purchased from Sigma-Aldrich Co., Ltd. (St Louis, MO, USA). Quant-iT™ PicoGreen® dsDNA quantitation reagent was obtained from Invitrogen by Life Technologies (Carlsbad, CA, USA). HeLa cells were obtained from the American type culture collection (Manassas, VA, USA). Other reagents were commercially available and were used as received.

Synthesis of Fa-PEG-PLA

Fa-PEG-PLA was synthesized using the following modified method (Tsai et al., 2010; Zhang et al., 2010; Wang et al., 2013): Fa (1 g) was dissolved in hydrous DMSO (30 mL). Then Fa, DCC, DMAP were added to the NH2-PEG-PLA containing DMSO solution to react with each other at 50°C water bath in dark for 36 h to get Fa-CO-NH-PEG-PLA (Fa-PEG-PLA). The raw materials molar ratio of Fa, DCC and DMAP to the initial of NH2-PEG-PLA was 1:1.2:1.2:1. The finished product Fa-PEG-PLA was obtained after purifying by dialysis against water and freeze drying.

Preparation and characterization of Fa-NPs/DNA

Fa-NPs/DNA was prepared by solvent evaporation-induced interfacial self-assembly method (Wang et al., 2013) (Figure 1). Briefly, Fa-PEG-PLA was dissolved in 1 mL of acetone, the solution was added dropwise into 20 mL pEGFP containing distilled water within 20 min under 400rpm of stirring at room temperature. Then the mixture was maintained with continuous stirring for 2 h, allowing the polymer to self-assemble to form gene loaded NPs while the organic solvent was evaporating. The NPs were separated from the remaining organic solvent through high speed centrifugation (100 g, 4°C, 10 min), and then washed with distilled water for 3 times and freeze dried. The blank Fa-NPs were prepared using the same method without the experience of pEGFP. Unmodified NPs/DNA was prepared as the same method using PEG-PLA without Fa ligands.

The mean diameter and zeta potential of the fresh made Fa-NPs and Fa-NPs/DNA were determined by photon correlation spectroscopy (PCS) with a Zetasizer 3000 (Malvern Instruments, Malvern, UK). The average particle size was expressed as volume mean diameter. The Gene loading capacity of the vectors was characterized by PicoGreen-fluorometry assay. Briefly, pEGFP was isolated from Fa-NPs/DNA and NPs/DNA by centrifugation at 1000 g, 4°C for 30 min. The concentration of pEGFP was determined by fluorescence, comparing with the supernatant from blank Fa-NPs. The amount of pEGFP loaded in the carriers was then calculated.

Cell culture and cytotoxicity test

For cytotoxicity studies, HeLa cells were seeded in 24 well plates at 3×104 cells/mL and cultured for 24 h allow cell attachment. The cells were incubated with Fa-NPs, NPs/DNA, and Fa-NPs/DNA various concentrations (25, 50, 100 μg/ml) for 48 h at 37°C and 5% CO_2 atmosphere, respectively. Cells without incubation were used as control. After incubation for 48 h, the culture medium was removed. Cellular viability was assessed using Cell Counting Kit-8 (CCK-8) according to the manufacturer's procedures and the absorbance at 450 nm was measured using a microplate reader (Bio-Rad 680, USA). Cells without the addition of CCK-8 were used as a blank control.

In vitro gene delivery efficiency

The HeLa cells were seeded into 24-well plates at a density of 3×104 cells/well in 1 mL of DMEM with 10% FBS, 24 h prior to transfection. When the cells were at about 80% confluence, the media were replaced with 300 μL of serum-free media containing Fa-NPs/DNA and NPs/DNA complexes at 37°C. Naked DNA was used as control. After incubation for 4 h at 37°C in a 5% CO_2 incubator, the original incubation medium was replaced with 1 mL of complete medium and then cells were incubated sequentially until 72 h post transfection (Yu et al., 2010). HUVEC were applied as the some method as HeLa cells mentioned above.

Flow cytometry was applied to quantitate the amount of cells that have been successfully transfected. The cells were washed with 1 mL of PBS (200 g, 4°C for 5 min) and were detached with trypsin/EDTA. The supernatant was discarded and resuspended with 300 μL of PBS and added into the flow cytometry to quantitate the successfully transfected HeLa cells and HUVEC.

