On the preparation of transferrin modified artesunate nanoliposomes and their glioma-targeting treatment in-vitro and in-vivo

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Abstract: Objective: To prepare transferrin modified artesunate nanoliposomes (Tf-ART-LPs) and study their glioma U87 cells-targeting treatment in-vitro and in-vivo. Methods: Ammonium sulfate transmembrane gradient method was used to prepare Tf-ART-LPs, whose size and stability was detected by a Nanosizer. Besides, the encapsulation efficiency and release rate of artesunate (ART) were tested by a ultraviolet spectrophotometer. Further, isothiocyanate (FITC) was used to label nanoliposomes and the cell-targeting property of Tf-ART-LP in-vitro was observed under a fluorescence microscope. In addition, CCK-8 method was used to detect the effect of single nanoliposomes and Tf-ART-LPs on the viability of glioma U87 cells. At last, a subcutaneously implanted tumor model in nude mouse was established for studying the in-vivo anti-tumor effect of Tf-ART-LPs by caudal vein injection. The tumor volume and mice weight were monitored and pathological sections of their major organs were analyzed. Results: Tf-ART-LPs were spherical with an average diameter of 94.2 nm. They showed no aggregation after being stored in a refrigerator for 14 days at 4 °C. The encapsulation efficiency and highest releasing rate (48 hours after being placed in normal saline under 37 °C) of ART was 85.9% and 58.7±2.9%, respectively. The uptake rate of U87 cells was 59.8±3.8% for Tf-ART-LPs and only 18.7±4.5% for ART-LPs. While single liposomes almost showed no toxicity, Tf-ART-LP had a concentration-dependent killing effect on U87 cells. Within 32 days of treatment, the growth of U87 cells was well inhibited by Tf-ART-LPs without significant toxicity. Conclusion: In this study, transferrin modified artesunate liposomes were prepared have a good targeting property to glioma U87 cells and good effect on glioma both in-vitro and in-vivo.

Keywords: Artesunate, transferrin, nanoliposome, ammonium sulfate transmembrane gradient method, targeting

Artesunate (ART) is one of the major derivatives of artemisinin with the structure of peroxide bridge. It was firstly isolated from Artemisia annua. feverfew by Chinese scientists in 1972. Being highly efficient in antimalaric treatment, it has been widely used for treating malignant warts, cerebral blisters and other chloroquine-resistant diseases [1, 2]. In recent years, investigators have found that ART is of good in-vitro anti-tumor effect, which is increasingly valued [3, 4]. It is reported that artemisinin-based drugs have many strengths in anti-tumor treatment, including its selective inhibiting effect on multiple tumor cells, good tolerance and little toxicity [5]. Although the anti-tumor effect of artemisinin derivatives has been recognized internationally, its clinical application is restricted because of poor compliance of patients due to its high frequency of administration required (since it both takes effect and is eliminated quickly, the effective half-life is only 30 minutes) [6]. Therefore, researches on sustained release preparation of artesunate are particularly important for its clinical use.

Nanoliposomes are drug carriers with cell-like structure. Drugs, once being encapsulated by liposomes, will be characterized by the following properties. Firstly, they have a certain passive targeting property. Besides, they can be modified by targeting molecules on the surface of liposomes, which can interact specifically with complementary molecules on the surface of target cells, like receptors, via ligand mole-
Transferrin modified artesunate nanoliposomes for neuroglioma targeting and therapy

cules in vivo. In this way, they can release drugs in target regions. Secondly, they have a long-term effect. Since drugs are encapsulated in liposomes, renal excretion and metabolism will be reduced. Thus, the residence time of drugs in blood will be prolonged and drugs can then be released slowly within human body, lengthening their acting time. Thirdly, they have tissue and cell compatibility. Since liposomes are vesicles resembling the structure of biological membrane, they are of good cell affinity and histocompatibility [7, 8]. Hereby, nanoliposomes are the first choice to be used as carriers for ART. Jin Meihua et al. found out that ART encapsulated in liposomes was of good killing effect on human hepatoma cells HepG2 [9]. However, the active targeting property of ART has rarely been investigated. Furthermore, whether it is effective for more tumor cells is still unknown.

