Original Article ENO1 monoclonal antibody inhibits invasion, proliferation and clone formation of cervical cancer cells

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Abstract: α -enolase (EN01), highly expressing in cell membranes, cytoplasm and nuclei of cervical cancer and other tumors, acts as a plasminogen receptor and a glycolytic enzyme. EN01 is found to be associated with tumorigenesis, invasion and migration, and proves to be an ideal target of tumor therapy. In this study, EN01 monoclonal antibodies (EN01mAb) was prepared to blockade EN01 and the therapeutic role was observed in cervical cancer cells. First, EN01mAb was prepared and screened by evaluating the inhibitory effect on migration and invasion of cervical cancer cells, which is supposed to block EN01 expressed on cell membrane. Second, folic acid (FA) conjugated PLGA nanoparticles (FA-SS-PLGA) targeting tumor cells were prepared to mediate EN01mAb entry into cells and its anti-tumor effects were investigated *in vitro*. We found that PLGA/FA-SS-PLGA nanoparticles-mediated EN01mAb could antagonize the activity of EN01 enzyme, significantly decreased the contents of lactic acid and pyruvate, and inhibited the proliferation, migration and clone formation of cervical cancer cells compared with the sham control (P < 0.05). In summary, EN01mAb could specifically block EN01 expressed on cell membrane and inhibit EN01 glycolysis enzyme activity inside tumor cells, and plays a therapeutic role against cervical cancer cells. It suggests that EN01mAb has promising anti-tumor effects.

Keywords: ENO1, monoclonal antibody, cervical cancer, glycolysis, PLGA nanoparticles

Introduction

Cervical cancer is one of the most common malignant tumors in gynecology, and still occupies the fourth place in the global female cancer mortality rate [1]. For early stage cervical cancer patients, operation and traditional treatment could improve the survival rate and prognosis to some extent. However, traditional treatment such as chemotherapy and radiotherapy would damage healthy tissue through obvious off-target accumulation, resulting in serious side effects. Moreover, for advanced patients with relapse, metastasis and drug resistance, who lost the opportunity of operation [2], there is no effective treatment until now. Therefore, it is urgent to develop novel therapeutic method against cervical cancer.

Aerobic glycolysis, also known as Warburg effects, is the main metabolic phenotype of

tumor cells, which is closely related to the proliferation, invasion, metastasis and drug resistance of tumor cells. The regulation of tumor cell metabolism is becoming a hot anti-tumor strategy [3-5]. In tumor cells, the expression of enzymes involved in glycolysis, such as Hexokinase-2 (HK2), Lactose dehydrogenase A (LDHA), Pyruvate kinase M2 (PKM2), Glucose-6-phosphate dehydrogenase (G6PD) and so on, were significantly higher than that in normal cells [6-8]. ENO1 in cytoplasm catalyzes the conversion between 2-phosphoglycerate (2-PGA) and phosphoenolpyruvate (PEP) [9]. In our previous study, we found that ENO1 was highly expressed in cervical cancer tissues, and its expression decreased after chemotherapy [10]. In addition, ENO1 is expressed on the cell surface and acts as a plasminogen receptor to mediate plasminogen activation and extracellular matrix degradation, which promotes cell migration and cancer metastasis [11-13].

Furthermore, ENO1 is also localized in the nucleus as a *c-Myc* promoter-binding protein (MBP-1) and regulates the expressions of some genes closely related to the occurrence and development of tumors [14-16]. It was found that over-expression of ENO1 in tumor cells could enhance the Warburg effect, cell growth and hypoxia tolerance, and was associated with tumor progression [17-21]. We also found that silencing of ENO1 expression in cervical cancer cells could reduce its tumorigenesis, invasion and metastasis, and enhance the sensitivity of cervical cancer cells to cisplatin and paclitaxel [10, 22].

Monoclonal antibody (mAb) has become a unique biological target drug because of its strong specificity and remarkable therapeutic effect. It was reported that Ab against human and mouse ENO1 suppressed cell-associated plasminogen and matrix metalloproteinase activation and inhibited tumor invasion and metastasis of lung cancer cells [13]. In this study, we prepared another novel ENO1mAbs against ENO1 protein expressed in insect cells. Due to large molecular weight and weak tissue penetration, monoclonal antibodies could not entry into cells to inhibit glycolysis. To overcome this shortcoming, we intended to develop nanoparticles (NPs) targeting tumor cells and used them to deliver ENO1mAb into tumor cells [23].

