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Anticancer efficacy of photodynamic therapy with hematoporphyrin-modified, doxorubicin-loaded nanoparticles in liver cancer

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ABSTRACT

Photodynamic therapy (PDT) in combination with chemotherapy has great potential for cancer treatment. However, there have been very few attempts to developing cancer-targeted co-delivered systems of photosensitizers and anticancer drugs.

We developed hematoporphyrin (HP)-modified doxorubicin (DOX)-loaded nanoparticles (HP-NPs) to improve the therapeutic effect of PDT in treating liver cancer.

HP is not only a ligand for low density lipoprotein (LDL) receptors on the hepatoma cells but also a well-known photosensitizer for PDT. *In vitro* phototoxicity in HepG2 (human hepatocellular carcinoma) cells and *in vivo* anticancer efficacy in HepG2 tumor-bearing mice of free HP and HP-NPs were evaluated.

The *in vitro* phototoxicity in HepG2 cells determined by MTT assay, annexin V-FITC staining and FACS analysis was enhanced in HP-NPs compared with free HP. Furthermore, compared with free HP-based PDT, *in vivo* anticancer efficacy in HepG2 tumor-bearing mice was markedly improved by HP-NPs-based PDT. Moreover, in both cases, the therapeutic effect was increased according to the irradiation time and number of PDT sessions.

In conclusion, the HP-NPs prepared in this study represent a potentially effective co-delivery system of photosensitizer (HP) and anticancer drug (DOX) which improved the effects of PDT in liver cancer.

1. Introduction

Photodynamic therapy (PDT) using photosensitizers has emerged as an effective therapeutic option for various cancers [1]. Upon irradiation with the proper wavelength of light, photosensitizers transfer energy to molecular oxygen, leading to the production of highly reactive singlet oxygen, which can damage adjacent tissues [2,3]. Moreover, photosensitizers remain effective even if they are conjugated to other molecules [4]. Among the various photosensitizers, hematoporphyrin (HP) is known as the first photosensitizer used in PDT [5]. However, the clinical application of PDT has been often hindered by the limited aqueous solubility and tumor specificity of photosensitizers [6]. Therefore, to overcome these limitations, various drug delivery systems such as lipid- or polymer-based nanoparticles and polymer conjugates have been extensively explored for the cancer-specific delivery of photosensitizers [7–9].

PDT in combination with chemotherapy has great potential for cancer treatment, because it may permit low doses of photosensitizer and anticancer drug, thereby diminishing undesirable side-effects [10]. Several studies have reported that the combination of PDT and chemotherapy could produce an additive or synergistic anticancer efficacy *in vitro* and *in vivo* [10–13]. To date, vast number of investigations have focused on developing cancer-targeted drug delivery systems for either photosensitizers or anticancer drugs alone. However, there have been very few studies on the co-delivery of photosensitizers and anticancer drugs in cancer.

Among various cancer-targeted drug delivery systems, we previously developed HP-modified bovine serum albumin nanoparticles (HP-NPs) for the cancer targeting of doxorubicin (DOX) [14]. DOX is one of the most widely used anticancer drugs for the

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treatment of lymphoma, sarcomas, carcinomas, and melanoma [15]. However, its therapeutic effects have been limited due to serious side effects such as dose-dependent irreversible cardiomyopathy and congestive heart failure [15]. HP is a ligand binding to low-density lipoprotein (LDL) receptors on tumor cell membrane [16], and albumin nanoparticles could accumulate more in the cancer without targeting [17]. The HP-NPs enhanced the delivery of DOX to liver tumors in rats and reduced the distribution of DOX to the heart, suggesting their usefulness as a cancer-specific delivery system for DOX [14]. Since HP is also a widely used photosensitizer, we hypothesized that the HP-NPs could combine HP-based PDT and DOX-based chemotherapy with enhanced cancer specificity, potentially leading to an improved anticancer efficacy and reduced toxicity. There are very few reports on photosensitizer-modified cancer-specific nanoparticulate systems for PDT in combination with chemotherapy.

Herein, we report on PDT using HP-NPs for cancer treatment. The HP-NPs were prepared as previously reported, and the *in vitro* phototoxicity of PDT using HP-NPs or free HP with varying light irradiation conditions in HepG2 (human hepatocellular carcinoma) cells was evaluated by MTT assay, annexin V-FITC staining and FACS analysis. Subsequently, the *in vivo* anticancer efficacy of PDT using HP-NPs or free HP with varying light irradiation conditions in HepG2 tumor-bearing mice was further evaluated in terms of tumor growth and immunohistology.

2. Materials and methods

2.1. Materials

Doxorubicin hydrochloride (DOX), bovine serum albumin (BSA, purity ⩾98%), glutaraldehyde 8% solution, N-hydroxysuccinimide (NHS, 98%), fetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin–streptomycin, thiazolyl blue tetrazolium bromide (MTT) and Dulbecco's Modified Eagle medium (DMEM) were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Hematoporphyrin dihydrochloride (HP) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). N,N-dicyclohexylcarbodiimide (DCC, purity >99%) was purchased from Fluka (Tokyo, Japan). Ethanol, dimethyl sulfoxide (DMSO), acetonitrile, and all other reagents were obtained from Fisher Scientific Korea Ltd. (Seoul, Korea).

