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• LIVER CANCER •

Preparation and characteristics of DNA-nanoparticles targeting to hepatocarcinoma cells

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Abstract

AIM: To prepare thymidine kinase gene (TK gene) nanoparticles and to investigate the expression of TK gene.

METHODS: Poly(D,L-lactic-co-glycolic acid) (PLGA), a biodegradable and biocompatible polymer, was used to prepare recombinant plasmid $P^{EGFP\text{-}AFP}$ nanoparticles by a double-emulsion evaporation technique. Characteristics of the nanoparticles were investigated in this study, including morphology, entrapment efficiency, and tissue distribution. The expression of TK gene was also investigated by MTT assay, by which the viable cells were determined after the addition of ganciclovir (GCV). The enhanced green fluorescent protein (EGFP) expression in human hepatocellular carcinoma SMMC-7721 cells and normal parenchymal Chang liver cells were assessed by flow cytometry.

RESULTS: The prepared plasmid-nanoparticles had regular spherical surface and narrow particle size span with a mean diameter of 72±12 nm. The mean entrapment efficiency was 91.25%. A total of 80.14% DNA was found to be localized in the livers after 1-h injection with ^{32}P-DNA-PLGA nanoparticles in mouse caudal vein. The expression of DNA encapsulated in nanoparticles was much higher than that in naked DNA, and human hepatocellular carcinoma SMMC-7721 cells were more sensitive to GCV than human normal parenchymal Chang liver cells.

CONCLUSION: The enhanced transfection efficiency and stronger ability to protect plasmid DNA from being degraded by nucleases are due to nanoparticles encapsulation.

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INTRODUCTION

In recent years, the gene delivery system has attracted much attention[1-3]. However, safe and efficient gene delivery remains a crucial barrier to successful gene therapy. Viral and retroviral vectors have been the most efficient and commonly used delivery modalities for *in vivo* gene transfer, but viral vector may provoke mutagenesis and carcinogenesis. Repeated administration of a viral vector induces an immune response which abolishes the transgene expression[4-7]. The non-viral delivery system has the potential to be non-immunogenic and stable *in vivo*[8-11]. Encapsulation of DNA in biodegradable polymer potentially offers a way to protect DNA from degradation and to control DNA release[12,13], and many examples of DNA incorporated in synthetic polymers have been developed in the micron scale. Recently, some studies have showen that intracellular biodistribution of particles with diameter less than 100 nm can be achieved[14].

Among all the present gene therapeutic protocols, combination of the administration of GCV with transfecting thymidine kinase gene of *Herpes simplex* virus (HSV-TK) into tumor cells is rather practical and potential in intra-tumoral gene therapy. The TK genes in the tumor cells can induce the metabolism of untoxicant prodrug GCV into cytotoxic parent drug, which can cause the suicide of cells. This protocol presents good potential in intra-tumoral gene therapy[15,16]. However, common TK genes (naked genes) do not have the abilities to target to specific organs and tissues, which can be harmful to the normal cells and tissues. In addition, they are easily degraded by nucleases *in vivo*.

To solve the problems mentioned above, a recombinant plasmid $P^{EGFP\text{-}TKAFP}$ was constructed, which can be specifically expressed in hepatocellular carcinoma cells. Furthermore, the plasmid was encapsulated in a biodegradable and biocompatible PLGA polymer to protect plasmid DNA from being digested by nucleases. The following characteristics of the nanoparticles were investigated, including *in vitro* anti-nuclease ability, tissue distribution in mice and the gene expression in hepatocellular carcinoma cells and normal parenchymal cells *in vitro*.

MATERIALS AND METHODS

Materials

Poly(D,L-lactic-co-glycolic acid) (PLGA; lactic-glycolic acid ratio: 75:25, M_r=30 000, batch number: 020112) was purchased from Chengdu Institute of Organic Chemistry, Chinese Academy of Science. Recombinant plasmid $P^{EGFP\text{-}TKAFP}$ was a gift from Dr. Liu Ji (Sichuan University). Human hepatocellular carcinoma SMMC-7721 cells[17] and normal parenchymal Chang liver cells[18] were provided by Shanghai Institute of Cell Biology, Chinese Academy of Science. DNase I was purchased from Chengdu Huamei Biochemicals Cooperation (Sichuan Province, China). Kunming mice, weighted 18-22 g, were provided by Experimental Animal Center of Sichuan University.