The biodegradable polymer nanoparticles as a macromolecular drug carrier has been widely developed. Nanoparticles can not only protect drugs from enzymatic degradation by encapsulating them to increase the half-life of drug, but also help macromolecules to cross physiological barriers such as cell membrane and bloodbrain barrier and achieve the controlled and sustained release of drugs, bringing new opportunities for application of macromolecular drugs [24]. Because of its good biodegradability, PLGA is used for delivery of antibodies and other types of drugs [25-28]. In addition, in order to achieve targeted delivery of drugs to tumor cells, the surface of nanoparticles is often modified with specific ligands like folic acid [29, 30]. Folic acid receptor was found highly expressed in many tumor cells including cervical cancer cells, and thus folic acid became a targeted ligand widely used in nanoparticle modification [31, 32]. In recent years, the emergence of environmentally responsive polymer nanoparticles had further promoted the development of PLGA-drug delivery systems [33-36].

In this study, ENO1 protein was expressed by eukaryotic cell, and five hybridoma cell lines (H1-H5) secreting high potency ENO1mAb were successfully prepared by hybridoma technique. The effective ENO1mAb was initially screened by investigating its blocking activity against cell membrane ENO1, the plasminogen receptor related to cell invasion and migration. The ENO1mAb had the ability to inhibit the migration and invasion of cervical cancer SiHa cells, which indicated that ENO1mAb had the capability to block ENO1, was screened for further study. Furthermore, folic acid-modified PLGA nanoparticles were prepared to deliver ENO1mAb into tumor cells. The inhibitory effect of ENO1mAb on glycolysis of cervical cancer cells and its anti-cervical cancer effect were investigated in vitro.

Materials and methods

Materials

The hybridoma cell lines H1-H5 were prepared by AtaGenix Laboratories (Wuhan, China) and stored in our lab. Special pathogen free BALB/c mice (6-8 weeks old, female) were purchased from the animal center of Lanzhou University (Lanzhou, China). Poly lactic-co-glycolic acid (PLGA-COOH, MW40,000) was from Dalian Meilun Biotech Co., Ltd (Dalian, China). Cysteamine Dihydrochloride (MW225.19), Polyvinyl alcohol, N, N-diisopropyl ethylamine were all purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd (Shanghai, China). Dicvclohexyl carbinimide (DCC, MW206.33). 1-(3-dimethylaminopropyl)-3-ethylcarbon-diimide hydrochloride, N-hydroxysuccinimide (NHS, MW115.09) were from Shanghai Macklin Biochemical Co., Ltd (Shanghai, China). Folic acid was from Sangon Biotech Co., Ltd (Shanghai, China). DMEM medium and RPMI-1640 medium were from Thermo Fisher Scientific (USA). The test kits of lactic acid, pyruvate, glucose and ATP were from Nanjing Jiancheng Biological Co., Ltd (Nanjing, China).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and bicinchoninic acid (BCA) protein assay kit were from Beijing Solarbio Technology Co., Ltd (Beijing, China). Protein A column was from GE Life Sciences (USA). Cell culture plates and bottles were from Corning Biological Co., Ltd (USA).

Methods

Preparation of ENO1mAb: The cDNA encoding human ENO1 gene was constructed into a baculovirus expression vector pFastBac1 to construct the recombinant plasmid pFastBac1-ENO1. Then, the plasmid was transfected into Sf9 insect cells, and the target protein was successfully expressed and purified. BALB/c mice were repeatedly immunized with ENO1 protein, and spleen cells of mice with higher antibody titer were taken out. The hybridomas were achieved by fusing the immunized spleen cells with the Sp2/0 myeloma cell line. The levels of IgG antibody against ENO1 protein (0.5 μ g/ml) in the culture supernatant of hybridoma cells were determined using enzyme-linked immunosorbent assay (ELISA) to evaluate its antibody titer. The positive clones were screened to obtain a hybridoma cell strain with high antibody titer. The hybridoma cells were injected intraperitoneally into BALB/c mice. Subsequently, the ENO1mAb were extracted from ascites by caprylic acid-ammonium sulfate, and further purified by protein A chromatography and verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Wound healing test: Cervical cancer cell line SiHa or Hela cells were plated in 6-well culture plates at a density of 5 × 10⁵ cells per well, and incubated overnight at 37°C with 5% CO,, and allowed to reach nearly 100% confluence. A scratch wound was created by a sterile pipet tip (200 µl). Then the culture medium was aspirated and exfoliated cells were washed 3 times with PBS . After that, the cells were exposed to five kinds of ENO1mAb, PLGA-ENO1mAb, FA-SS-PLGA-ENO1mAb, and 3-BrPA in serumfree DMEM medium and incubated in incubators at 37°C and 5% CO₂. Then, the cell movement into the wound area was monitored and photographed at 24 h using a inverted microscope. Image J software was used to calculate the scratch area, and the average migration rates were analyzed.