Results and Discussion

Size and zeta potential of Fa-NPs/DNA

Mean diameter, polydispersity index (PDI), and Zeta potential of Fa-NPs, NPs/DNA, Fa-NPs/DNA, F-SLN/pEGFP, and D-SLN/pEGFP as well as gene loading capacity were characterized and summarized in Table 1. The volume mean diameter of Fa-NPs and gene loaded Fa-NPs/DNA is around 97 and183 nm respectively. Both Fa-NPs and Fa-NPs/DNA demonstrated narrow particle size distribution (below 0.1), narrow size-range of NPs

Table 1. Particle size and Zeta Potential of Different NPs in Water

Sample	Mean diameter (nm)	PDI	Zeta potential (mV)
Fa-NPs	96.5	0.05	-15.3
NPs/DNA	138.9	0.08	-29.5
Fa-NPs/DNA	182.8	0.09	-32.6

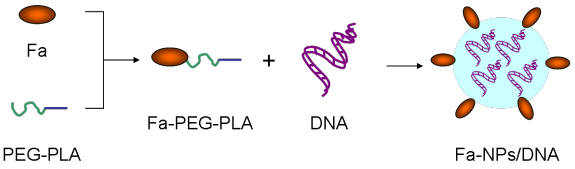

Figure 1. General Scheme for the Preparation of Fa-NPs/DNA

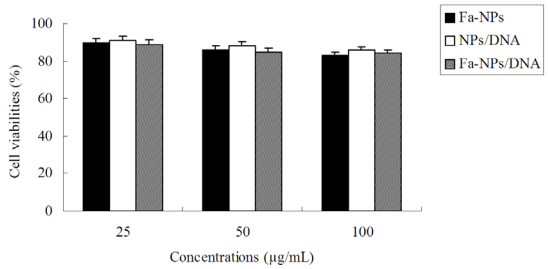

Figure 2. Cytotoxicity Test Results in HeLa Cells

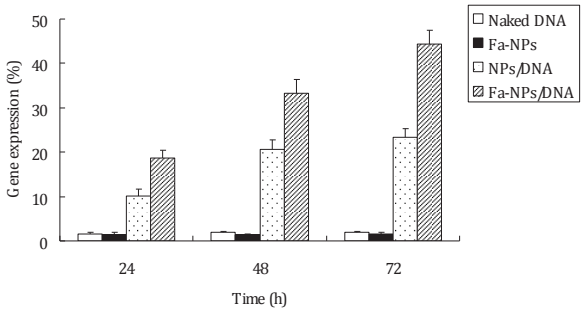

Figure 3. Quantitation Results of In Vitro Gene Delivery in HeLa Cells

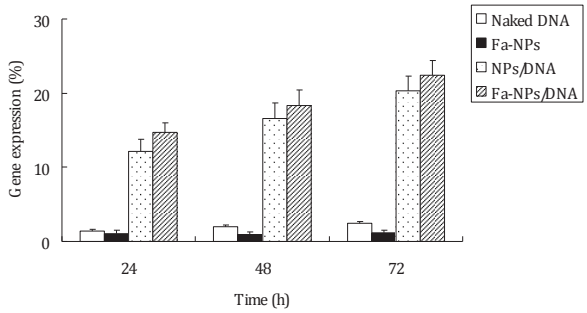

Figure 4. Quantitation Results of In Vitro Gene Delivery in HUVEC

acts as a crucial factor in determining the transport and retention of NPs in tissues showing enhanced permeability and retention (EPR) effect (Parveen et al., 2012). Fa-NPs/DNA has a zeta potential of -33 mV, it is in the stable range of electric charge of the nanoparticles. These results could demonstrate the uniformity and stability of the dual ligands system.

In vitro cytotoxicity test

Figure 2 showed the in vitro cytotoxicity of NPs/DNA and Fa-NPs/DNA evaluated by CCK-8 in HeLa cells at different concentrations. The cell viabilities of the vectors over the studied concentration range (25~100 μg/ml) were over 80% compared with controls. Fa-NPs/DNA showed no obvious cytotoxicity at all concentrations.

Cytotoxicity was a major hurdle for clinical feasibility of gene carriers (Wang et al., 2012; Yang et al., 2013). The nanocarriers showed no higher cytotoxicity at all concentrations. This could facilitate its usage as gene delivery vectors without unexpected damage to the cells.

