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Polybutylcyanoacrylate nanoparticles loaded with antisense oligodeoxynucleotide of hTERT inhibit glioma growth.

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Abstract

Objective: To prepare hTERT antisense oligodeoxynucleotide (ASODN) loaded polybutylcyanoacrylate nanoparticles (PBCA-NP) and evaluate the efficacy to inhibit glioma growth *in vitro*.
Methods: hTERT ASODN-PBCA-NP was prepared using the emulsion polymerization method. The size and morphology were observed by transmission electron microscopy. The drug loading and entrapment efficiency of ASODN in the nanoparticles were measured by UV spectra. The viability of SHG44 glioma cells was detected by MTT assay, cell cycle was determined by flow cytometry. The expression of hTERT mRNA was measured by RT-PCR, telomerase protein expression was measured by immunocytochemical analysis.
Results: The nanoparticles were discrete and uniform spheres with average diameter of 120 nm, with drug loading and entrapment efficiency of 71.17% and 95.20%, respectively. Zeta potential was +41.3 mV. SHG44 cells treated by ASODN-NP showed reduced hTERT mRNA and reduced telomerase protein expression levels, reduced cell viability, increased percentages of cells at G0/G1 and G2/M phases and decreased percentages of cells at S phase compared to control groups.
Conclusion: PBCA nanoparticles loaded with antisense oligodeoxynucleotide of hTERT can inhibit the expression of hTERT and inhibit the viability of glioma cells.

Keywords: hTERT, Polybutylcyanoacrylate, Nanoparticles, Antisense oligodeoxynucletide, Emulsion polymerization.

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Introduction

Glioma is the most common aggressive primary malignant brain tumors. Although significant advances have been made by neurosurgical operative techniques, adjuvant chemotherapy and radiotherapy, the prognosis of glioma remains unfavourable and median survival is within 2 years. The blood-brain barrier (BBB) consists of endothelial cells which are connected by tight junction, and many enzymes, receptors, transporters and efflux pumps of multidrug resistance pathways. As a result, most chemotherapy agents are unable to reach the glioma in therapeutic concentrations [1]. Nanoparticles made by poly (butyl cyanoacrylate) (PBCA) or poly (lactic-co-glycolicacid) (PLGA) with surface modified by polysorbate 80 enable the transport of chemotherapeutic agents such as doxorubicin across BBB and enter the tumor [2]. After intravenous injection of nanoparticles loading doxorubicin to intracranial glioma bearing rats, the life span of the animals significantly increased. Moreover, these particles considerably reduced the Dox induced cardiotoxicity and testicular toxicity.

It has been found that more than 50% gliomas have increased telomerase activity, and the expression of telomerase is correlated with the grade of malignancy [3]. The level of hTERT mRNA is predictive of glioma patient survival. Antisense oligodeoxynucleotide (ASODN) could inhibit or block the expression of targeting gene, resulting to the loss of activity. It is difficult for ASODN to penetrate biomembrane as a hydrophilic anionic polymer, which can be biodegraded by enzymes *in vivo*, and its low biostability limited the applications. PBCA nanoparticles are considered to have relatively low toxicity, moreover, it is among the most rapidly biodegrading synthetic polymers [4]. In this study, we prepared hTERT ASDO loaded PBCA nanoparticles (NP) by emulsion polymerization method and evaluated the efficacy to inhibit glioma *in vitro*.

Material and Methods

Preparation of PBCA-NP and SODN-NP/ASODN-NP

The PBCA-NP was prepared using the emulsion polymerization method as described previously [5]. Briefly, 0.12 g DEAE-Dextran and 0.18 g Dextran70 were dissolved in 25 ml hydrochloric acid solution, and pH is 2.0. 0.19 ml

BCA monomer was slowly dropped into the mixed solution and continued stirring for 6 h, and then filtered by a sintered glass funnel with diameter 0.45 μm. 1 mol/L NaOH was added into such solution until pH reached 7.0 to get PBCA-NP. A certain amount of sense or antisense oligonucleotides was added into the above nanoparticle fluid and continue to stir for 3 h to get SODN-NP/ASODN-NP. hTERT ASODN was designed according to the sequences of hTERT mRNA (AF015950), the sequences were as follows: ASODN1 5'-GCA CGG CTC GGC AGC GGGA-3'; ASODN2 5'-GGAGCG CGC GGC ATC GCGGG-3'; ASODN3 5'-GGT AGA GAC GTG GCT CTGA-3; Sequence of 5' end FITC labeled antisense oligonucleotides (FASODN) was same as ASODN1; Antisense oligonucleotides sequence (SODN) was designed as control: 5'-CCC GCG ATG CCGCGC GCT CC-3'.