Transwell assay

Cell migration experiment: DMEM medium 500 µl containing 15% FBS was plated in the lower

chamber of the Transwell chamber. Cervical cancer cell line SiHa cells were plated in the upper chamber of the Transwell chamber at a density of 4 \times 10⁴ cells per well with 100 µl serum-free DMEM medium and the five monoclonal antibodies (10 µg/ml) were added respectively, and incubated together for 24 h at 37°C and 5% CO₂. Then, The upper chamber was taken out, and fixed the cells with 500 µl of 4% paraformaldehyde, stained with 600 µl of 0.1% crystal violet, rinsed 3 times with PBS buffer. Finally, the center of the chamber and the four fields of view (× 200) from the top and bottom of the chamber were selected under a inverted microscope (Nikon) to allow for counting the number of migrating cells.

Cell invasion test: The Matrigel was taken out from -80°C in advance, and placed at 4°C overnight, diluted it according to the 1:8 ratio of Matrigel and serum-free DMEM medium, and 50 μ l of diluted Matrigel was plated in the upper chamber at 37°C for 1 h. SiHa cells were plated in the upper chamber of Transwell chamber at a density of 1 × 10⁴ cells per well with 100 μ l serum-free DMEM medium. The other procedures were the same as the cell migration experiment.

Synthesis and characterization of PLGA-SS-FA copolymer

FA-SS-PLGA copolymer was prepared according to previous report [37, 38]. First, PLGA-Cys was synthesized through the amidation reaction between the carboxyl group at one end of PLGA and the amino group at the other end of cysteine hydrochloride. Activated PLGA-COOH (1.5 g or 0.0375 mmol), cystine dihydrochloride (84.5 mg or 0.375 mmol), DCC (77 mg or 0.375 mmol) were dissolved in dimethylformamide (DMF). Appropriate amount of N, N-diisopropyl ethylamine (DIEA) was added to the mixture and stirred for 24 h at room temperature under the protection of nitrogen. After filtration, the product was precipitated with ice anhydrous ethyl ether and washed with ice methanol 3 times to remove the unreactants, and the PLGA-Cys copolymer was dried by freezing vacuum dryer.

Second, FA-SS-PLGA copolymer was synthesized by amidation reaction between the terminal carboxyl group of folic acid (FA) and the terminal amino group of PLGA-Cys. Copolymer 200 mg, activated folic acid 15 mg and DCC 10 mg were dissolved in dimethylformamide (DMF). The mixture solution was stirred at room temperature for 24 h under the protection of nitrogen, and the reaction was taken place in the darkness. After the end of this reaction, it was precipitated with ice anhydrous ethyl ether and redissolved in dichloromethane, repeated 2-3 times. At last, the reaction product was dried in vacuum, and stored at -20°C.

The chemical structure of the copolymer was characterized by fourier infrared spectrum (FT-IR) and nuclear magnetic hydrogen spectrum (¹H-NMR). In detail, a small amount of the copolymer was dissolved in dichloromethane (DCM) and determined by infrared spectrometer. For the ¹H-NMR, the copolymer was dissolved in CDCL3 or DMSO-d6, and analyzed by Bruker AVANCE 400 NMR spectrometer (Billerica, MA, USA).

Preparation and characterization of ENO1m-Ab-loaded nanoparticles

ENO1mAb-loaded PLGA nanoparticles were prepared by double emulsion solvent evaporation [39, 40]. In detail, 200 mg PLGA was dissolved in 4 ml dichloromethane; Consequently, 4 ml ENO1mAb-1 (6 mg) solution was added into the PLGA solution, and the liquid colostrum was emulsified by ultrasonic crushing machine under ice bath. The colostrum was slowly poured into 50 ml 2% PVA solution to form milky white reemulsion by homogenizer. The solution was stirred overnight at 500 rpm on a magnetic agitator to completely volatilize dichloromethane. The precipitate was washed with deionized water for 3 times and dried in vacuum. The FA-SS-PLGA nanoparticles with or without ENO1mAb were prepared separately according to the same method. The encapsulation rate and drug loading rate were calculated by BCA protein assay kit. Dry nanoparticles were dispersed in deionized water to prepare a solution with a concentration of 2-3 mg/ml. The particle size distribution, multi-dispersion coefficient and Zeta potential of the nanoparticles were measured by a dynamic light scatterer after filtration with a 0.45 µm filter. Dried nanoparticles were dispersed in anhydrous ethanol, and the morphology was observed by scanning electron microscopy (SEM).

Cell uptake of nanoparticles

Hela cells were plated at a density of 1×10^5 cells per well, incubating overnight at 37°C with 5% CO₂, and then the cells were incubated with FITC-labeled nanoparticles for 4 h. The supernatant was absorbed, and washed with PBS for 3 times, and the cells were fixed with 4% paraformaldehyde for 15 min. After washing with PBS for 3 times, DAPI, with the final concentration of 10 µg/ml was added and incubated for 30 s. After incubation, the fluorescence content in cytoplasm and nucleus were observed by fluorescence microscope.

