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Focused ultrasound and interleukin-4 receptor-targeted liposomal doxorubicin for enhanced targeted drug delivery and antitumor effect in glioblastoma multiforme

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ABSTRACT

The clinical application of chemotherapy to brain tumors has been severely limited because the blood–brain barrier (BBB) often prevents therapeutic levels from being achieved. Here we show that pulsed HIFU and human atherosclerotic plaque-specific peptide-1 (AP-1)-conjugated liposomes containing doxorubicin (AP-1 Lipo-Dox) act synergistically in an experimental brain tumor model. We developed an intracranial brain-tumor model in NOD-*scid* mice using human brain glioblastoma multiforme (GBM) 8401 cells. Pulsed HIFU was used to transcranially disrupt the BBB in these mouse brains by delivering ultrasound waves in the presence of microbubbles. Prior to each sonication, AP-1 Lipo-Dox or unconjugated Lipo-Dox was administered intravenously, and the concentration in the brains was quantified by fluorometer. Compared to control animals treated with injections of AP-1 Lipo-Dox or unconjugated Lipo-Dox, animals receiving the drug followed by pulsed HIFU exhibited enhanced accumulation of the drug in tumor cells. Drug injection with sonication increased the tumor-to-normal brain doxorubicin ratio of the target tumors by about twofold compared with the control tumors. Moreover, the tumor-to-normal brain ratio was highest after the injection of AP-1 Lipo-Dox with sonication. Combining sonication with AP-1 Lipo-Dox also significantly inhibited tumor growth compared with chemotherapy alone. There was a modest but significant increase in the median survival time in mice treated with AP-1 Lipo-Dox followed by pulsed HIFU, compared to those treated with AP-1 Lipo-Dox without sonication. The use of AP-1-conjugated liposomes carrying cytotoxic agents followed by pulsed HIFU represents a feasible approach for enhanced targeted drug delivery in brain tumor therapies.

1. Introduction

The concentration of chemotherapeutics required to achieve clinically effective cytotoxicity in tumors is limited by the associated tissue toxicity and by physiologic barriers that prevent the delivery of drug to the tumor [1]. Liposome-based drug-delivery systems have been designed to elevate tumor drug levels while limiting systemic drug exposure [2]. It is thought that targeted delivery of liposomes encapsulating cytotoxic drugs should increase the accumulation and retention of drugs at the tumor site. The employment of liposomal chemistry, such as liposomes conjugated to antibodies or targeting ligands, can optimize and enhance the local delivery and better drug cell internalization compared with the free drug [3,4].

Glioblastoma multiforme (GBM) is an aggressive, high-grade brain tumor in humans, and patients have a poor prognosis even after chemotherapy and radiation therapy. Surgical resection is difficult due to the diffuse nature of the glioma. Current chemotherapies are either ineffective in treating the glioma completely or display a series of toxic side effects to normal tissues, limiting potentially effective treatments. New therapeutic strategies are therefore vital to improving the life expectancy of these patients. Previous work has shown that human brain tumor cell lines express high levels of plasma membrane interleukin-4 receptors (IL-4R) [5]. Furthermore, human brain tumors *in situ* overexpress IL-4R compared with normal brain tissues [6]. IL-4R-targeted cytotoxin has been shown to mediate a remarkable antitumor effect in immunodeficient xenograft models of human GBM tumors [7]. These observations show that selective

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drug delivery may be achievable by binding chemotherapeutic agents to IL-4R. It is noteworthy that IL-4R is also up-regulated in some diseases, such as ovarian cancer and lung cancer, and has already been used as a target for tumor treatment [8,9].

Our previous study demonstrated that the concentration of Evans blue dye (EB) in tumors and the tumor-to-normal brain ratio of EB in the brain are increased after blood–brain barrier (BBB) disruption (BBB-D) induced by pulsed high-intensity focused ultrasound (pulsed HIFU) with an ultrasound contrast agent (UCA) [10]. This disruption to the BBB is transient and reversible and does not damage neurons [11–14]. Pulsed HIFU provides a more attractive approach for increasing local concentrations of therapeutic agents in the brain for the treatment of GBM than do other methods involving modified chemicals. It has been shown that the use of focused ultrasound achieves noninvasive targeted delivery of liposomal doxorubicin (Lipo-Dox) in the normal brain [15]. Another strategy is to use receptor-targeted Lipo-Dox to improve the therapeutic efficacy, as demonstrated in an intracranial brain tumor model [16,17].

A novel peptide we designed as a ligand from atherosclerotic plaque-specific peptide-1 (AP-1) was selected from phage display libraries that can locate atherosclerotic plaque tissue and bind to the IL-4 receptor, since it has the same binding motif to the IL-4 protein [18]. AP-1-labeled nanoparticles were used for the targeted drug delivery to tumor [19,20]. In the present study, pulsed HIFU exposures were combined with AP-1-conjugated liposomes to enhance the targeted delivery of doxorubicin (Dox) into tumors (Fig. 1), and the results were compared with that of the cytotoxic agent alone. The experiments were carried out *in vivo* to evaluate the local drug accumulation and therapeutic efficacy in a human brain tumor model. This technology combines pulsed HIFU and targeted nanoparticles as a synergistic delivery system for treating central nervous system diseases.

