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Effect of high intensity focused ultrasound (HIFU) in conjunction with a nanomedicines-microbubble complex for enhanced drug delivery

Hyounkoo Han[a,c], Hohyeon Lee[a], Kwangmeyung Kim[c,d,**], Hyuncheol Kim[a,b,*]

[a] Department of Chemical and Biomolecular Engineering, Sogang University, 35 Baekbeom-ro, Mapo-gu, Seoul 121-742, Republic of Korea
[b] Interdisciplinary program of integrated Biotechnology, Sogang University, 35 Baekbeom-ro, Mapo-gu, Seoul 04107, Republic of Korea
[c] Center for Theragnosis, Biomedical Research Institute, Korea Institute of Science and Technology, Seoul 02456, Republic of Korea
[d] KU-KIST Graduate School of Converging Science and Technology, Korea University, 145 Anam-ro, Seongbuk-gu, Seoul 02841, Republic of Korea

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ABSTRACT

Although nanomedicines have been intensively investigated for cancer therapy in the past, poor accumulation of nanomedicines in tumor sites remains a serious problem. Therefore, a novel drug delivery system is required to enhance accumulation and penetration of nanomedicines at the tumor site. Recently, high-intensity focused ultrasound (HIFU) has been highlighted as a non-invasive therapeutic modality, and showed enhanced therapeutic efficacy in combination with nanomedicines. Cavitation effect induced by the combination of HIFU and microbubbles results in transiently enhanced cell membrane permeability, facilitating improved drug delivery efficiency into tumor sites. Therefore, we introduce the acoustic cavitation and thermal/mechanical effects of HIFU in conjunction with microbubble to overcome the limitation of conventional drug delivery. The cavitation effect maximized by the strong acoustic energy of HIFU induced the preferential accumulation of nanomedicine locally released from the nanomedicines-microbubble complex in the tumor. In addition, the mechanical effect of HIFU allowed the accumulated nanomedicines to penetrate into deeper tumor region. The preferential accumulation and deeper penetration of nanomedicines by HIFU showed enhanced therapeutic efficacy, compared to low frequency ultrasound (US). These overall results demonstrate that the strategy combined nanomedicines-microbubble complex with HIFU is a promising tools for cancer therapy.

1. Introduction

High-intensity focused ultrasound (HIFU) is a novel therapeutic tool that has emerged in recent years as an accurate, non-invasive, and safe modality for the treatment of various cancers. An ultrasound beam is focused on a specific location of small volume (*e.g.*, about 1 mm in diameter and about 10 mm in length), resulting in irradiation with high intensity acoustic energy, causing thermal ablation [1] and mechanical effects [2] for cancer therapy. Besides being exploited as a stand-alone therapeutic tool, HIFU has emerged as a promising technology in combination with numerous therapeutic agents such as anti-cancer drugs [3], liposomes [4], microbubbles [5], and siRNA [6] for intractable diseases including cancer, stroke [7], diabetes [8], and obesity [9]. HIFU in combination with nanomedicines has been intensively studied for cancer therapy, and remarkable results in preclinical studies [10] and clinical trials [11] have been reported. Due to the thermal ablation and mechanical effects of HIFU, a significant amount of intravenously administered nanoparticles are extravasated from blood vessels and accumulate in the tumor site, resulting in enhanced therapeutic efficacy [12,13].

It is known that intravenously administered nanoparticles accumulate within a tumor preferentially because of the enhanced permeability and retention (EPR) effect, and then release their therapeutic payloads in the tumor site [14,15]. The EPR effect indicates that a nanomedicine could accumulate more specifically in tumor tissue than in normal tissue by passive extravasation. However, regardless of advances in nanomedicine, only about 0.7% of the intravenously administered dose of nanomedicine is delivered to the tumor [16]. Most intravenously administered nanomedicines are distributed in normal tissues, causing side effects. Therefore, many studies have been performed to minimize the exposure of nanomedicines to normal tissues and to improve the delivery efficiency to the tumor site. Moon et al. developed an ultrasound-sensitive complex by conjugating nanomedicines onto the surface of a microbubble to improve drug delivery

* Correspondence to: Hyuncheol Kim, Department of Chemical and Biomolecular Engineering, Sogang University, 35 Baekbeom-ro, Mapo-gu, Seoul 04107, Republic of Korea.
** Correspondence to: Kwangmeyung Kim, Center for Theragnosis, Biomedical Research Center, Korea Institute of Science and Technology (KIST), Hwarang-ro 14-gil 6, Seongbuk-gu, Seoul 02456, South Korea.
E-mail addresses: kim@kist.re.kr (K. Kim), hyuncheol@sogang.ac.kr (H. Kim).