Determination of glucose metabolites

Hela cells were plated in 6-well culture plates with a density of 1×10^5 cells per well, and incubated overnight at 37°C with 5% CO₂. The nanoparticle solution and 3-bromopyruvate (3-BrPA) prepared by the phenol red-free DMEM containing 5% fetal bovine serum (FBS) were added, and the culture supernatant was collected 24 h later. The contents of lactic acid and pyruvate in the supernatant were determined according to the instructions of lactic acid kit and pyruvate kit respectively. The total protein in cells was extracted and the concentration was determined by BCA protein assay kit.

The inhibitory effect of ENO1mAb-loaded NPs on the proliferation of cervical cancer cells in vitro

Hela cells were plated in triplicate in 96-well culture plates at the density of 5×10^3 cells/100 µl per well and incubated overnight. The cells were incubated with the FA-SS-PLGA-ENO1mAb. PLGA-ENO1mAb and 3-BrPA at 37°C for 24 h. The formulations were replaced with the DMEM containing MTT (5 mg/ml) and cells were then incubated at 37°C for 4 h. MTT was aspirated off and DMSO was added to dissolve the formazan crystals. Absorbance was measured at 490 nm using a microplate reader. Untreated cells were taken as a negative control with 100% viability and cells without addition of MTT were used as blank to calibrate the spectrophotometer to zero absorbance. Cell survival rate was calculated: cell survival rate = (experimental group OD-blank group OD)/(control group ODblank group OD) × 100%. The inhibitory effect on the proliferation of Hela cells was analyzed.

Experiment of plate cloning formation

Hela cells were plated at a density of 300 cells per well, 400 cells per well and 600 cells per well respectively in 6-well culture plates, and incubated overnight at 37°C with 5% CO. PLGA-ENO1mAb, FA-SS-PLGA-ENO1mAb and 3-BrPA were added, and a group without drugs was set up as negative control. The cells were cultured in a cell incubator at 37°C and 5% CO, for 2-3 weeks. When visible clones appeared in the culture plate, the cultivation process was terminated. The culture supernatant was discarded, the cells were fixed with methanol for 15 min, and the fixative solution was removed. Then 0.1% crystal violet dye was added for 10-20 min, and washed slowly with running water. After air-dried, the number of clones were counted by the naked eye, and the clone formation rate was calculated: clone formation rate = (number of clones/number of inoculated cells) × 100%.

Statistical analysis

All experiments were repeated at least three times. The experimental data were expressed as mean \pm SEM. Statistical analysis was performed using SPSS statistical package (SPSS 25.0). Differences between two groups were analyzed by Student's *t* test. The difference between three or more groups was analyzed by one-way ANOVA using SPSS13.0 software. A value of *P* < 0.05 was considered statistically significant.

Results

Preparation and purification of ENO1mAb

ENO1 protein was expressed in Sf9 insect cells by a baculovirus expression vector pFastBac1. BALB/c mice were immunized with purified ENO1 protein, and the monoclonal antibodies were prepared by hybridoma technology. Five positive hybridoma cell lines (H1-H5) with high ENO1mAb titer was successfully screened by traditional ELISA method. ENO1mAbs were purified by caprylic acid-ammonium sulfate and protein A chromatography. The results of SDS-PAGE showed that the molecular weight of the proteins were about 50 KDa and 25 KDa, which conformed to the size of the heavy and light chains of the antibody. The ENO1mAbs had high purity and could be further used in subsequent experiments.

Evaluation of the inhibitory effect of ENO1mAb on migration and invasion of cervical cancer cells

The inhibition effects of the five monoclonal antibodies on migration and invasion of tumor cells were initially evaluated using wound healing test and transwell chamber assay. The results showed that five ENO1mAbs all could inhibit the migration and invasion of cervical cancer SiHa cells. Among them, ENO1mAb-1 had the strongest inhibitory effect (**Figures 1** and **2**). Therefore, the ENO1mAb-1 was selected for further investigation.