In this study, ART was enveloped by nanoliposomes, the surface of which was modified by a targeting protein-transferrin. This protein can specifically bind with transferrin receptors highly expressed on the surface of tumor cells. In this way, anti-tumor drugs can then be transferred to the tumor site specifically, achieving an active targeting effect [10, 11]. By using glioma U87 cells as the object of study, we explored the anti-tumor effect of artesunate nanoliposomes both in-vitro and in-vivo.

Materials and methods

Major reagents

Artesunate (ART, CAS: 88495-63-0), the crude drug, was purchase from Wuhan Dong Kangyuan Technology Co., Ltd. (≥99.5% [HPLC]), lecithin, cholesterol, DSPE-PEG_{2000} and fluorescein isothiocyanate (FITC) from Sigam (US), MEM culture medium, trypsin, fetal calf serum and phosphate buffer (PBS) from Gibco (US), penicillin-streptomycin mixed solution (100× double antibody) from Beijing Leagene Biotech. Co., Ltd., Cell Counting Kit (CCK-8) from YEASEN (Shanghai) and transferring (Tf) from Invitrogen (US).

Main instruments

Cell incubator HERAcell240i was purchased from Thermo (US), automatic inverted fluorescence microscope Axio Observer Z1 from Carl Zeiss AG (German), rotary evaporator YRE-2020Z and lyophilizer LGJ-1 from Henan Yuhua Instruments Co., Ltd., microplate reader MLDEL680 from Bio-Rad (US) and laser particle size analyzer Nanosizer from Malvern Instruments Ltd.

Cell lines and cell culture

U-87 cells were star-shaped human brain glioblastoma cells (hereafter referred to as glioma cells in short). Epithelioid cells were obtained from the cell resource center of Shanghai Institutes for Biological Sciences, Chinese Academy of Science. These cells were cultured in a MEM culture medium with 10% fetal calf serum and 1% double antibody in an incubator under 37°C.

Laboratory animals

4-6 weeks old BALB/c-nu nude mice (half males and half females) with their body weight ranging from 20 to 25 g were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. The test began after they were fed on SPF for a week.

Test methods

The preparation of Tf-ART-LPs: In this study, ammonium sulfate transmembrane gradient method was adopted to prepare Tf-ART-LP according to the following three steps. Step one: A prescribed mixture of lecithin, cholesterol and DSPE-PEG_{2000} with mass ratio of 60:20:3 was dissolved in chloroform; The resultant substance was then placed in a rotary evaporator and was evaporated at 50°C until film formation was observed; Afterwards, chloroform was removed by reduced pressure evaporation, followed by the addition of 300 mM ammonium sulfate solution to hydrate properly; The resultant milk white suspension was crushed by an ultrasonic cell crusher for 10 mins and the blank liposome suspension was obtained. Step two: Excessive ART was added into the liposome obtained; The mixture was then heated to 50°C until film formation was observed; Afterwards, chloroform was removed by reduced pressure evaporation, followed by the addition of 300 mM ammonium sulfate solution to hydrate properly; The resultant milk white suspension was crushed by an ultrasonic cell crusher for 10 mins and the blank liposome suspension was obtained. Step three: Sulfated transferrin was added into ART liposomes; The resultant mixture was reacted overnight at room temperature; Then, free transferrins were removed by Sephadex CL-48
Transferrin modified artesunate nanoliposomes for neuroglioma targeting and therapy

gel-filtration; Tf-ART-LPs were thus obtained and stored at 4°C.

Characterization of Tf-ART-LPs: Particle size distribution and stability of Tf-ART-LPs were detected by a Nanosizer. Their encapsulation efficiency was measured as follows. A certain amount of liposome solution was put into a dialysis bag. The bag was then immersed in PBS buffer solution. After magnetic stirring for 3 hs, the absorbance at 238 nm was detected with an ultraviolet spectrophotometer. The encapsulation efficiency was then calculated according to ambert-beer’s law. The release rate of ART in normal saline was detected by the same method.

