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Advanced therapeutic approach for the treatment of malignant pleural mesothelioma via the intrapleural administration of liposomal pemetrexed

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ABSTRACT

Malignant pleural mesothelioma (MPM) is an aggressive cancer that proliferates in the pleural cavity. Pemetrexed (PMX) in combination with cisplatin is currently the approved standard care for MPM, but a dismal response rate persists. Recently, we prepared various liposomal PMX formulations using different lipid compositions and evaluated their *in vitro* cytotoxicity against human mesothelioma cells (MSTO-211 H). In the present study, we investigated the *in vivo* therapeutic effect of our liposomal PMX formulations using an orthotopic MPM tumor mouse model. PMX encapsulated within either cholesterol-containing (PMX/Chol CL) or cholesterol-free (PMX/Non-Chol CL) cationic liposome was intrapleurally injected into tumor-bearing mice. PMX encapsulated in cholesterol-free liposomes (PMX/Non-Chol CL) drastically inhibited the tumor growth in the pleural cavity, while free PMX and PMX encapsulated in cholesterol-containing liposomes (PMX/Chol CL) barely inhibited the tumor growth. The enhanced *in vivo* anti-tumor efficacy of PMX/Non-Chol CL was credited, on the one hand, for prolonging the retention of cationic liposomes in the pleural cavity via their electrostatic interaction with the negatively charged membranes of tumor cells, but on the other hand, it was charged with contributing to a higher drug release from the "fluid" liposomal membrane following intrapleural administration. This therapeutic strategy of direct intrapleural administration of liposomal PMX, along with the great advances in CL-guided therapeutics, might be a promising therapeutic approach to conquering the poor prognosis for MPM.

1. Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor that disseminates in the pleural cavity [1–3]. MPM is often caused by exposure to asbestos with a long-term latency period of about 2 decades [4]. Currently, the incidence of MPM is predicted to increase globally, particularly in developing countries, where the use of asbestos is still prevalent. For the treatment of MPM, a combination therapy of intravenous pemetrexed (PMX) with cisplatin is the clinical standard care [5–8]. In addition, intrapleural cisplatin-based chemotherapy has been used in the treatment of patients with malignant pleural effusions and MPM [9,10]. However, the benefit for the treatment of MPM remains insufficient [11]. Accordingly, new therapeutic strategies to treat MPM are in urgent demand.

PMX is a novel anti-folate agent that exerts its anti-cancer activity via the inhibition of multiple folate-dependent enzymes (thymidylate synthase (TS), dihydrofolate reductase (DHFR) and glycinamide ribonucleotide formyltransferase (GARFT)), and thereby, it tends to interfere with DNA synthesis and repair [12]. PMX is recognized to be taken up by the cells via reduced folate carriers (RFC) that are expressed on the cell membrane, which means that PMX is transferred into the cells in a concentration-dependent manner [13,14]. After being taken up by the cells, PMX undergoes glutamate-conjugation by folylpolyglutamate synthetase (FPGA) and then exhibits its anti-cancer effect in a time-dependent manner [12,15]. For the treatment of MPM, direct administration of PMX into the pleural cavity is expected to achieve a therapeutic effect that is superior to intravenous administration, since it is assumed that direct injection will increase PMX concentration in the cavity confining the tumor cells. However, low-molecular compounds such as cisplatin or PMX are known to be quickly eliminated from the

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pleural cavity to blood circulation after intrapleural administration [16]. Therefore, any technique that could help maintain a higher PMX concentration in the pleural cavity for an extended period of time following intrapleural administration is expected to enhance the overall therapeutic efficacy of PMX.

Liposomes are membrane vesicles that are composed of phospholipid bilayers and show a high degree of biocompatibility. In addition, they enable the encapsulation of both hydrophilic and hydrophobic drugs, and can be directly administered into the pleural cavity. Furthermore, their physicochemical properties, which strongly determine their *in vivo* fate, can be manipulated on demand. For example, surface modification with polyethylene glycol (PEG) prolongs their circulation time [17]; the inclusion of a cationic lipid into the liposomal membrane enhances cellular uptake/internalization [18]; and, employing saturated-phospholipids as membrane-forming lipids increases the stability of liposomal membranes [19].

Recently, we developed various formulations of PEG-coated cationic liposomes encapsulating PMX using different types of phospholipids [20]. In addition, we revealed that manipulation of the physicochemical properties of the prepared liposomes, via the use of different membrane-forming phospholipids and/or incorporating cholesterol in the liposomal membrane, has significantly affected the PMX release from liposomes, and consequently, the *in vitro* cytotoxicity against the human pleural mesothelioma cell line (MSTO-211 H) [20]. However, the *in vivo* therapeutic efficacy of our liposomal PMX formulations has not been evaluated.

In the present study, therefore, we investigated the therapeutic activity and the *in vivo* fate of different liposomal PMX formulations following their intrapleural administration in an orthotopic tumor mouse model. Those results suggested that a combination of the manipulation of the physicochemical properties of liposomes and direct local injection of liposomal PMX into the pleural cavity could be a promising strategy for the treatment of MPM.