2.2. Preparation and characterization of HP-NPs

HP-NPs were prepared in the same way as previously reported [14]. Briefly, first, DOX-loaded nanoparticles (NPs) were prepared by a desolvation technique. 10 mg of DOX and 200 mg of BSA were dissolved in 10 mL of double-distilled water (DDW). The solution was then stirred and ethanol was added under constant stirring at room temperature. For the desolvation process, 8% glutaraldehyde solution was added as a crosslinking agent. The resulting nanoparticles were purified by centrifugation (Centrifuge 5415R, Eppendorf AG Hamburg, Germany) at 16,000*g* for 12 min; then redispersion in DDW was repeated 3 times to eliminate the ethanol and glutaraldehyde.

Secondly, HP was conjugated to prepare NPs. HP, NHS and DCC were added to DMSO, and the mixture was filtered. HP-NHS complex was isolated by mixing the filtrate with excess diethyl ether and washed with methanol. This step was repeated once. The sample was lyophilized and stored at −20 °C. HP-NHS was dissolved in an aqueous suspension of NPs and stirred overnight. Centrifugation and redispersion in DDW were repeated 3 times.

The mean particle size and zeta potential of prepared HP-NPs were measured using an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka Electronics Co., Ltd. Osaka, Japan).

The morphology of HP-NPs was then observed by field-emission scanning electron microscopy (FE-SEM) (SUPRA 55VP, Carl Zeiss, Oberkochen, Germany) and transmission electron microscopy (TEM) (JEM 1010, JEOL, Tokyo, Japan).

2.3. In vitro phototoxicity

HepG2 (human hepatocellular carcinoma, ATCC, Manassas, VA, USA) cells were seeded in 12-well cell culture plates at a density of 1×10^5 cells/well in DMEM medium containing 10% (v/v) FBS and 1% (w/v) penicillin–streptomycin and incubated for 1 day at 37 °C in a humidified 5% CO_2 and 95% air atmosphere. After cell attachment, the medium was washed off and replaced with serum-free media containing free HP or HP-NPs (2 μM as HP). After 4-h incubation, the cells were washed twice with cold PBS to eliminate the remaining drugs, and then fresh culture medium was added.

For MTT assay, the cells were illuminated once or twice with a PDT laser (Diomed Inc, Andover, MA, USA) (630 nm, 400 mW/cm^2) for 0, 5, 10, 25, 35, and 50 s (0, 2, 4, 10, 14, and 20 J/cm^2, respectively.). For the double irradiation, the interval time was 2 h and the conditions of first and second irradiation session were the same. The irradiated cells were incubated for 1 day, and cell viability was performed by MTT assay [18]. The cells were incubated for 4 h with 50 μL/well of medium containing 1 mg/mL of MTT agent, and 500 μL/well of DMSO was added. The plates were shaken gently, and absorbance was read at a wavelength of 540 nm using an absorbance microplate reader (Spectramax plus384, Molecular Devices Corporation, Sunnyvale, CA, USA). Cytotoxicity was represented as the percentage of the control. All the experiments were performed in triplicate.

For microscopic analysis, the cells were illuminated with a PDT laser (630 nm, 400 mW/cm^2) for 50 s (20 J/cm^2). The irradiated cells were incubated for 1 day and washed with cold PBS twice. Apoptotic cells were identified by using an annexin V-FITC fluorescence microscope kits (BD bioscience, San Jose, CA, USA) [19]. The cells were stained with both annexin V-FITC and DAPI, and the apoptotic cells were examined by light microscope (Axioskop 40, Carl Zeiss, Gottingen, Germany) with a X-Cite 120Q excitation light source (Lumen Dynamics Group Inc., Mississauga, Ontario, Canada).

For flow cytometry analysis, the cells were illuminated single or double with a PDT laser (630 nm, 400 mW/cm^2) for 25 or 50 s (10 or 20 J/cm^2). For the double irradiation, the interval time was 2 h and the conditions of first and second irradiation were the same. The irradiated cells were incubated for 1 day and washed with cold PBS twice. Then, the cells were stained with both annexin V-FITC and PI, and the apoptotic cells were measured by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA) using CellQuest software (BD Immunocytometry Systems, Mountain View, CA, USA).

2.4. In vivo anticancer efficacy in tumor bearing mice

All animal study protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University Bundang Hospital.

BALB/C male nude mice (6–7 weeks, 20–22 g) were purchased from Orientbio (Kyungki-Do, Korea). 1×10^6 HepG2 cells in 0.1 mL DMEM medium were injected subcutaneously into the left flanks of the mice. When the tumors grew to approximately 200 mm^3 in volume, the mice were randomly divided into 12 groups (n = 5 each) and injected with PBS, free HP or HP-NPs once or twice (on day 0 and 7). Each injection was made into a tail vein at an HP dose of 2 mg/kg in PBS. At 24 h post-injection, tumor sites were irradiated with a PDT laser (630 nm, 400 mW/cm^2) once or