Test on the targeting property of Tf-ART-LPs: ART-LPs and Tf-ART-LPs, marked by FITC, were incubated with glioma U87 cells for 3 h. After extracellular liposomes were washed away by PBS, they were imaged under a fluorescence microscope. The excitation wavelength was 488 nm.

Cytotoxicity test: First of all, the impact of blank LPS and Tf-LPs on cell viability was detected. Cells in logarithmic phase were transferred into a 96-well plate. After 24 hours, 0-100 µg/ml LPS and Tf-LPs were added and the mixture was incubated for another 24 hours. Then, 10% CCK-8 culture solution was added and the resultant solution was incubated for 30 mins. Finally, it was placed into a microplate reader to detect the absorbance at 450 nm (OD450 nm). Next, the same method was used to detect the impact of ART-LPs and Tf-ART-LPs on the viability of U87 cells.

The establishment of a subcutaneously implanted tumor model in nude mouse with glioma and its in-vivo treatment: U87 cells in logarithmic phase collected were prepared as single-cell suspension (10⁷ cells/ml). For each nude mouse, 200 ul single-cell suspension was injected subcutaneously at the right lower back. The condition of these mice was observed every day. Ever four days, tumor’s major axis (a) and minor axis (b) was measured with a vernier caliper. Tumor volume (V) was then calculated according to the following equation: V=axb²/2. At the same time, their body weight was also weighed. When their tumor volume became about 300 mm³, these mice were randomized into five groups with 8 mice in each group, which were normal saline, Tf-LP, ART, ART-LP and Tf-ART-LP group, respectively. All reagents
Transferrin modified artesunate nanoliposomes for neuroglioma targeting and therapy

were administered via caudal vein. According to tumor volume, a relative growth curve was drawn. After one month’s treatment, these mice were euthanized. Pathological changes of major organs like heart, liver, spleen, lung and kidney collected were observed by HE staining.

**Statistical analysis**

Measurement data were expressed by mean ± SD. Comparison results between two samples were expressed by LSD test. P<0.05 indicated that there was statistically significant difference, while P<0.01 suggested that there was statistically extremely significant difference. All statistical analyses were completed by the statistical software SPSS17.0.

**Results**

**Characterization of Tf-ART-LPs**

As shown in Figure 1, the diameter of Tf-ART-LPs ranged from 84.5 nm to 100 nm, with a mean diameter of 94.2 nm. Tf-ART-LPs were placed at room temperature for 14 days and their average diameter was measured on day 0, 1, 3, 5, 7, 9 and 14, respectively. The results presented in Figure 2 showed that the average diameter of Tf-ART-LPs was about 94 nm. It indicated that no aggregation happened to Tf-ART-LPs during a certain period of time. Tf-ART-LPs were of good stability. The encapsulation efficiency of ART was found to be 85.9%. In normal saline at 37°C, ART could be released from liposomes slowly. The highest release rate was 58.7±2.9% after 48 hs (Figure 3).

**Test on the targeting property of Tf-ART-LPs**

ART-LPs and Tf-ART-LPs marked by FITC were incubated with U87 cells at 37°C for 3 h, re-
Transferrin modified artesunate nanoliposomes for neuroglioma targeting and therapy

spectively. Their fluorescent microphotograph and statistical results were shown in Figures 4 and 5. These figures suggested that Tf-ART-LPs were uptaken by U87 cells significantly. The uptake rate reached up to 59.8±3.8%. By contrast, fewer ART-LPs were uptaken and fluorescence positive cells accounted for only 18.7±4.5%. These results showed that the modification of Tf markedly increased the affinity of liposomes for U87 cells.

