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Protocols

Preparation of artificial red cell and its application on alleviation of tumor hypoxia

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ABSTRACT

Hemoglobin-based oxygen carriers were developed as an alternative for blood transfusion. However, the research progress for their further clinic applications was slow in recent several years. Hypoxia is found in most solid tumors, which is responsible for the tumor formation, increased metastasis, drug resistance during therapeutic process as well as poor patient survival. In this work, novel hemoglobin (Hb) loaded nanoliposomes, as artificial red cells for oxygen delivery, were optimized by screening various types of phospholipids and analyzing different mole ratio of phospholipid to cholesterol. The nanoliposomes presented a high encapsulating efficiency to hemoglobin and also significantly enhanced its stability. The obtained hemoglobin loaded nanoliposome (HLL) could be lyophilized for long term storage. HLL did not cause significant cell death in the concentration range of 0–100 μg equivalent Hb/mL under normoxia and hypoxia incubation conditions, suggesting the low cytotoxicity and high biocompatibility of HLL. Importantly, HLL could efficiently accumulate into subcutaneous and deep orthotopic tumors, inducing a significant decrease of hypoxia-inducible factors 1α subunits (HIF-1α) in the tumors and remarkably reduced expression of vascular endothelial growth factor (VEGF). The study of acute and chronic toxicity indicated that HLL did not induce obvious damage to main organs of mice after intravenous injections with total Hb dose of 120 mg/kg. We presented a promising method for relieving the hypoxia degree in solid tumors and down-regulating HIF-1α protein by directly delivering oxygen into tumors, which will be very helpful for subsequent cancer therapy.

1. Introduction

Tumor microenvironment supports unique proliferation of cancer cells, further inducing tumor development [1]. Changes in the tumor microenvironment have profound effects on cancer progression [2]. The reduction of oxygen level (hypoxia) in the tumor region is one of main features in tumor microenvironment, which is found to be responsible for tumor formation, increased metastasis, drug resistance as well as poor patient survival [3]. The hypoxia in tumor is induced mainly because of the inadequate perfusion of blood as a result of severe structural and functional abnormalities of the tumor microcirculation. In order to adapt to hypoxia, tumors develop their own blood vessel network [4] and tumor cells can mutate adaptively for hypoxic microenvironment to resist hypoxia-induced cell death and tissue necrosis. It was found that tumor cells in hypoxic region exhibit the higher expression of MDR1 (multidrug resistance gene) and P-glycoprotein genes, responsible for the development of multidrug resistance to anticancer therapies[5].

Hypoxia-inducible factors (HIF) are the master regulators of gene expression in tumor cells under hypoxia [6]. HIF-1α is found to play a predominant role in regulating the transcriptional targets of HIF [7,8], and can translocate to the nucleus to regulate cell metabolism, angiogenesis and erythropoiesis [9]. The

Abbreviation: HBOCs, Hemoglobin (Hb)-based oxygen carriers; HLL, Hb loaded nanoliposome; HIF-1α, hypoxia-inducible factors 1α subunits; VEGF, vascular endothelial growth factor; RBC, red blood cell; PEG-Hb, poly (ethyleneglycol)-conjugated Hb; LEH, liposome encapsulated Hb; HLL, Hb loaded nanoliposome; CT-26-luc, luciferase-expressing mouse colon adenocarcinoma cells.

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increase of HIF-1α induces tumor angiogenesis by up-regulating the expression of vascular endothelial growth factor (VEGF) and further promotes the malignant progression of tumors [10]. Thus, reduction of HIF-1α in tumor cells will lead to effective blockage of HIF-1α-mediated "switch-on" function for those malignant-pathway related genes and suppression of HIF-1α/VEGF-mediated signaling pathway that promotes cancer progression and metastasis [11]. These cause the current increasing interest in HIF-1α as a cancer therapy target [12].

Blood transfusion has been wildly used in the clinic for saving lives and keeping human organs in normal function [13]. However, donated blood from human is always accompanied by the problems of the risk of blood type matching, shortage of blood source, strict preservation conditions and the short shelf life low to three weeks. Thus, clinical application of blood substitutes has been expected as an alternative for blood transfusion [14]. A blood alternative, especially a red blood cell (RBC) substitute such as hemoglobin (Hb)-based oxygen carriers (HBOCs), has been widely investigated. Hb can bind or release oxygen according to the conditions in vessel or tissue (i.e., the pressure of oxygenor carbon dioxide, pH and temperature). In the last few decades, various kinds of HBOCs, such as glutaraldehyde-polymerized Hb [15,16] and poly (ethyleneglycol)-conjugated Hb (PEG-Hb) [17], have been developed. However, a main side-effect of them is vasoconstriction, which elicits an acute increase in blood pressure. Liposome encapsulated Hb (LEH) was investigated as a universal oxygen-carrier with nanometric dimensions, which significantly decreased the side-effect and enhanced circulation half-life of Hb [18,19]. However, further clinic applications of LEH presented a slow progress in recent several years.