Synthesis and characterization of PLGA copolymers

PLGA-Cys and FA-SS-PLGA copolymers were generated by two-step amidation reactions as shown in Figure 3. First, PLGA-Cys was synthesized through the amidation reaction between the carboxyl group of PLGA and the amino group of cysteine hydrochloride. Second, FA-SS-PLGA copolymer was synthesized by amidation reaction between the terminal carboxyl group of FA and the terminal amino group of PLGA-Cys. The infrared spectrum (Figure 4) and nuclear magnetic hydrogen spectrum (Figure 5) were used to detect the characterization of PLGA copolymers. In Figure 4, 1544.5 cm⁻¹ was the N-H bending vibration peak, 1673.8 cm⁻¹ was the C = O bending vibration peak, 1759.1 cm⁻¹ was the carbonyl peak, and 2998.3 cm⁻¹ and 2952.7 cm⁻¹ were the CH absorption peaks. In addition, 3415.3 cm⁻¹ was the N-H telescopic vibration peak. The absorption peaks of FA-SS-PLGA were shown in Figure 4C, in which 1542.8 cm⁻¹ was the N-H bending vibration peak, 1673.4 cm⁻¹ was the C = O bending vibration peak, 1758.8 cm⁻¹ was the carbonyl peak, and 2997.4 cm⁻¹ and 2950.3 cm⁻¹ were the CH absorption peaks. In addition, 3422.7 cm⁻¹ was the N-H stretching vibration peak, and 1607.9 cm⁻¹ was the characteristic absorption peak of folic acid benzene ring structure. The ¹H-NMR verification results of FA-SS-PLGA copolymer were shown in Figure 5, where 2.496 ppm was the solvent DMSO-d6 peak, and the characteristic proton peaks of PLGA were detected at 1.482 ppm, 4.870 ppm and 5.232 ppm (Figure 5A). The characteristic proton peaks of the disulfide bonding part in the copolymer structure were d (2.719 ppm) and e (2.878 ppm) (Figure 5B). In addition, the





Figure 1. Inhibitory Effects of ENO1mAbs on migration of SiHa cells. The effect of the five monoclonal antibodies (10 μ g/ml) on SiHa cells migration was evaluated by wound healing test. The results showed that ENO1mAb-1 and ENO1mAb-5 could inhibit the migration at 24 h. Mean ± SEM, n = 3, **P < 0.01.

chemical displacement of the disulfide bond parts in FA-SS-PLGA at 2.8-3.0 ppm, 3.126 ppm, 3.340 ppm, as well as the water absorption peak of solvent DMSO-d6 at 3.487 ppm were also detected (**Figure 5B**, **5C**). The characteristic proton peaks of the aromatic rings in the folic acid structure were shown as h (8.670 ppm), g (7.670 ppm) and f (6.657 ppm) (**Figure 5C**). In summary, the results of infrared spectrum and nuclear magnetic hydrogen spectrum all showed that the FA-SS-PLGA copolymers were successfully synthesized.

Characterization of ENO1mAb-loaded PLGA nanoparticles

ENO1mAb-loaded nanoparticles were prepared by conventional w/o/w double emulsion method. The size and morphology of particles were detected by dynamic light scattering (DLS) and scanning electron microscopy (SEM), respectively. The results showed that the blank PLGA

nanoparticles, PLGA-ENO1mAb nanoparticles and FA-SS-PLGA-EN01mAb nanoparticles were all round, with the average particle size being less than 200 nm (Figure 6). And the polydispersity index (PDI) was less than 0.25, presenting a narrow distribution. After ENO1mAb was coated, the Zeta potential of nanoparticles did not change significantly. The Zeta potential of nanoparticles modified with folic acid increased slightly, and remained negative (Table 1). As for PLGA-EN01mAb nanoparticles, the encapsulation rate and drug loading rate were 74.39 ± 1.54% and 8.86% respectively. As for FA-SS-PLGA-ENO1mAb nanoparticles, the encapsulation rate and drug loading rate were 70.48 ± 1.01% and 5.25% respectively.

Cell uptake and in vitro cytotoxicity of nanoparticles

To test the cell uptake efficacy of PLGA nanoparticles to human cervical cancer cells, the



Figure 2. The inhibitory effect of ENO1mAbs on invasion and migration of cervical cancer cells. The inhibition effects of the five monoclonal antibodies (10 μ g/ml) on invasion and migration of SiHa cells were evaluated using transwell chamber assay. A, B. The inhibition effects of the five monoclonal antibodies (10 μ g/ml) on invasion of SiHa cells. C. The inhibition effects of the five monoclonal antibodies (10 μ g/ml) on migration of SiHa cells. C. The inhibition effects of the five monoclonal antibodies (10 μ g/ml) on migration of SiHa cells. Mean ± SEM, *n* = 3, ***P* < 0.01.



Figure 3. Preparation of FA-SS-PLGA copolymer. In the presence of EDC and NHS, the copolymer was synthesized by two-step amidation reaction.



Figure 4. FT-IR spectra of PLGA copolymer. (A) PLGA. (B) PLGA-Cys. (C) FA-SS-PLGA. The 1544.5 cm⁻¹ is the N-H bending vibration peak; 1673.8 cm⁻¹ is the C = 0 bending vibration peak; 1759.1 cm⁻¹ is the carbonyl peak; 3415.3 cm⁻¹ is the N-H telescopic vibration peak; 1542.8 cm⁻¹ is the N-H bending vibration peak; 1673.4 cm⁻¹ is the C = 0 bending vibration peak; 1758.8 cm⁻¹ is the carbonyl peak; 3422.7 cm⁻¹ is the N-H stretching vibration peak; 1607.9 cm⁻¹ is the characteristic absorption peak of folic acid benzene ring structure.

nanoparticles were labeled with FITC, and the uptake of nanoparticles by Hela cells was verified by fluorescence microscope. The results showed that the group of PLGA nanoparticles modified with folic acid had stronger fluorescence in cytoplasm than that of PLGA nanoparticles (data not shown), which meant that folic acid modification could increase the uptake of nanoparticles, indicating that folate receptormediated endocytosis could enhance the uptake of nanoparticles by cervical cancer cells [41, 42].