2. Materials and methods

2.1. Intracranial glioma xenograft model

All procedures were performed according to the guidelines of and were approved by the Animal Care and Use Committee of the National Yang-Ming University. Male 6- to 8-week-old NOD-*scid* mice were anesthetized via an intraperitoneal administration of pentobarbital at a dose of 40 mg/kg body weight. Their heads were shaved above the nape of the neck, scrubbed with Betadine/alcohol, and immobilized in a Cunningham Mouse/Neonatal Rat Adaptor stereotactic apparatus (Stoelting, Wood Dale, IL, USA). A 5-mm skin incision was made along the sagittal suture and a burr hole drilled into the skull. Then, 2×10^5 human brain malignant glioma cells (GBM8401) in 2 μl of culture medium were injected stereotactically into a single location in each left hemisphere (0.14 mm anterior and 2.0 mm lateral to the bregma) of each mouse at a depth of 3.5 mm from the brain surface. The burr holes in the skull were then sealed with bone wax and the wound was flushed with iodinated alcohol. Biophotonic imaging was used to determine that a tumor was established.

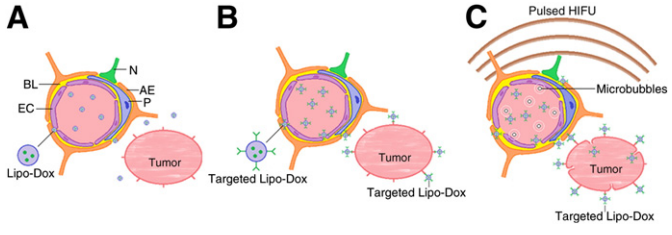

Fig. 1. Schematic depiction of the synergistic treatment strategy. (A) Tumor treated with liposomal doxorubicin (Lipo-Dox); Lipo-Dox diffusion was easier than in tumors treated with free doxorubicin (Dox). (B) Lipo-Dox actively targeted the chemotherapeutics to the tumor via conjugation with human atherosclerotic plaque-specific peptide-1 (AP-1). (C) Combining targeted Lipo-Dox (AP-1 Lipo-Dox) with pulsed high-intensity focused ultrasound (pulsed HIFU) in the presence of microbubbles significantly increases the amount of chemotherapeutics that reaches the tumor site. EC = endothelial cell, P = pericyte, BL = basal lamina, N = neuron, AE = astrocyte endfoot.

2.2. Preparation of Lipo-Dox and AP-1-labeled Lipo-Dox

Lipo-Dox was prepared using a solvent injection method plus remote loading procedures. Briefly, hydrogenated soybean L-α-phosphatidylcholine (95.8 mg, Avanti Polar Lipids), cholesterol (31.9 mg, Sigma-Aldrich), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000, 31.9 mg, Avanti Polar Lipids) were dissolved and well mixed in 1 ml of absolute ethanol at 60 °C. The lipid and ethanol mixture was then injected into a 9-ml solution of 250 mM ammonium sulfate and stirred for 1 h at 60 °C. The mixture was then extruded five times through polycarbonate membranes (Isopore Membrane Filter, Millipore) with pore sizes of 0.4, 0.2, 0.1, and 0.05 μm, consecutively, at 60 °C with high-pressure extrusion equipment (Lipex Biomembranes) to produce small liposomes. The liposome suspension was then dialyzed five times against large amounts of 10% sucrose containing 5 mM NaCl to remove the unentrapped ammonium sulfate and ethanol. After dialysis, the liposome suspension was placed in a 50-ml glass bottle in a 60 °C water bath and mixed with Dox, to a final Dox concentration of 2 mg/ml in 10% sucrose solution. The bottle was intermittently shaken in a 60 °C water bath for 1 h and then immediately cooled down to 4 °C, culminating in the production of Lipo-Dox.

Due to the presence of a thiol group on each cystine of the AP-1 peptide (CRKRLDRNC), it is possible to couple AP-1 to liposomes via the thiol-maleimide reaction. Briefly, AP-1 peptide was conjugated to 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2000-MAL, Avanti Polar Lipids) by adding AP-1 to the DSPE-PEG2000-MAL micelle solution at a 2:1 molar ratio while mixing at 4 °C overnight. The free thiol groups were measured with 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent, Sigma-Aldrich) at 420 nm to confirm that most of the AP-1 was conjugated with DSPE-PEG2000-MAL after the reaction. AP-1-conjugated DSPE-PEG2000 was transferred into the preformed Lipo-Dox at a 1.5% molar ratio of total lipid components and incubated at 60 °C for 1 h to obtain AP-1-labeled Lipo-Dox (AP-1 Lipo-Dox; Fig. 2).

The resulting unconjugated Lipo-Dox and AP-1 Lipo-Dox were found to have particle diameters of 100–120 nm, as measured by a dynamic light-scattering apparatus (Coulter N4 plus, Beckman), as well as a surface zeta potential of between −20 and −30 mV, as measured by electrophoretic light scattering (ZetaPlus, Brookhaven).

2.3. Pulsed HIFU and the treatment protocol

Pulsed HIFU exposures were generated by a 1.0-MHz, single-element focused transducer (A392S, Panametrics, Waltham, MA, USA) with a diameter of 38 mm and a radius of curvature of 63.5 mm. The focal zone of the therapeutic transducer was in the shape of an elongated ellipsoid, with a radial diameter (−6 dB) of 3 mm and an axial length (−6 dB) of 26 mm. The ultrasound-driving system and equipment setup were the same as used in our previous study [21]. UCA (SonoVue, Bracco International, Amsterdam, The Netherlands) was injected into the tail vein of the mice about 10 s before each sonication. This agent contains phospholipid-coated microbubbles at a concentration of $1–5\times10^8$ bubbles/ml, with the bubbles having a mean diameter of 2.5 μm. The sonication was precisely targeted using a stereotaxic apparatus that utilized the bregma of the skull as an anatomical landmark. The ultrasound beam was delivered to one location in the left brain hemisphere, centered on the tumor injection site. The following sonication parameters were used: an acoustic power of 2.86 W (corresponding to a peak negative