In this work, a novel Hb loaded nanoliposome (HLL) was designed, and its formulation was further optimized by evaluating the stability of HLL with various compositions. DiR-labeled HLL was further prepared to investigate its biodistribution and accumulation in tumors. Although HLL was originally used for short-term blood perfusion as a blood alternative, we investigated its increased application after oxygen saturation, as a carrier to deliver oxygen molecules into tumor and thus alleviate the hypoxic microenvironment in tumors.

2. Materials and methods

2.1. Materials

Human hemoglobin (Hb) and 3-(4,5-dimethy lthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent) were purchased from Sigma (St Louis, MO). DiD and DiR were supplied by TCI (Tokyo, Japan). Soya lecithin-100 (S100), hydrogenated soybean phosphatidylcholine (HSPC), egg yolk lecithin-80 (E80), dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG_{2000}) and cholesterol were purchased from Lipoid GmbH (Ludwigshafen, Germany). All of the chemicals and solvents were of analytical grade.

2.2. Cell cultures

The mouse colon carcinoma cells (CT-26), luciferase-expressing mouse colon adenocarcinoma cells (CT-26-luc) and mouse sarcoma cells (S180) were purchased from Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI 1640) medium containing 10% fetal bovine serum (Life Technologies, Inc., Carlsbad, CA), 1% penicillin and 1% streptomycin at 37 °C in an environment containing 5% CO_2.

2.3. Animals

Male ICR mice (18–22 g) and BALB/c (18–22 g) mice were supplied by the Laboratory Animal Center of Zhejiang University (Hangzhou, China). The animals were fed with a standard diet and allowed water ad libitum. All animal procedures were approved by the Animal Ethics Committee of Zhejiang University.

2.4. Preparation of HLL

Thin-film hydration method was selected to prepare HLL. The parameters, such as various molar ratios(1:1,1:2,1:5,1:10) of phospholipids(S100, HSPC, E80 and DPPC) to cholesterol and various weight ratios of Hb to total lipid (phospholipids and cholesterol), were optimized by comparing the particle size and the stability of different formulation. Briefly, phospholipid, cholesterol, DSPE-PEG_{2000} were dissolved in chloroform, and the mixture was warmed to 40 °Cin a round-bottomed flask. The solvent in the mixture was evaporated under vacuum in a rotary evaporator until a thin lipid film was formed. Then, a certain volume of PBS solution (pH 7.4) containing Hb (2.0 mg/mL) was added into the resulting lipid thin-film with a water bath, followed by stirring until the formation of a crude dispersion of nanoliposome. HLL was obtained by extruding through filter membranes with 0.8, 0.45 and 0.22 μm pore size in sequence. For the purification, HLL went through SephadexG-100 to remove the free Hb. Hb concentration was determined by spectrophotometry at the wavelength of 415 nmThe obtained HLL was further lyophilized for the long-term storage. The ability of re-suspension of lyophilized powder to obtain the solution containing HLL was investigated by measuring the size distribution of HLL.

The average size of HLL was measured by dynamic light scattering (DLS) method using a Malvern Zetasizer (Malvern, UK) at 25 ± 1 °C. Refractive index was set to 1.33 and the samples were appropriately diluted with PBS (pH 7.4) before the measurement. Experiments were carried out in triplicate. HLL was stored at 4 °C and the size was tested once every other day for one week. While the lyophilized powder of HLL was stored at room temperature and the particle size was measured once a week for two months. The UV–vis spectrum measurements of Hb packaged in the nanoliposome were made at room temperature with a UV–vis spectrophotometer (Agilent Cary 60 UV–vis, Santa Clara) once every other day for one week, in the wave length range of 200–800 nm at 1 nm resolution. The morphology of HLL was investigated with transmission electron microscope (TEM; JEOL JEM-1230 microscope, JEOL, Japan). Samples were dried on copper grid, negatively stained with phosphotungstic acid (1%, g/mL), and were viewed at an accelerating voltage of 120 kV.

2.5. *Cytotoxicity* in vitro

CT-26 cells were incubated in normoxia (20% O_2 concentration) or hypoxia environment (~0.1% O_2concentration) with a low oxygen incubator (FormaSteri-Cycle i160, Thermo, US). MTT assay was used to test the cytotoxicity of HLL according to the manufacturer's suggested procedures.CT-26 cells were seeded at 7×10^3 cells per well in 96 well plates at 37 °C and incubated overnight for adherence of the cells. Then the cells were incubated with HLL with various concentrations (0, 0.1, 1, 10, 100, 200 μg/mL) under normal or hypoxia condition for 48 h. The cells incubated in medium without any drug or nanoliposomes were used as controls. After incubation, 15 μL of MTT (5 mg/mL) reagent were added to each well, including controls. Another 4 h later, the supernatant liquor of each well were removed before DMSO (150 μL) were added to dissolve the formazan crystals, and jolt 15 min for its ample dissolution. Finally, the absorbance value was determined at wave