The cytotoxicity of PLGA nanoparticles on Hela cells was evaluated by MTT assay. After coincubation of Hela cells with blank nanoparticles for 24 h, the PLGA nanoparticles and FA-SS-PLGA nanoparticles at the concentration of 50-400 μ g/ml showed no obvious cytotoxicity on Hela cells, and the cell survival rates were maintained at about 100%, which was consistent with the reports in the literatures. It suggests that both kinds of nanoparticles prepared in our study have a good biocompatibility.

Inhibitory effect of ENO1mAb NPs on glycolysis of Hela cells

Following ENO1mAb mediated by FA-SS-PLGA or PLGA nanoparticles entering cervical cancer cells, ENO1mAb is supposed to inhibit the activity of ENO1 in cytoplasm. We determined the effect of ENO1mAb nanoparticles on glycolysis in cervical cancer cell line Hela cells by detecting the metabolism products using glycolysis kits. 3-BrPA is an analog of pyruvic acid and inhibits key glycolytic enzymes including hexokinase II. 3-BrPA exhibits strong cytotoxic activity towards most cancer cells and maked as a promising candidate for anticancer therapy



Figure 5. ¹H-NMR spectra of PLGA copolymer. (A) PLGA. The characteristic proton peaks of PLGA in the copolymer are a (1.482 ppm), b (4.870 ppm) and c (5.232 ppm). (B) PLGA-Cys. d (2.8-3.0 ppm), e (3.126 ppm) and f (3.340 ppm) are the disulfide bond parts in the copolymer. (C) FA-SS-PLGA. The characteristic proton peaks of the aromatic rings in the folic acid structure are shown as h (8.670 ppm), g (7.670 ppm) and f (6.657 ppm). Spectrum A was obtained in CDCI3, Spectrum B and C was obtained in DMSO-d6.



Figure 6. SEM diagrams of FA-SS-PLGA-ENO1mAb NPs. A. SEM of PLGA NPs. B. SEM of PLGA-ENO1mAb NPs. C. SEM of FA-SS-PLGA-ENO1mAb NPs (5.0 kv, × 30000).

Table 1. The size	and Zeta potential o	of different nanopartic	cles (n
$=$ 3, mean \pm SD)			

Sample	Size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
PLGA	155.3 ± 0.93	0.098 ± 0.032	-14.85 ± 1.54
PLGA-ENO1mAb	150.77 ± 1.09	0.159 ± 0.037	-14.35 ± 2.49
FA-SS-PLGA-ENO1mAb	175.14 ± 4.29	0.244 ± 0.026	-11.56 ± 1.99

[43]. In this study, 3-BrPA was used as a positive control. After co-incubation for 24 h, the contents of pyruvate in the groups of FA-SS-PLGA-ENO1mAb, PLGA-ENO1mAb, and 3-BrPA were 0.145 \pm 0.031 µmol/mL, 0.22 \pm 0.07 µmol/mL and 0.27 \pm 0.07



Figure 7. Inhibitory effect of ENO1mAb NPs on glycolysis of Hela cells. Different ENO1mAb nanoparticles and 3-BrPA were co-incubated with Hela cells at 37 °C for 24 h, and the contents of pyruvate and lactic acid were determined by glycolysis kits. A. Pyruvate content. B. Lactic acid content. Mean \pm SEM, n = 3, *P < 0.05, **P < 0.01, ***P < 0.001.

µmol/mL, significantly lower than that of the control group (0.39 ± 0.02 µmol/L) (**Figure 7A**, P < 0.05). The contents of lactic acid in the groups of FA-SS-PLGA-EN01mAb, PLGA-EN01mAb, and 3-BrPA were 11.30 ± 1.13 mmol/gprot, 7.85 ± 0.84 mmol/gprot, and 12.19 ± 1.87 mmol/gprot respectively, significantly lower than that of the control group (16.83 ± 1.16 mmol/gprot) (**Figure 7B**, P < 0.05). These results indicated that EN01mAb had entered into the cytoplasm of Hela cells through the mediation of nanoparticles and obviously inhibited the glycolysis of cells.