Characterization of NP and SODN-NP/ASODN-N-NP

The NP or SODN-NP/ASODN-N-NP was diluted with distilled water and measured by Laser diffraction particle size analyzer (Mastersizer 3000HS) to assess size and size distribution of nanoparticles. The NP or Dox-NP Suspension was diluted appropriately and dropped on the carbon film coated copper net, stained by 2% phosphate tungsten, dried naturally for 30 min, then observed by Transmission electron microscope (TEM, JEM-100CX, Japan) for its shape and size. The freeze dried SODN-NP/ASODN-N-NP powder which was kept for three months under normal temperature was dissolved by distilled water to detect its stability.

The Drug loading and entrapment efficiency of ODN in the nanoparticles were measured by means of UV spectra at weave length of 260 nm. ODN standard solution was prepared with the concentration gradient of 0.25, 0.5, 0.75, 1.0, 1.25, 2.5, 5.0 and 10 μg/ml. Then 5 ml ODN-PBCA-NP emulsion was centrifuged by speeding cold Freeze (LABCONCO Free Zone 12 L freeze dryer, USA) at 20,000 r/min for 30 min, then the supernatant was taken and the absorbance was measured at the wavelength of 481 nm by UV Spectrophotometry. The drug loading ratio (DL) and encapsulation efficiency (EE) were calculated as follows [6]: $DL\%=ODN_{total}-ODN_{supernatant}/BCA \times 100\%$; $EE=ODN_{total}-ODN_{supernatant}/ODN_{total} \times 100\%$.

NP-ODN was suspended in a dialysis system and the released ODN was detected by UV Spectrophotometry.

In vitro cytotoxicity assay

SHG44 glioma cells were seeded into 96-well culture plates at a density of 5×10^4 cells/well and maintained in 100 μL of DMEM medium supplemented with 10% FBS at 37°C in 5% CO_2 for 24 h. After exposure to different NP formulations (ASODN1-3, ASODN1-3-NP, SODN, NP and DMEM) or untreated as control for 24 h at 37, cell viability was evaluated by MTT assay (Yes Service Biotech, Inc.), and the absorbance was read on a micro-plate reader at 540 nm. The survival percentages were calculated as: Survival %=(A540 nm for treated cells/A540 nm for control cells) × 100%. Each assay was conducted in triplicate. Finally, concentration-viability curves were made and IC_{50} values were calculated.

Analysis of cell cycle

SHG44 glioma cells were seeded into 6-well culture plates at a density of 1×10^5 cells/well and cultured in DMEM medium supplemented with 10% FBS at 37°C in 5% CO_2 for 24 h. The cells were treated with SODN-NP, ASODN or ASODN-NP for 48 h. The cells were collected and washed by PBS, and then cell cycle distribution was analyzed by flow cytometry.

RT-PCR

Total RNA was extracted from different groups of SHG44 cells using TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol. cDNA was synthesized by reverse transcription using RT kit (Promega, Madsion, WI, USA) following the manufacturer's protocol. PCR was performed using the primers as follows: hTERT 5'-GTTTGGAAGAACCCCACAT-TT-3, and 5'-CGAGTCAGCTTGAGCAGGAAT-3'; β-actin 5'-TGACGTGGACTCCGCAAAG-3' and 5'-CTGGAAGGTGGACAGCGAGG-3'. The amplification condition was as follows: 94°C for 4 min; 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec (35 cycles). The relative mRNA levels of hTERT were normalized to GAPDH and calculated by 2-ΔΔCt method.

Immunocytochemical analysis

SHG44 cells were treated by different NP. The cells were fixed by 3% paraformaldehyde for 30 min and washed by PBS twice. The telomerase protein was detected by EnVision Kit (DAKO) according to the manufacturer's instructions.

Data analysis

Data analysis was performed by using SPSS 12.0 software. The results were expressed as means ± standard error (SE). ANOVA was used for the comparison among the groups. Statistical significance was set at $P<0.05$.

