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Oleic acid-embedded nanoliposome as a selective tumoricidal agent

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ABSTRACT

HAMLET (Human Alpha-lactalbumin Made LEthal to Tumor cell), a molecular complex of human α-lactalbumin and oleic acid, is known to have selective cytotoxic activity against certain types of tumors. This cytotoxicity is known to stem from water-insoluble oleic acid. In this study, we manufactured an alternative complex using liposome as an oleic acid delivery vesicle. We named this nanolipoplex LIMLET (LIposome Made LEthal to Tumor cell). The LIMLET vesicle contained approximately 90,200 oleic acid molecules inserted into its lipophilic phospholipid bilayer and had a nominal mean diameter of 127 nm. Using a WST-1 assay, its cytotoxicity against two cancer cell lines, MDA-MB-231 (human breast cancer) and A549 (human lung cancer), were tested. The results were compared with that of a normal cell line, Vero (from monkey kidney). We found that (1) LIMLET showed distinctive cytotoxicity against A549 and MDA-MB-231 cells, whereas bare liposomes (containing no oleic acid) had no toxicity, even at high concentrations, and (2) LIMLET demonstrated selective, concentration-dependent toxicity against the cancer cells: the LD_{50} values of MDA-MB-231 and A549 cells were 1.3 and 2.2 nM LIMLET, respectively, whereas the LD_{50} of Vero was 5.7 nM. The strength of the tumoricidal effect appeared to stem from the number of oleic acid molecules present. Our result suggests that LIMLET, like HAMLET, is an interesting nanolipoplex that can potentially be developed into tumor treatments.

1. Introduction

HAMLET (Human α-lactalbumin Made LEthal to Tumor cell) is a molecular complex of human α-lactalbumin derived from human milk and oleic acid, a naturally occurring unsaturated fatty acid (see Fig. 1). It induces apoptosis through autophagy in certain tumor cells such as skin, breast, and lung cancers, as well as in some incompletely differentiated cells, but does no damage to normally differentiated cells [1,2]. The cytotoxicity of HAMLET is known to be accompanied by mitochondrial damage and a decrease in the activity of mTOR (mammalian target of rapamycin), which modulates cell growth, division, motility, survival, and protein synthesis and transcription [3].

The role of α-lactalbumin in HAMLET's cytotoxicity has not been fully elucidated; some reports have suggested that its cytotoxicity might stem from aggregates of partially unfolded α-lactalbumin [4–6], while others have suggested that α-lactalbumin itself does not cause cytotoxicity but simply acts as a delivery vehicle for oleic acid [7,8]. The exact biochemical mechanisms of α-lactalbumin and oleic acid complexes are still under investigation [9]; however, recent studies have shown that oleic acid is responsible for cytotoxicity [10,11].

To study the feasibility of replacing α-lactalbumin with liposome, we prepared a nanolipoplex in which oleic acid molecules were embedded into the phospholipid bilayer by hydrophobic interactions. Borrowing from the idea of HAMLET, we named this nanolipoplex LIMLET (LIposome Made LEthal to Tumor cell). We experimentally tested LIMLET's cytotoxic properties from two perspectives. First, oleic acid's cytotoxicity was confirmed by comparing LIMLET's cytotoxicity with that of bare liposome containing no oleic acid. Second, we assessed its specificity for tumor cells by comparing its cytotoxicity against cancer cells (MDA-MB-23, human breast cancer cells, and A549, human lung cancer cells) and normal cells (Vero; monkey kidney cells).

2. Materials and methods

2.1. Materials

DPPC (1,2-dipalmitoyl-sn-*glycero*-3-phosphocholine) was used as the phospholipid for LIMLET. DPPC, cholesterol, a polycarbonate membrane filter, and a mini extruder were purchased from Avanti Polar, Inc. (Albaster, USA). Chloroform and phosphate buffered saline (PBS) were obtained from Welgene Inc. (Gyeongsan, Korea),

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and oleic acid was purchased from Sigma (St. Louis, MO, USA). Oleic acid concentration was measured with an HPLC system (Agilent, HPLC 1260, Santa Clara, CA, USA) equipped with a reverse phase silica column (Shim-pack GIS-ODS 5 μm; 250 × 4.6 mm ID., Dong-il ShimazuSpeChrom Corp., Seoul, Korea). The mobile phase was composed of acetonitrile, methanol, and *n*-hexane (all HPLC-grade and purchased from Daejung Chemicals [Siheung, Korea]) in a ratio of 90: 8: 2 and a flow rate of 0.3 ml/min [12,13]. Cell lines were purchased from Korea Cell Line Bank; MDA-MB-231 (No.30026, breast cancer), A549 (No.10185, lung cancer), and Vero (No.10081, monkey kidney). Cell culture media and ingredients were purchased from Welgene Inc. (Gyeongsan, Korea); RPMI (Rosewell Park Memorial Institute) 1640 (catalog # LM011-03), fetal bovine serum (FBS, catalog # S101-01), P/S (penicillin-streptomycin, catalog # LS202-01), and DPBS (Dulbecco's phosphate-buffered saline, catalog # LB001-02). DMSO (dimethyl sulfoxide, catalog # D2650) was from Sigma.

2.2. LIMLET preparation procedure

LIMLET was prepared by the same method as previously described [14]. Briefly, DPPC (117.6 mg) and cholesterol (26.9 mg) were mixed at a molar ratio of 7: 3 in 10 ml chloroform. DPPC (dipalmitoylphosphatidylcholine,1,2-dipalmitoyl-sn-*glycero*-3-phosphocholine) was used as the main phospholipid for liposome preparation. It is relatively uncharged and inert so that electrostatic interactions between liposomes become minimal, making it less prone to aggregation. Because it is saturated, phosphatidylcholine (PC) has a relatively high phase transition temperature (T_m = 41.5 °C) [14]. Cholesterol is known to provide the physical influence on the membrane properties such as fluidity, permeability, and width. It is frequently added to liposome preparation to improve its stability [15].