ENO1mAb NPs inhibiting the proliferation of cervical cancer cells

At first, after free ENO1mAb at different concentrations (50-400 μ g/ml) incubated with Hela cells at 37°C for 24 h, the inhibitory effect of free ENO1mAbs on proliferation of Hela cells was evaluated by MTT assay. The results showed that the survival rates of Hela cells remained at 100% and did not change significantly (data not shown). It indicated that free ENO1mAb did not inhibit the proliferation of cervical cancer cells. The main reason was that ENO1mAb could not enter into cells freely.

Consequently, PLGA/FA-SS-PLGA nanoparticles were prepared and loaded with EN01mAb.

The proliferation of Hela cells after incubated with nanoparticles coating ENO1mAb were observed. As shown in Figure 8, compared with control, PLGA and FA-SS-PLGA groups, both PLGA-ENO1mAb and FA-SS-PLGA-ENO1mAb (50 µg/ml) could obviously inhibited the proliferation of Hela cells (Figure 8A, P < 0.01). Moreover, the viability of Hela cells treated with FA-SS-PLGA-ENO1mAb, PLGA-ENO1mAb gradually decreased along with the increasing concentrations of ENO1mAb (Figure 8B). The results also showed that the inhibitory effect of ENO1mAb loaded PLGA/FA-SS-PLGA nanoparticles on the proliferation of cervical cancer cells was weaker than that of the 3-BrPA (Figure **8A**, P < 0.001).

Inhibitory effect of ENO1mAb NPs on cell migration of cervical cancer cells

Accumulated evidences show that metabolism is a major driver of cancer metastasis. Once the glycolysis is inhibited by ENO1mAb-loaded NPs, it would lead to the reduction of extracellular acidification, which consequently has an impact on tumor cell invasion and migration [44]. Therefore, the effect of ENO1mAb nanoparticles on cell migration of Hela cells was evaluated *via* wound healing assay. The results showed that compared with the PBS control, PLGA-ENO1mAb nanoparticles, FA-SS-



Figure 8. The inhibitory effect of ENO1mAb NPs on the proliferation of Hela cells. Different ENO1mAb nanoparticles were co-incubated with Hela cells at 37 °C for 24 h, the cell viability was evaluated by MTT assay. A. The inhibitory effect of ENO1mAb nanoparticles (50 μ g/ml) on the proliferation of Hela cells. B. The inhibitory effect of ENO1mAb nanoparticles at different concentrations (50-400 μ g/ml) on the proliferation of Hela cells. Mean ± SEM, *n* = 3, ****P* < 0.001.



Figure 9. Inhibitory Effects of ENO1mAb loaded PLGA on migration of Hela cells. The effect of ENO1mAb nanoparticles on Hela cell migration at 12 h and 24 h was evaluated by wound healing test. Mean \pm SEM, n = 3, ***P < 0.01.

PLGA-ENO1mAb nanoparticles and 3-BrPA all inhibited the Hela cells migration ability at 24 h (Figure 9). The mean mobility of PLGA-EN-01mAb nanoparticles, FA-SS-PLGA-ENO1mAb nanoparticles and 3-BrPA group at 24 h were 31.84 \pm 3.51%, 24.04 \pm 5.08%, 29.55 \pm 2.12%, respectively, being significantly decreased compared with the PBS group (57.49 \pm 8.03%) (P < 0.001).

The effect of ENO1mAb-NPs on the colony forming ability of cervical cancer cells

To investigate whether inhibiting glycolysis of tumor cells would inhibit the colony forming ability of tumor cells, the effect of ENO1mAb on the colony forming ability of Hela cells was detected by plate clone formation experiment. The clone formation rates of FA-SS-PLGA-ENO1mAb, PLGA-ENO1mAb and 3-BrPA group were 21.56 \pm 2.06%, 19.67 \pm 3.02% and 24.88 \pm 1.53%, respectively, significantly lower compared with the PBS group (29.58 \pm 1.23%) (Figure 10, *P* < 0.05). The results showed that ENO1mAb-loaded nanoparticles had certain inhibitory effect on the colony forming ability ability of cervical cancer cells.

Discussion

Cervical cancer is one of the tumors with high incidence in women. In recent years, blocking energy metabolism of tumor cells is becoming a popular therapeutic idea. In our study, ENO1 protein was prepared from insect cells by baculovirus expression system. Using ENO1 protein



to immune mice, the ENO1mAbs with higher titer were successfully prepared by hybridoma technology. Through evaluation their ability to inhibit the migration and invasion of cervical cancer cells, the most effective ENO1mAb was screened. Furthermore, to introduce ENO1mAb entry into tumor cells, we modified PLGA nanoparticles with folate to construct the novel nanoparticle FA-SS-PLGA. The NPs were loaded with ENO1mAb and the therapeutic effects were investigated *in vitro*. Our studies found that ENO1mAb inhibited migration, glycolysis, proliferation and clone formation of cervical cancer cells.