In vitro gene delivery effect

The in vitro gene delivery effect of NPs/DNA and Fa-NPs/DNA were evaluated against HeLa cells (Figure 3) and HUVEC (Figure 4). Fa-NPs/DNA achieved displayed over 20% higher transfection efficiency than NPs/DNA and 40% higher than naked DNA in HeLa cells at 72h ($P < 0.01$). At the same incubation time (24, 48, and 72 h), the uptake of the Fa-NPs/DNA for HeLa cells was significantly higher than of NPs/DNA. To further determine the effect of Fa receptor mediated targeted delivery, one kind of common cell line, HUVEC were used for the transfection. The appearance in HUVEC showed no significant difference between Fa-NPs/DNA and NPs/DNA. The studies of cervical cancer gene diagnosis and therapy in Asia countries have been carried out by several researches and gained excellent results (Kahla et al, 2014; Lin et al, 2014; Ma et al, 2014; Zhao et al, 2014). The results in this study suggesting much higher affinity and selecting capability of the Fa containing gene loaded NPs to cervical cancer cells. It is known that the cellular uptake of Fa in physiological environments is mainly controlled by receptor-mediated endocytosis (Wang et al., 2013). The more Fa receptor (FR) in cell membrane allows more uptake of Fa into cells. HeLa cell line is one of the types of tumor cells over-express Fa receptors compared to normal cells. So in this section, Fa in the surface of Fa-NPs/DNA would target to HeLa cells surfaces through receptor-ligands interactions and facilitate the entry of the particles to the cells by means of endocytosis. In fact, EPR effect due to the leaky vasculature of tumor tissues is another effective way to target NPs to tumor tissues rather than normal tissues. Thus, it may be expected that both receptor-mediated endocytosis and particle size-dependent EPR effects would lead to the much stronger selecting capability of Fa-NPs/DNA to cervical cancer parts than to normal tissues and cells of the body.

In conclusion: In this study, a novel Fa containing lipid Fa-PEG-PLA was used as a polymeric carrier for tumor targeting gene delivery. The results from the CCK-8 assay revealed that the obtained Fa-NPs/DNA is non-toxic to tumor cells HeLa. Transfection study against cervical cells demonstrated that the existence of folate on the surface of the carriers significantly increased the selecting capability of the NPs to the tumor cells, suggesting the receptor-mediated endocytosis pathway of Fa-NPs/DNA. Therefore, it can be concluded that the resulting gene loaded polymeric nanoparticles could be used as a safe and effective nanomedicine for the treatment of cervical cancer.

Acknowledgements

The authors declare that they have no competing interests.

References

AAntic LG, Djikanovic BS, Antic DZ, et al (2014). Differencies in the level of knowledge on cervical cancer among health care students, midwives and patients in Serbia. *Asian Pac J Cancer Prev*, **15**, 3011-5.

Buyens K, De Smedt SC, Braeckmans K, et al (2012). Liposome based systems for systemic siRNA delivery: stability in blood sets the requirements for optimal carrier design. *J Control Release*, **158**, 362-70.

Chen Y, Zhu X, Zhang X, et al (2010). Nanoparticles modified with tumor-targeting scFv deliver siRNA and miRNA for cancer therapy. *Mol Ther*, **18**, 1650-6.

Elfinger M, Maucksch C, Rudolph C. (2007). Characterization of lactoferrin as a targeting ligand for nonviral gene delivery to airway epithelial cells. *Biomaterials*, **28**, 3448-55.

Elzoghby AO (2013). Gelatin-based nanoparticles as drug and gene delivery systems: Reviewing three decades of research. *J Control Release*, **172**, 1075-91.

Filipi K, Xhani A (2014). Assessment of cervical cytological data in Albanian females. *Asian Pac J Cancer Prev*, **15**, 2129-32.

Gao X, Tao W, Lu W, et al (2006). Lectin-conjugated PEG-PLA nanoparticles: preparation and brain delivery after intranasal administration. *Biomaterials*, **27**, 3482-90.

Geiger J, Aneja MK, Hasenpusch G, et al (2010). Targeting of the prostacyclin specific IP1 receptor in lungs with molecular conjugates comprising prostaglandin I2 analogues. *Biomaterials*, **31**, 2903-11.

Gersch ED, Gissmann L, Garcea RL (2012). New approaches to prophylactic human papillomavirus vaccines for cervical cancer prevention. *Antivir Ther*, **17**, 425-34.

Kahla S, Kochbati L, Maalej M, et al (2014). Situation of HPV16 E2 gene status during radiotherapy treatment of cervical carcinoma. *Asian Pac J Cancer Prev*, **15**, 2869-73.

Karadag G, Gungormus Z, Surucu R, et al (2014). Awareness and practices regarding breast and cervical cancer among Turkish women in Gazientep. *Asian Pac J Cancer Prev*, **15**, 1093-8.

Lee H, Kim Y, Schweickert PG, et al (2014). A photo-degradable gene delivery system for enhanced nuclear gene transcription. *Biomaterials*, **35**, 1040-9.

Lin C, Huang F, Zhang YJ, et al (2014). Roles of MiR-101 and its target gene Cox-2 in early diagnosis of cervical cancer in Uygur women. *Asian Pac J Cancer Prev*, **15**, 45-8.

Luu HN, Dahlstrom KR, Mullen PD, et al (2013). Comparison of the accuracy of Hybrid Capture II and polymerase chain reaction in detecting clinically important cervical dysplasia: a systematic review and meta-analysis. *Cancer Med*, **2**, 367-90.