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efficiency to the tumor site [17]. When ultrasound-sensitive complexes were administered intravenously into MCF-7 tumor-bearing mice, the complexes circulated inside the blood stream without extravasation to normal tissues because of its micron-size. As low-frequency ultrasound (US) (1 MHz) was applied at the tumor site, the complexes circulating around the tumor site were disrupted, and the conjugated nanomedicines were released locally at the tumor site. In addition, due to the cavitation effect induced by low frequency US and microbubbles, higher amounts of nanomedicine accumulated in the tumor site compared to the group with no US exposure, leading to improvement in chemotherapeutic effect. Although the combined strategy of using low frequency US and US-sensitive complexes partially overcame the limitations of conventional drug delivery, the majority of extravasated nanomedicines were distributed superficially at the tumor site, and could not penetrate into deeper regions efficiently. Therefore, a new drug delivery system is required to improve the accumulation and penetration of nanomedicines into the tumor site.

In general, both low frequency US and HIFU have ultrasound waves with 0.2–10 MHz frequencies. However, the crucial difference between low frequency US and HIFU is acoustic pressure amplitude due to differently shaped transducers. HIFU transducers produce much larger acoustic pressure amplitudes at the focus of the transducer than low frequency US transducers do [18]. This leads to a difference in strength (W/cm^2). Higher intensity HIFU is capable of producing both thermal and mechanical effects on tissue, and sufficient acoustic energy to collapse microbubbles more completely than low frequency US. The difference in intensity between low frequency US and HIFU indicates that the overall effects of HIFU in conjunction with microbubbles are more powerful than those of US in conjunction with microbubbles. Therefore, the overall effects due to the combination of HIFU and microbubbles can be expected to maximize the accumulation and penetration of nanomedicines in the tumor site, resulting in maximized therapeutic efficacy.

We developed a low frequency US- and HIFU-sensitive complex comprised of microbubbles and therapeutic agent-loaded nanoparticles. Paclitaxel-loaded thiolated human serum albumin nanoparticles (PTX-tHSA-NPs)-conjugated microbubbles (MBs) complexes were developed for enhanced local drug delivery as applied to low frequency US-mediated drug delivery [17,19]. To investigate the enhanced effects of drug delivery of large acoustic pressure amplitudes and thermal/mechanical effects generated by HIFU, we focused on studying the higher acoustic energy of HIFU compared to that of US as an advantage in local drug delivery, deeper accumulation of nanomedicines at the tumor site, and enhanced therapeutic efficacy. In particular, *in vitro* cancer cell uptake, cytotoxicity, and *in vivo/ex vivo* distribution of nanomedicine-microbubble complexes in tumor-bearing mice were extensively analyzed to determine the maximized cavitation effects on drug delivery efficacy (Fig. 1).

2. Materials and methods

2.1. Materials

1,2-Distearoyl-sn-glycero-phosphatidylcholine (DPSC) was purchased from NOF Corporation (Tokyo, Japan) and 1,2-distearoyl-sn-glycero-3-phosphoethanolaminen-polyethyleneglycol-N-hydroxysuccinimide (DSPE-PEG_{2000}-NHS) was purchased from Nanocs Inc. (Boston, USA). Human serum albumin (HSA), 2-Iminothiolane hydrochloride (2-IT), and chloroform were purchased from Sigma-Aldrich (St. Louis, USA). Paclitaxel was purchased from Samyang Biopharmaceuticals (Daejeon, Korea). DiI-C18 was purchased from Molecular Probes Inc. (Eugene, OR, USA). A549 human lung carcinoma cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cell culture products including fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's phosphate buffered saline (DPBS) were provided by Invitrogen (Carlsbad, CA). All chemicals and reagents were of analytical grade and used without further purification.