Oleic acid (50 μl, or 44.8 mg) was added and fully dissolved. Chloroform was completely removed by rotary evaporation at 45 °C and freeze drying for 24 h. The lipid film that formed on the flask wall was hydrated by adding 5 ml PBS (pH 7.4) at 45 °C for 30 min. Using a mini extruder, the hydrated solution was extruded 20 times through polycarbonate membrane (mean pore size of 100 nm) at 45 °C [14]. Free oleic acid molecules not embedded into liposomes were removed through dialysis against a PBS buffer using a Slide-A-Lyzer® dialysis cassette (G2, 3.5 kD MWCO) at ambient temperature for 24 h. The LIMLET solution recovered was added to the cell culture broth after appropriate dilution.

2.3. Cell culture procedure

Culture medium (RPMI1640 90%, FBS 9%, P/S 1%) and cell culture broth were equilibrated at 37 °C. To a 15 ml tube, 5 ml medium and 1 ml cell culture broth were added and centrifuged at 1,000 rpm for 3 min. The cell pellet that was recovered was suspended in 1 ml medium, added to a Petri dish containing 9 ml medium, and evenly distributed by gentle mixing. Cells were cultured in a 5% CO_2 incubator at 37 °C, and the medium was replaced every 2 days.

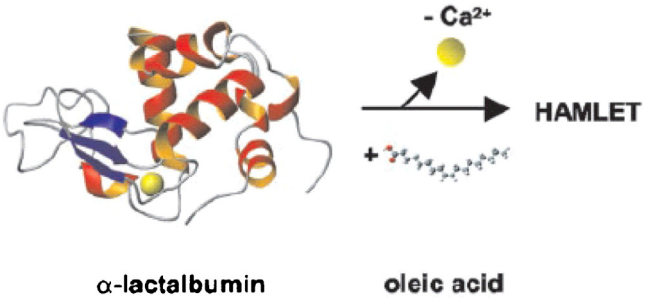

Fig. 1. A diagram of HAMLET (Human α-lactalbumin Made LEthal to Tumor cell) formation [29].

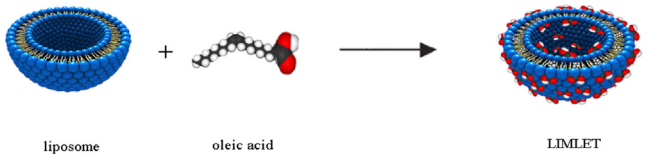

Fig. 2. A diagram of LIMLET formation.

2.4. Cell viability measurement using the WST-1 assay

The cellular toxicity of LIMLET or bare liposome was determined by measuring cell viability using the WST-1 assay. The WST-1 cell proliferation assay was used to measure the activity of a mitochondrial dehydrogenase present only in metabolically active and viable cells for its ability to generate, a color body that is linearly proportional to viable cell count [16]. The WST-1 assay can be used to replace MTT assay, a traditional cellular apoptosis assay, because the results of the assays show a strong correlation (R^2 = 0.93) while WST-1 assay takes only 1–4 h compared to 3–4 days needed for MTT assay [17].

The EZ-Cytox® kit (EZ-3000) was purchased from Daeil Lab Service Co., Ltd (Seoul, Korea). The detailed WST-1 assay procedure is as follows: 1 ml cell culture broth and 5 ml medium were added to a 15 ml tube and centrifuged at 1,000 rpm for 3 min to recover the cell pellet. The cell pellet was suspended in 10 ml medium. Ten μl of the cell suspension was added to 10 μl of trypan blue in a microtube. The cell number was measured by a hemocytometer in order to dilute the cell suspension to approximately 20,000 cells per 100 μl. Fifty μl of the diluted cell suspension was transferred to a 96-well plate and incubated at 37 °C for 24 h. Another 50 μl of various concentrations of LIMLET and bare liposome (0.045, 0.225, 0.45, 2.25, 4.5, 22.5, 45, 90 nM each) or medium (as a control) were added. Here, one molecule of LIMLET was equivalent to one liposomal vesicle. After 4 h cultivation, 10 μl of EZ-Cytox® was added. After another 1 h, absorbance at 450 nm was measured by a plate reader, and cell viability was calculated [18]. All assays were performed in triplicate.

3. Results and discussion

3.1. Characteristics and oleic acid content of LIMLET

Fig. 2 illustrates LIMLET's vesicular structure. The phospholipid bilayer was used as a depot for water-insoluble oleic acid to be embedded through hydrophobic interactions. By this mechanism, oleic acid can be delivered through the systemic circulatory system without leakage [19]. LIMLET diameter was measured by dynamic light scattering method. The Z-average diameter was 127 nm, and the size distribution according to intensity was relatively wide with a standard deviation of 41.8 nm (n = 3) (data not shown). LIMLET was kept in the PBS buffer (pH 7.4) at room temperature. It was very stable; for about a month's storage, it was clear with no sign of aggregation or precipitation.

During LIMLET vesicle formation, the oleic acid molecules already integrated into the lipid film are hardly released outside the vesicles as a free molecule. Therefore, it is very unlikely for them to form micelles or other aggregates in the bulk liquid phase as well as to change the equilibrium state after the dialysis.

The number of oleic acid molecules embedded in one LIMLET vesicle was calculated as follows: First, from a sample of LIMLET solution (10 μl) with a known molar concentration (45 nM) (see