Results

Characterization of NP and SODN-NP/ASODN-N-NP

The nanoparticles were discrete and uniform spheres with average diameter of 120 nm (Figure 1A). Its size distribution was narrow. Its drug loading and entrapment efficiency for ASODN was 71.17% and 95.20%, respectively, and its Zeta potential was +41.3 mV.

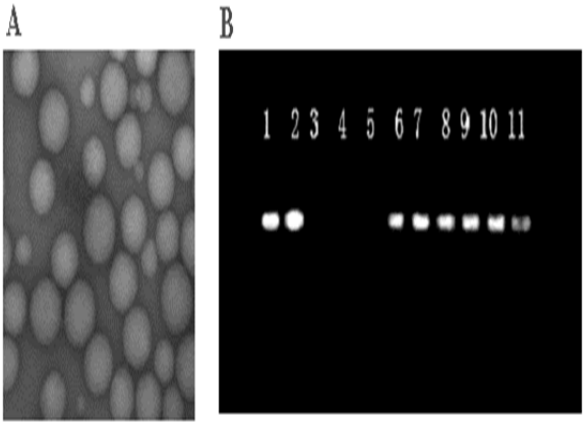

Figure 1. A. *TEM showed that PBCA-NP was spherical with average diameter of 120 nm. (× 50,000).* ***B.*** *Electrophoresis, 1-2 without restriction digestion of free oligonucleotide, 3-5 oligonucleotide without NP, 6-11 oligonucleotide encapsulated by PBCA-NP.*

Agarose gel electrophoresis showed that the control sample without restriction in the positive direction were free ODN, but we found no band in the positive direction of strip for ODN-NP, which indicated the protection effect of nanoparticles for oligonucleotide (Figure 1B).

Cumulative release of ASODN from ASODN-NP was analyzed by drawing release curve. As shown in Figure 2, ASODN release increased with the increase of the time. Furthermore, the freeze dried SODN-NP/ASODN-NP powder were kept for three months under normal temperature and then dissolved in distilled water. The drug loading ratio (DL) and encapsulation efficiency (EE) were calculated, and they showed no significant difference compared to those of freshly prepared SODN-NP/ASODN-NP, indicating the stability of ASODN-NP.

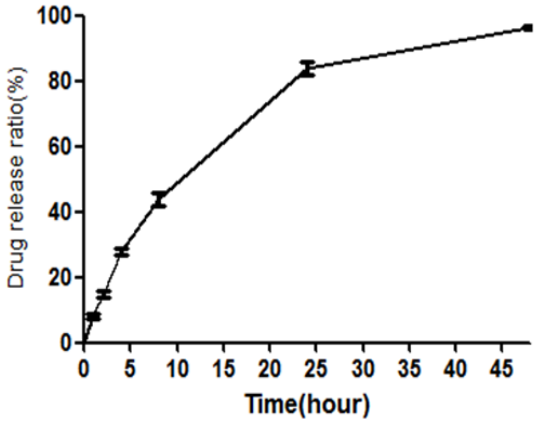

Figure 2. *The release of ASODN from ASODN-PBCA-NP. 5 ml of ASODN-PBCA-NP were kept at room temperature, stirred for 1, 2, 4, 8, 24 and 48 hours at 20,000 r/min, and then the content of ASODN in the supernatant was determined by HPLC, respectively.*

ASODN-NP inhibited SHG44 cell viability

MTT assay showed that 48 hours after transfection, there was no significant difference in SHG44 viability among NP, SODN-NP and ASODN groups. Compared with control group, ASODN-NP exhibited significant inhibitory effect on SHG44 cell viability ($P<0.01$).

ASODN-NP inhibited SHG44 cell cycle progression

Flow cytometry analysis showed that 48 hours after transfection, there was no significant difference in cell cycle progression among NP, SODN-NP and ASODN group. However, the percentages of cells at G0/G1 and G2/M phases increased significantly while the percentages of cells at S phase decreased significantly in ASODN-NP group compared with other groups ($P<0.01$, Table 1).