Methods

Nanoparticles preparation A double-emulsion evaporation technique[19] was used to prepare the nanoparticles. Briefly, plasmid DNA (200 μg) in 100 μL Tris-EDTA (TE) buffer was emulsified in 1 mL methylene chloride solution containing 100 mg of PLGA using a probe sonicator for 5 s. Polyvinyl alcohol (2 mL) was added to the primary emulsion and sonicated for another 5 s to form a double emulsion. The emulsion was added into the same concentration of polyvinyl alcohol and agitated by a magnetic stirrer for 3 h at room

temperature to remove methylene chloride.

Particle size and morphology analysis The PLGA nanoparticles were sized by laser diffractometry using a Malvern 2 000 laser sizer. The morphology was observed by the scanning electron microscope (JEM-100SX, Akishima, Japan). The samples were placed on to special copper grids and then stained with 20 mL/L phosphato-tungstic acid prior to visualization.

Entrapment ratio analysis The entrapment ratio was determined by measuring the total amount of added DNA and that of DNA being not encapsulated. In detail, colloid solution of DNA-PLGA nanoparticles was centrifuged at 45 000 *g* for 1 h. Then, the concentration of DNA in the supernatant was assessed by fluorescence spectrophotometry after stained with ethidium bromide. The exciting and emission wavelengths were 546 nm and 590 nm, respectively. The entrapment rate (ER) was calculated as follows: ER (%)=DNA_{added}-$DNA_{in\ the\ supernatant}$/$DNA_{added}$×100%

Protection from DNase The PLGA nanoparticles were incubated with DNase I (0.1 unit) at 37 ℃ in a shaking water bath. The nanoparticles were collected by centrifugation after 4, 8, and 16 h incubation, and then chloroform was added to solubilize the nanoparticles. An equal volume of PBS solution was added, and the mixture was rotated end-over-end to facilitate the extraction of DNA from the organic phase into the aqueous phase. The samples were then centrifuged at 15 000 *g* for 15 min. The resulted supernatant was transferred to another tube and DNA was precipitated with the addition of isopropanol. Precipitate was obtained after centrifugation at 5 000 r/min for 15 min. Then, the resulted pellet was rinsed with 700 mL/L ethanol and resuspended in sterile TE buffer. The purified DNA was analyzed by gel electrophoresis.

Tissue distribution One hundred Kunming mice weighed 18-22 g were randomly divided into 10 test groups and 10 control groups with 5 in each group. The nanoparticles of ^{32}P-DNA-PLGA at a dose of 10 μL/g was intravenously administered to each mouse in test groups, and ^{32}P-DNA at the same dose was intravenously administered in control groups. At predetermined intervals, mice were sacrificed for blood collection. Then, heart, livers, spleen, lungs, and kidneys were removed from mice. The radioactivity of each organ was measured by a liquid scintillation analyzer.

The cpm_t was the total value of cpm in each organ (cpm_i) at a certain time point. The ratio of cpm_i/cpm_t×100% represented the relative content of DNA in viscera and blood.

MFI assay Human hepatocellular carcinoma SMMC-7721 cells and normal parenchymal Chang liver cells (5×10^5) were cultured in the DMEM medium containing 100 mL/L fetal bovine serum (FBS) in 12-well plates. The cells were transfected with plasmid DNA or nanoparticles containing DNA, and maintained at 37 ℃ in an incubator at a 50 mL/L CO_2 humidified atmosphere. After incubation for 12 h, the medium was removed and replaced with fresh DMEM containing 100 mL/L FBS for further 48 h incubation. The mean fluorescence intensity (MFI) of the cells was measured by flow cytometry.

Cytotoxicity assay Cells were cultured in the same way as the MFI assay. After incubation for 12 h, the medium was removed, replaced with DMEM containing 100 mL/L FBS, and incubated with 0.1, 1, or 10 μg/mL GCV. The cytotoxicity of GCV was detected by MTT assay.

RESULTS

Size and morphology

The resulted plasmid-nanoparticles had regular spherical surface (Figure 1) and a narrow size distribution with a mean diameter of 72±12 nm.