Ma JQ, Kurban S, Zhao JD, et al (2014). Epigenetic regulation of human riboflavin transporter 2 (hRFT2) in cervical cancers from Uighur women. *Asian Pac J Cancer Prev*, **15**, 2485-9.

Meads C, Auguste P, Davenport C, et al (2013). Positron emission tomography/computerised tomography imaging in detecting and managing recurrent cervical cancer: systematic review of evidence, elicitation of subjective probabilities and economic modelling. *Health Technol Assess*, **17**, 1-323.

Narayan G, Murty VV (2010). Integrative genomic approaches in cervical cancer: implications for molecular pathogenesis. *Future Oncol*, **6**, 1643-52.

Parveen S, Misra R, Sahoo SK (2012). Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging. *Nanomedicine*, **8**, 147-66.

Podolska K, Stachurska A, Hajdukiewicz K, Małecki M (2012). Gene therapy prospects--intranasal delivery of therapeutic genes. *Adv Clin Exp Med*, **21**, 525-34.

Schädlich A, Caysa H, Mueller T, et al (2011). Tumor accumulation of NIR fluorescent PEG-PLA nanoparticles: impact of particle size and human xenograft tumor model. *ACS Nano*, **5**, 8710-20.

Shi Y, Huang W, Liang R, et al (2013). Improvement of in vivo efficacy of recombinant human erythropoietin by encapsulation in PEG-PLA micelle. *Int J Nanomedicine*, **8**, 1-11.

Takai N, Kira N, Ishii T, et al (2011). Novel chemotherapy using histone deacetylase inhibitors in cervical cancer. *Asian Pac J Cancer Prev*, **12**, 575-80.

Tsai HC, Chang WH, Lo CL, et al (2010). Graft and diblock copolymer multifunctional micelles for cancer chemotherapy and imaging. *Biomaterials*, **31**, 2293-301.

Tungsrithong N, Kasinpila C, Maneenin C, et al (2014). Lack of significant effects of *Chlamydia trachomatis* infection on cervical cancer risk in a nested case-control study in North-East Thailand. *Asian Pac J Cancer Prev*, **15**, 1497-500.

Vachani A, Moon E, Wakeam E, et al (2011). Gene therapy for lung neoplasms. *Clin Chest Med*, **32**, 865-85.

Wang S, Luo Y, Zeng S, et al (2013). Dodecanol-poly (D, L-lactic acid)-b-poly (ethylene glycol)-folate (Dol-PLA-PEG-FA) nanoparticles: evaluation of cell cytotoxicity and selecting capability in vitro. *Colloids Surf B Biointerfaces*, **102**, 130-5.

Wang YQ, Su J, Wu F, et al (2012). Biscarbamate cross-linked polyethylenimine derivative with low molecular weight, low cytotoxicity, and high efficiency for gene delivery. *Int J Nanomedicine*, **7**, 693-704.

Xia H, Gao X, Gu G, et al (2012). Penetratin-functionalized PEG-PLA nanoparticles for brain drug delivery. *Int J Pharm*, **436**, 840-50.

Xiao RZ, Zeng ZW, Zhou GL, et al (2010). Recent advances in PEG-PLA block copolymer nanoparticles. *Int J Nanomedicine*, **5**, 1057-65.

Yang S, Chen Y, Gu K, et al (2013). Novel intravaginal nanomedicine for the targeted delivery of saquinavir to CD4+ immune cells. *Int J Nanomedicine*, **8**, 2847-58.

Yu DH, Lu Q, Xie J, et al (2010). Peptide-conjugated biodegradable nanoparticles as a carrier to target paclitaxel to tumor neovasculature. *Biomaterials*, **31**, 2278-92.

Yu W, Liu C, Liu Y, et al (2010). Mannan-modified solid lipid nanoparticles for targeted gene delivery to alveolar macrophages. *Pharm Res*, **27**, 1584-96.

Zhao LW, Zhong XH, Yang SY et al (2014). Inotodiol inhabits proliferation and induces apoptosis through modulating expression of cycline, p27, bcl-2, and bax in human cervical cancer Hela cells. *Asian Pac J Cancer Prev*, **15**, 3195-9.

Zhang L, Hu CH, Cheng SX, Zhuo RX (2010). Hyperbranched amphiphilic polymer with folate mediated targeting property. *Colloids Surf B Biointerfaces*, **79**, 427-33.

Zhou X, Gu Y, Zhang SL (2012). Association between p53 codon 72 polymorphism and cervical cancer risk among Asians: a HuGE review and meta-analysis. *Asian Pac J Cancer Prev*, **13**, 4909-14.