2.2. Preparation of PTX-tHSA-NPs-MBs

PTX-tHSA-NPs-MBs were synthesized using the following sequential procedures for HIFU-triggered drug delivery. First, PTX-tHSA-NPs were fabricated by the emulsification method [20,21]. Briefly, HSA was dissolved in deionized water at 40 mg/ml, and then mixed with a 2-IT (2 mg/ml) solution to substitute primary amines for sulfhydryl (−SH) groups. After mixing for 1 h, the HSA solution was adjusted to pH 8.0–8.5 with 0.01 M NaOH and then filtered twice using a Centricon filter (Mw cut off: 30,000) at 4000 rpm for 5 min to remove the unbound 2-IT. After purification, 100 μl of PTX in chloroform (30 mg/ml) was added to the thiolated HSA solution. The solution was mixed well by vortexing, and sonication with a probe sonicator (Duty cycle 100%, Amplitude 50%) for 30s. The residual chloroform in the mixture was removed completely by drying with a rotary evaporator for 1 h. After evaporation, the mixture was centrifuged three times at 4 °C and 13,200 rpm for 10 min. Then, the mixture was resuspended in PBS and centrifuged at 4 °C and 3000 rpm for 5 min to obtain submicron-sized tHSA-NPs. 0.5 mg of the fluorescence dye DiI-C18 was loaded onto tHSA-NPs to fabricate DiI-tHSA-NPs using the emulsification procedure described above.

MBs with *N*-hydroxysuccinimide (NHS) functional groups were synthesized using two types of phospholipids and a reverse-phase evaporation method [22]. DSPC and DSPE-PEG_{2000}-NHS (molar ratio 9:1) were briefly dissolved in chloroform. The organic solvent was evaporated using a stream of nitrogen gas to prepare a lipid film. The lipid film was then dried under vacuum for 30 min to remove residual chloroform solvent completely. The lipid film was hydrated in 1 ml of PBS (0.01 M, filtered). The final concentration of the lipid mixture was 0.5 mg/ml. After hydration with PBS, the headspace of the vial was filled with perfluoropropane gas. Then, the vial was placed in a water bath at the phase-transition temperature of the lipid mixture (65 °C). The lipid mixture was then sonicated for 30 s (40 kHz, 100 W). To achieve homogeneity in the lipid mixture, the hydration process was repeated three times. After the hydration process, the vial was filled with perfluoropropane gas, and then MBs were formed by mechanical agitation using a VialMix™ agent activator (Lantheus Medical Imaging, MA, USA).

To conjugate PTX-tHSA-NPs onto the surface of MBs, PTX-tHSA-NPs (20 mg) were mixed with NHS-activated MBs, and then the mixture was stored at room temperature for 1 h. Following the incubation, the newly synthesized PTX-tHSA-NPs-MBs were washed twice to remove unconjugated PTX-tHSA-NPs.

2.3. Characterization of PTX-tHSA-NPs-MBs

The size distributions of PTX-tHSA-NPs, free MBs, and PTX-tHSA-NPs-MBs were analyzed by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, Westborough, MA). All measurements were performed in triplicate and are presented as the mean ± SD. The morphologies of the PTX-tHSA-NPs were determined by transmission electron microscopy (TEM) (Tecnai TEM, FEI, The Netherlands). To visually confirm the attachment of PTX-tHSA-NPs onto the surface of MBs *via* NHS-amine conjugation, DiI-C18-stained MBs and Cy5.5-labeled PTX-tHSA-NPs (Cy5.5-PTX-tHSA-NPs) were used. Conjugation was confirmed under a confocal laser scanning microscope (TCS SP8, Leica, Germany). Laser light at 532 nm and 675 nm wavelength was used to excite DiI-C18 and Cy5.5, respectively, and detector settings were adjusted to avoid interference between emission signals.

To measure *in vitro* release rate of PTX from PTX-tHSA-NPs, 20 mg of PTX-tHSA-NPs was dispersed in 1 ml of PBS and placed into a dialysis membrane (MWCO 10,000, Spectrum®, Rancho Dominguez, CA). The dialysis membrane was then immersed in 10 ml of PBS with 0.01%