2. Materials and methods

2.1. Materials

Palmitoyloleoyl phosphatidylcholine (POPC), dioleoyl phosphatidylethanolamine (DOPE), dimyristoyl phosphatidylcholine (DMPC), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanol-amine-*n*-[methoxy (polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) were generously donated by NOF (Tokyo, Japan). Cholesterol (Chol) and D-luciferin potassium salt were purchased from Wako Pure Chemical (Osaka, Japan). A cationic lipid, O,O'-ditetradecanoyl-N-(alpha-trimethyl ammonio acetyl) diethanolamine chloride (DC-6-14) was purchased from Sogo Pharmaceutical (Tokyo, Japan). Pemetrexed disodium (PMX; Alimta®) was purchased from Eli Lilly (Indianapolis, IN, USA). 1,1′-Dioctadecyltetramethylindotricarbocyanine iodide (DiR) was purchased from Life Technologies (Carlsbad, CA, USA). [^{3}H]-cholesterylhexadecyl ether ([^{3}H]-CHE) was purchased from PerkinElmer Japan (Yokohama, Japan). All other reagents were of analytical grade.

2.2. Animals and tumor cells

BALB/c *nu/nu* mice (male, 5 weeks old) and ddY mice (male, 5 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The experimental animals were allowed free access to water and mouse chow, and were housed under controlled environmental conditions (constant temperature, humidity, and a 12-h dark–light cycle). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of Tokushima University.

A human pleural mesothelioma cell line (MSTO-211 H) expressing firefly luciferase (MSTO-211 H-Luc) that was generated by stable transfection with the firefly luciferase gene (pGL3 Basic plasmid; Promega, WI, USA) was generously supplied by Dr. Masashi Kobayashi (Department of Thoracic Surgery, Faculty of Medicine, Kyoto University, Kyoto, Japan). MSTO-211 H-Luc was cultured in RPMI-1640 medium (Wako Pure Chemical) supplemented with 10% of heat-inactivated FBS (Corning, Corning, NY, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin (ICN Biomedicals, CA, USA) in a 5% CO_2/air incubator at 37 °C.

2.3. Preparation of liposomes

Neutral liposomes (NL), cholesterol-containing cationic liposomes (Chol CL), and cholesterol-free cationic liposomes (Non-Chol CL) were composed of POPC/DOPE/Chol/DMPC/$mPEG_{2000}$-DSPE (2/3/3/2/0.5 as molar ratio), POPC/DOPE/Chol/DC-6-14/$mPEG_{2000}$-DSPE (2/3/3/2/0.5), and POPC/DOPE/DC-6-14/$mPEG_{2000}$-DSPE (5/3/2/0.5), respectively. DiR-labeled liposomes were prepared by the addition of DiR to the lipid mixture (0.1% to total lipid) before the formulation of a thin-film layer. [^{3}H]-labeled liposomes were prepared by the addition of a trace amount of [^{3}H]-CHE (40 μCi/μmol lipid), as a non-exchangeable lipid phase marker, into the lipid mixture. Liposomes were prepared using the thin-film hydration technique, as previously described [21]. The hydrated liposomes were sized by extrusion through a polycarbonate membrane filter with pore sizes of 200, 100 and 80 nm. The particle sizes and zeta-potentials of liposomes were determined using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK).

The liposomal PMX formulation (PMX/Chol CL or PMX/Non-Chol CL) was prepared by the Bangham method as to hydrate the lipid film of Chol CL or Non-Chol CL with 25 mg/ml PMX solution at a total lipid concentration of 50 mM. The encapsulation efficiency (EE) of PMX into the liposomes was calculated from the free amount of PMX after separating the liposomal PMX via column chromatography using Sepharose CL-4B (GE Healthcare Bioscience, Buckinghamshire, UK), and the free PMX was determined via HPLC, as described previously [20]. The physicochemical properties and encapsulation efficiency of the liposome preparations are summarized in Table 1.

2.4. In vitro release study of PMX from the liposomes

PMX/Chol CL and PMX/Non-Chol CL were mixed with mouse serum (1:1 *v/v* (%)) and incubated at 37 °C for 48 h. Free PMX released from PMX/Chol CL or PMX/Non-Chol CL was eliminated by gel filtration chromatography with Sepharose CL-4B. The liposome fractions were collected, and the PMX that remained in the liposomes was determined via HPLC, as described previously [20].

2.5. Orthotopic mesothelioma mouse model

An orthotopic mesothelioma mouse model was prepared by direct injection of MSTO-211 H-Luc cells (1 x 10^6 cells/mouse) into the left pleural cavity of BALB/c *nu/nu* mice. The development of the implanted tumor was monitored using an *in vivo* imaging system (IVIS, Xenogen, CA, USA). For *in vivo* imaging, mice were intraperitoneally injected with 100 μl of 7.5 mg/ml D-luciferin potassium salt and were subsequently anesthetized with isoflurane inhalation. At 5 min post injection, bioluminescence was observed with a CCD camera (exposure time was fixed at 30 s). The region of interest (ROI) for the bioluminescence was calculated and shown as photon counts (photons/s/cm^2/sr).

2.6. In vivo pharmacokinetic study

2.6.1. Bio-distribution of the liposomes following intrapleural administration

To visualize the elimination of liposome from the pleural cavity, fluorescently DiR-labeled Chol CL or DiR-labeled NL were intrapleurally injected into BALB/c *nu/nu* mice at a dose of 2 μmol phospholipids/mouse. At selected time points post injection (10, 30 min, 1, 2, 4, 6, 12,