The effect of Tf-ART-LPs for treating tumors in-vitro

First of all, the impact of blank LPs and Tf-LPs on cell viability was detected. As shown in Figures 6, 7, no reduction of cell viability was observed after 0-100 µg/ml LPs and Tf-LPs were incubated with cells for 24 hours. It proved that blank lipid carriers almost had no toxicity. However, when ART was encapsulated, a concentration-dependent reduction of cell viability was observed after cells were treated with 0-40 µg/ml ART-LPs (P<0.05). Compared with ART-LPs of the same concentration, Tf-ART-LPs modified by Tf had a better killing effect. These results showed that Tf-ART-LPs could transport more ART actively into cells and thus took effect.

The in-vivo study on the therapeutic effect of Tf-ART-LPs

A subcutaneously implanted tumor model of human glioma cells in nude mouse was established and the tumor formation rate reached up to 100%. Figure 8 showed the ratio of tumor volume after and before administration. Four
days after administration, tumor volume of mice in the normal saline, Tf-LP, ART and ART-LP groups tended to increase gradually, while that of Tf-ART-LP group decreased instead. As time went by, tumor volume of mice in the normal saline, Tf-LP, ART and ART-LP groups further increased day by day, whereas that of Tf-ART-LP group was inhibited or even eliminated. These results showed that Tf-ART-LPs, after entering into mouse body, could enable more ART to target tumor and give full play of their anti-tumor effect. As shown in Figure 9, no obvious reduction of body weight was observed during administration, which suggested that all drugs had no evident toxicity. Photographs of pathological sections of major organs by HE staining were presented in Figure 10. No significant damage was found after caudal injection of LPs and ART-LPs.

Discussions

Human glioma is the most common intracranial primary malignant tumor with high mortality rate. Glioma has an infiltrative growth and ill-defined site compared with surrounding normal brain tissues. It is hard to be removed thoroughly by surgery and tends to have postoperative residual. Further, it is insensitive to radiotherapy. Thus, the prognosis of patients with malignant glioma is very poor. The recurrence rate is very high even after comprehensive treatments like combined radiotherapy after operation [12]. Moreover, chemotherapy, due
Transferrin modified artesunate nanoliposomes for neuroglioma targeting and therapy

to a lack of good targeting property, can not achieve good therapeutic effect owing to low local drug concentration. Besides, high concentration drugs have higher toxicity. Therefore, it is imperative to find out a new treatment method. Nanoliposomes, as a new type of drug carrier, can be released slowly as well as reduce drug dosage and adverse reactions. Furthermore, they are of no toxicity and immunosuppressive effect on human body. Thus, they can be used as carriers of anti-tumor drugs to prolong the circulation time of drugs in blood and enable drugs to aggregate at tumor sites to take effect [13, 14].

Transferrin is a member of the family of iron-binding proteins. Transferrin receptors are essential for cell proliferation. They are over-expressed on the surface of malignant tumor cells, including glioma. In normal tissue cells, their expression level is extremely low. Thus, it has always been considered as an ideal therapeutic target [15, 16]. Transcytosis mediated by transferrin has been proved to be able to transport across the blood brain barrier, so nano-carriers coupled by transferrin has a potential glioma targeting property. Hereby, transferrin is believed to be a very potential molecular therapeutic target for treating glioma [17, 18].

In this study, an active targeting nano-scale anti-tumor drug was prepared by using liposomes modified with transferrin as the carrier of artesunate. With fluorescence microscopy, it was observed that artesunate nanoliposomes modified with transferrin (with an average diameter of 95 nm) could target glioma U87 cells effectively and enter into cytoplasm. The results of an in-vitro test on cell viability indicated that artesunate nanoliposomes modified with transferrin had a better killing effect compared with those without modification of transferrin. This should be the result of active targeting of transferrin, which transported artesunate into cells efficiently and took effect. The anti-tumor effect in-vivo also indicated their good tumor inhibitive effect.

In conclusion, we prepared spherical artesunate nanoliposomes modified with transferrin in this study. With good active targeting property and anti-tumor effect both in-vitro and in-vivo, they will be a good alternative for treating human glioma.

Disclosure of conflict of interest

None.

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References

Transferrin modified artesunate nanoliposomes for neuroglioma targeting and therapy