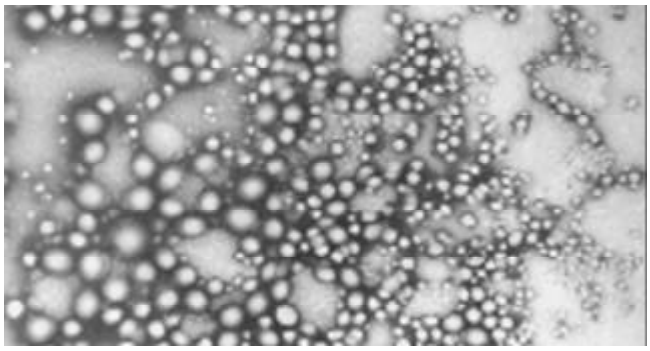

Figure 1 Transmission electron microphotography of TK-PLGA nanoparticles.

Entrapment efficiency

The mean entrapment efficiency was 91.25%, which was rather high in the nanoparticles preparation with PLGA as a carrier.

Protection from DNase

Plasmid DNA encapsulated in nanoparticles remained intact in the presence of DNase I for up to 16 h incubation. On the other hand, control plasmid DNA was completely digested within 1 h incubation with the equal amount of DNase I. This result demonstrated that PLGA nanoparticles could protect encapsulated plasmid DNA from nuclease digestion (Figure 2).

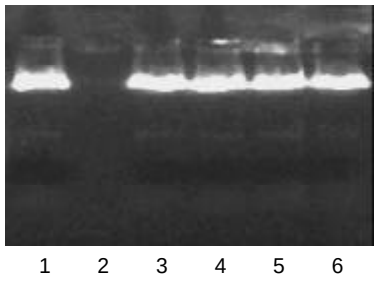

Figure 2 Agarose gel electrophoresis of DNA extracted from nanoparticles after treatment with sonication and Dnase I. Lane 1 represented untreated control plasmid DNA, lane 2 represented plasmid DNA incubated with Dnase I at 37 ℃ for 1 h, and lane 3, 4, 5, 6 indicated DNA extracted from PLGA nanoparticles incubated with Dnase I at 37 ℃ for 0, 4, 8, or 16 h, respectively.

Measurement of DNA in blood and viscera of mice

The tissue distribution of DNA-PLGA nanoparticles was investigated by the technique of gamma scintigraphy. The results showed that 1 h after injection with ^{32}P-DNA-PLGA nanoparticles in mouse caudal vein, the ratio of radioactivity in livers against total radioactivity was more than 80%, which was 1.5-fold of that after injection with ^{32}P-DNA alone (Table 1 and Table 2).

Cytotoxicity assay and MFI assay

The EGFP expression in human hepatocellular carcinoma SMMC-7721 cells and normal parenchymal Chang liver cells were assessed by flow cytometry. The expression of TK gene was also investigated by MTT assay, by which the viable cells were quantitated after the addition of parent drug GCV. The results showed that the EGFP and TK expression in human hepatocellular carcinoma SMMC-7721 cells was much higher than that in human normal parenchymal Chang liver cells ($P<0.05$) (Table 3). It also showed that plasmid DNA encapsulated in nanoparticles could enhance the expression of TK or EGFP gene compared with naked plasmid DNA ($P<0.05$) (Table 4).

Table 1 Distribution (%) of ^{32}P-DNA in mice after intravenous administration of ^{32}P-DNA (n=5)

Time	Heart	Liver	Spleen	Lung	Kidney	Blood
5 min	3.14±0.60	36.67±2.65	3.56±1.58	16.32±0.79	23.52±1.48	16.79±1.62
15 min	3.15±0.97	41.26±2.89	5.25±0.86	14.16±0.99	20.61±1.62	15.57±1.58
30 min	6.94±1.57	52.59±4.02	10.46±0.75	7.95±0.58	13.10±0.85	2.95±0.65
1 h	6.11±1.95	54.62±3.12	14.85±1.23	7.82±0.67	13.50±0.94	3.10±0.78
2 h	4.56±1.01	51.34±2.88	14.93±0.68	9.65±1.41	15.03±1.34	4.49±0.28
6 h	5.12±0.79	48.66±1.87	13.15±0.84	9.66±1.27	18.26±1.58	5.15±1.67
12 h	5.48±0.99	44.52±2.02	11.32±1.33	11.36±1.34	21.83±2.54	5.49±0.58
24 h	3.93±0.65	43.31±1.67	11.48±1.11	10.12±0.64	25.01±3.12	6.16±0.94
48 h	3.43±0.84	36.75±1.65	11.56±0.82	13.36±1.60	28.52±2.15	6.38±0.83
72 h	4.52±0.77	34.48±1.85	12.03±1.73	13.88±1.63	28.31±3.01	6.78±0.91