The metabolism of solid tumor cells is known as the Warburg effect [45]. ENO1 is one of the speed limiting enzymes of glycolysis and closely related to the occurrence and development of various tumors [3, 16, 18]. Blocking energy metabolism of tumor cells by inhibiting its activity with ENO1mAb provides a new idea for antitumor therapy.

ENO1 is expressed in different parts of the cells and plays different roles. Studies have shown that ENO1 expressed in cytoplasm is mainly involved in the glycolysis pathway as a glycolytic rate-limiting enzyme. In this experiment, the free ENO1mAb failed to exert its inhibitory effect on glycolysis, and showing no inhibitory effect on cell proliferation, the reason is that ENO1mAb is a macromolecule drug and cannot cross the cell membrane barrier. PLGA-EN01mAb nanoparticles could enter Hela cells through endocytosis, and PLGA would be hydrolyzed in the cytoplasm and ENO1mAb released, which could inhibit glycolysis of Hela cells by inhibiting the activity of ENO1, and reduced the energy and intermediate products required for

cell proliferation, therefore inhibited cell proliferation. FA-SS-PLGA-EN01mAb nanoparticles could enter Hela cells through folate receptormediated endocytosis. Under the action of high concentration of reducing GSH in the cytoplasm, the disulphide bond in the copolymer broke, and ENO1mAb was released into the cytoplasm rapidly. In this study, there was no significant different in therapeutic effects observed between FA-SS-PLGA-ENO1mAb nanoparticle and PLGA-ENO1mAb nanoparticle. The main reason might be that the therapeutic effects were performed on tumor cell lines in vitro, in which all cells applied were tumor cells. Further investigations need to be done in vivo.

After the application of ENO1mAb, the contents of lactic acid and pyruvate of glycolysis products decreased significantly. It proved that ENO1mAb could inhibit the activity of glycolytic enzyme ENO1 directly and exert an inhibitory effect on glycolysis. Studies have found that overexpression of ENO1 could up-regulate the expression of glycolytic related gene LDHA by regulating the FAK/PI3K/AKT signaling pathway, promoting glycolysis, and consequently promoting the proliferation, invasion and metastasis of tumor cells [19]. It suggests that blocking ENO1 by monoclonal antibody could play a anti-tumor role.

Besides in cytoplasm, ENO1 is expressed on the cell membrane surface and acts as a plasminogen receptor and binds with plasminogen [11], which is then activated under the action of urokinase-type plasminogen activator, and promotes plasmin-mediated degradation of extracellular matrix barriers and contributes to cell adhesion and migration [46]. The wound healing test in our study showed that free ENO1mAb significantly reduced the migration of cervical cancer cells. It indicated that ENO1mAb blocked the activation of fibrinolytic enzyme by binding to the ENO1 receptor on the cell membrane, and thus inhibited the migration of tumor cells. Epithelial to mesenchymal transition (EMT) and tumor cell migration, invasion and metastasis are closely related. Some studies showed that knockdown of ENO1 resulted in the restoration of E-cadherin expression, which consequently suppressed EMT, and promoted tumor cell migration and invasion [18]. It suggests that ENO1mAb might also inhibit Hela cells migration by inhibiting EMT.

It is found that most tumor stem cells, such as colon cancer stem cells and breast cancer stem cells, show an enhanced Warburg effect to promote their growth and maintain stemness [47-49]. It is reported that glucose uptake, lactic acid production, glycolytic enzyme expression and ATP content in tumor stem cells increased significantly, while inhibition of glycolysis or glucose deprivation led to a decrease in Cancer stem cell (CSCs) population [50]. Our work showed that the colony forming ability in PLGA-ENO1mAb nanoparticle and the 3-BrPA groups were significantly lower than that in PBS control group, and they both could inhibit the clone formation of Hela cells.

ENO1 also plays a role as c-Myc promoter-binding protein and regulates the development of tumors [14-16]. It was reported that ENO1 functioned as an oncogene in bladder cancer via regulating cell cycle and apoptosis [51]. Some studies also found that silencing of ENO1 could induce apoptosis and inhibit the proliferation and clone formation of breast cancer cells [52] and lung adenocarcinoma cells [53]. These data suggested that ENO1mAb might inhibit the proliferation of cervical cancer cells via the induction of apoptosis. The mechanism of ENO1mAb inhibiting the clone formation, proliferation of cervical cancer stem cells remains to be further explored.

In summary, we developed an effective ENO1mAb, which blocked the plasminogen receptor highly expressed in cell membrane of cervical cancer cells and inhibited the migration and invasion of tumor cells. Furthermore, PLGA nanoparticles could mediate ENO1mAb entry into cytoplasm and inhibit the glycolysis, contributing to reducing proliferation and clonal formation of cervical cancer cells. The therapeutic effects of ENO1mAb against tumor *in vivo* will be further studied.

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Disclosure of conflict of interest

None.

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