Table 1. *Flow cytometry analysis of cell cycle in each group.*

Group Percentage of cells in each phase (%)		
G0/G1	S	G2/M
Control (DMEM) 34.2 ± 1.4	60.1 ± 0.3	5.6 ± 0.1
NP 35.3 ± 0.8	57.7 ± 1.6	6.9 ± 0.3
SODN-NP 33.7 ± 1.6	61.4 ± 1.7	4.8 ± 0.5
ASODN1 36.4 ± 0.3	56.3 ± 0.8	7.1 ± 0.4
ASODN2 33.8 ± 1.2	62.2 ± 1.1	3.3±0.2
ASODN3 36.9 ±1.9	55.8 ± 0.7	6.9 ± 0.6
ASODN1-NP 62.8 ± 0.5	24.5 ± 0.6	12.5 ± 0.7
ASODN2-NP 60.6 ± 2.2	27.3 ± 2.1	12.3 ± 0.3
ASODN3-NP 63.4 ± 1.1	25.7 ± 1.4	10.1 ± 0.2

ASODN-NP inhibited hTERT mRNA expression in SHG44 cells

The level of hTERT mRNA in control group, NP group, SODN group, SODN-NP and ASODN-NP1-3 groups were 2.23 ± 0.12, 2.31 ± 0.14, 2.26 ± 0.19, 2.33 ± 0.16, 1.15 ± 0.13, 1.17 ± 0.21 and 1.08 ± 0.32 respectively. hTERT mRNA expression in SHG44 cells treated by ASODN-NP1-3 was significantly lower than that in control groups ($P<0.05$).

ASODN-NP inhibited telomerase protein expression in SHG44 cells

Telomerase protein expression was detected by immunocytochemical analysis. The results showed that the positive ratio of blank control group, NP group and SODN-NP were 94.6 ± 1.3%, 93.2 ± 3.1% and 93.7 ± 2.6%, respectively, showing no significant difference among three groups ($P>0.05$). However, positive ratio of ASODN1-NP, ASODN2-NP and ASODN3-NP group was 53.1 ± 1.8%, 56.7 ± 1.5% and 59.6 ± 4.3%, respectively, showing significant difference compared with blank control group ($P<0.05$).

Discussion

Telomerase is a reverse transcriptase enzyme which maintains telomeres by adding telomeric TTAGGG repeats to the ends of chromosomes [7]. High expression of telomerase is believed to be an essential step during malignant tumor progression [8,9]. Furthermore, high telomerase activity is usually associated with high tumor aggressiveness. It has been found that more than 50% gliomas have high telomerase activity, and the level of hTERT mRNA was predictive of glioma patient survival. Antisense treatment induced a decrease in hTERT mRNA expression, telomerase activity, cell viability, and an increase in apoptosis [10]. However, antisense oligonucleotide is difficult to penetrate cell membranes and blood-brain barrier, and prone to be degraded by endonuclease in the blood or CSF, which limits the use of antisense oligonucleotides in the clinic.

In this study, ASODN encapsulated in PBCA-NP was protected from the degradation by the nuclease. Maksimenko et al. reported that antisense oligonucleotides against EWS-Fli-1 oncogene inhibited the growth of EWS-Fli-1 dependent Ewing's tumor grafted to nude mice with high specificity after being encapsulated by nanocapsules or nanospheres [11]. The main advantages of using nanoparticles for brain drug targeting include their ability to deliver drugs to the brain without changing their original characteristics, decrease drug escape in the brain and reduce peripheral toxicity. It was found that oligonucleotides encapsulated into polyisohexylcyanoacrylate nanoparticles are protected from nuclease attack and increase cellular uptake of oligonucleotides by an endocytic/phagocytic pathway [12].

In this study, after being encapsulated into nanoparticles, the stability of ASODN increased significantly, which was confirmed by the electrophoretic mobility shift assay. After SHG44 glioma cells were treated with ASODN-NP, we found reduced hTERT mRNA and reduced telomerase protein expression levels, reduced cell viability, increased percentages of cells at G0/G1 and G2/M phases and decreased percentages of cells at S phase compared to control groups.

In conclusion, nanoparticles obtained by emulsion polymerization method are steady with high drug loading and entrapment efficiency of oligodeoxynucletide. Polybutylcyanoacrylate nanoparticles loaded with antisense oligodeoxynucleotide of hTERT can enter into glioma cells, block the expression of hTERT, and inhibit cell viability. Thus they are promising agent for the treatment of glioma.

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