Table 2 Distribution (%) of ^{32}P-DNA in mice after intravenous administration of ^{32}P-DNA-PLGA nanoparticles (n=5)

Time	Heart	Liver	Spleen	Lung	Kidney	Blood
5 min	2.22±0.51	57.32±2.36	2.96±0.69	8.29±1.33	16.58±2.02	12.63±1.24
15 min	3.52±0.64	69.69±3.32	3.19±0.58	5.77±0.55	13.87±0.99	3.96±0.36
30 min	2.38±0.67	73.37±3.62	7.55±1.03	3.64±0.41	11.28±1.28	1.78±0.65
1 h	2.08±0.54	80.14±4.56	3.37±0.67	3.08±0.62	9.34±0.68	1.99±0.54
2 h	2.59±0.28	78.45±4.02	3.73±0.76	5.35±0.58	8.36±0.94	1.47±0.23
6 h	3.18±0.60	75.03±3.69	7.38±1.03	3.74±0.39	9.61±0.96	1.06±0.24
12 h	2.21±0.51	69.34±3.96	6.17±1.24	7.88±0.64	10.17±1.04	4.22±0.32
24 h	1.94±0.32	65.59±3.25	6.46±1.04	7.95±0.57	12.10±1.23	5.96±0.59
48 h	2.21±0.29	61.18±2.69	7.01±0.48	9.27±0.71	12.87±0.86	6.46±0.86
72 h	2.51±0.37	60.56±2.98	6.45±0.69	9.98±0.65	13.54±1.11	6.96±0.75

Table 3 Inhibition ratio of different concentrations of GCV on SMMC-7721 and Chang liver cells

GCV (μg / mL)	SMMC-7721		Chang liver	
	DNA	NP	DNA	NP
1	2.0	6.6[a]	1.2	3.7
10	3.0	10.0[a]	2.8	5.8

[a]P<0.05 *vs* Chang liver NP group; NP: nanoparticles.

Table 4 Mean fluorescence intensity in SMMC-7721 and Chang liver cells

Cell	MFI	
	DNA	NP
SMMC-7721	0.6±0.1[a]	2.1±0.3[c]
Chang liver	0.6±0.2	0.7±0.2

[a]P<0.05 *vs* NP group; [c]P<0.05 *vs* Chang liver NP group; NP: nanoparticles.

DISCUSSION

Recombinant plasmid P$^{EGFP-AFP}$ was constructed, which could be specifically expressed in hepatocellular carcinoma cells because of alpha-fetoprotein-albumin (AFP-alb) promoter[20-27]. Meanwhile, EGFP as the reporter gene of plasmid DNA, can be assessed by confocal laser scanning microscopy and flow cytometry[28-32]. A polymer, PLGA, was selected due to its biocompatible and biodegradable properties, which was already approved for *in vivo* applications[33-39]. Non-toxicity of the carrier may permit repeated administration of the nanoparticles to compensate for transient transgene expression.

Our results showed that plasmid DNA could be encapsulated in PLGA nanoparticles without compromising its structural and functional integrity. Additionally, PLGA nanoparticles could protect plasmids from nuclease degradation, and therefore offer an effective approach for gene delivery *in vivo*. However, the relatively low transfection efficiency was obtained in comparison with viral vector, which still remains to be a problem.

The DNA nanoparticles probably permeate the cells through endocytotic mechanism due to their small size and negative charged surface[40]. The encapsulation of plasmid DNA in cationic liposomes offers another choice to be protected from DNases. However, cationic liposomes may be toxic to cells due to an excess of positive charge[41,42], and can be easily influenced by the substances in plasma. In recent years, nanoparticles attract more and more attention because of many advantages, including high stability at room temperature, favorable safety, the ability to deliver plasmid DNA at a controllable rate, and easy adaptability. Unlike most viral vector, there is no limit on the size of plasmids encapsulated into the nanoparticles[43,44].

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