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Evaluation of drug penetration with cationic micelles and their penetration mechanism using an in vitro tumor model

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ABSTRACT

Elevated interstitial fluid pressure (IFP) and abnormal extracellular matrix (ECM) are major factors causing significant barriers to penetration of nanomedicines in solid tumors. To better understand the barriers, various in vitro tumor models including multicellular spheroids and multilayered cell cultures (MCCs) have been developing. Recently, we have established a unique in vitro tumor model composed of a MCC and an Ussing chamber system which is modified to add a hydraulic pressure gradient through the MCC. In this study, we evaluated the drug penetration ability of cationic micelles using the unique in vitro tumor model. The doxorubicin (DOX)-loaded cationic micelles, which are formed from a triblock copolymer of poly(D,L-lactide-co-glycolide)-*block*-branched polyethyleneimine-*block*-poly(D,L-lactide-co-glycolide), deeply penetrated and released the DOX throughout the MCC against convectional flow caused by a hydraulic pressure gradient. The studies using endocytosis markers and inhibitors showed that the micelles utilized mainly macropinocytosis as an internalization pathway. Furthermore, the penetration was apparently inhibited by treatment with exocytosis inhibitor Exo1. These results suggest that the active penetration of the cationic micelles is induced by iterative transcytosis via macropinocytosis and exocytosis. Our findings could be beneficial information to improve intratumoral penetration in new nanomedicines for solid tumors.

1. Introduction

A large body of nanomedicines for cancer therapy is now being developed to improve efficacy and minimize toxicity. The strategy is based on the enhanced permeability and retention (EPR) concept [1], which provides a neat rationale for targeting macromolecules to the tumor tissues; however, the impact of these formulations on efficacy is rarely significant as anticipated [2]. In tumor tissues, there are several physiological features distinguished from those of normal tissues: first, the fluid leakage from blood vessels and the lack of functional lymphatic vessels, which contribute to elevated interstitial fluid pressure (IFP) [3]; second, abnormal interstitial properties such as increased stiffness of extracellular matrix (ECM) [4]. The high IFP and abnormal ECM structure are thought to be major barriers to penetration of nanomedicines into the core of the tumor mass. The limited intratumoral penetration of nanomedicines could be responsible for clinical resistance of solid tumors. To bridge the gap between preclinical research and clinical practice, many research groups have been developing tumor models to mimic tumor microenvironment. Multicellular spheroids [5] and multilayered cell cultures (MCCs) [6] are commonly used to evaluate the drug penetration as an in vitro three-dimensional (3D) tumor model which can share several properties with solid tumors derived from the same cell type, including a similar but not identical ECM and tight junctions between epithelial cells [7]. Recently, we have established a unique in vitro tumor model composed of a MCC and an Ussing chamber system to simulate tumor microenvironment and control over certain conditions [8]. The model is distinctive in that a hydraulic pressure gradient can be applied to the MCC by adjusting the height of a water column in the reservoir (Fig. 1). Our approach has the advantage of being able to observe the drug distribution as an isolated event without complicating factors such as pharmacokinetics and hepatic metabolism [9]. In previous studies, we have demonstrated that cationic micelles, which are formed from a triblock copolymer of poly(D,L-lactide-co-

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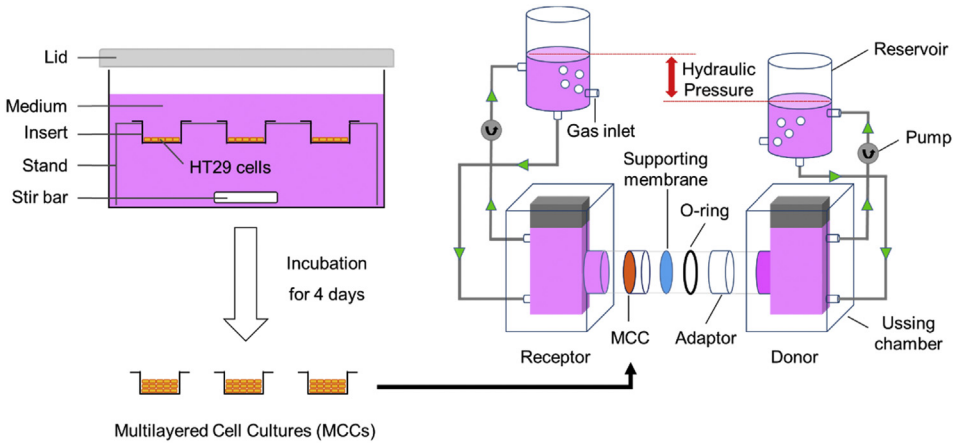

Fig. 1. The apparatus used for growing multilayered cell cultures (MCCs) and modified Ussing chamber system. The culture inserts were placed on the stand and suspended in stirred medium. The set-up was placed in a 37 °C/5%CO_2 for 4 days. After growth, the MCC was mounted into the Ussing chamber by an O-ring, supporting membrane and an adaptor. The top of the MCC was faced the donor chamber. Each chamber was connected to the reservoir with silicone tubing. The culture medium in each chamber was circulated by a pump. A probe (drug or micelle) was added into the donor chamber and incubated for predetermined time.

glycolide)-*block*-branched polyethyleneimine-*block*-poly(D,L-lactide-co-glycolide) (PLGA-*b*-bPEI-*b*-PLGA), penetrated into the MCC against a hydraulic pressure gradient, suggesting active transport mechanisms [8]. Similarly, several studies have been suggesting high potential of cationic nanoparticles for intratumoral penetration [10–13]. However, their abilities and mechanisms for drug penetration into the depth of tumor still need to be clarified.

In this study, we prepared DOX-loaded cationic micelles formed from PLGA-*b*-bPEI-*b*-PLGA to evaluate the ability of drug penetration using the modified Ussing chamber system. Furthermore, we investigated the penetration mechanism of the cationic micelles using endocytosis markers and inhibitors. The mechanisms governing endocytosis and exocytosis of nanoparticles have been extensively investigated with 2D monolayer cell cultures which are over-simplified and might result in inaccurate conclusions [14,15]. By contrast, we used 3D multilayered cell cultures as well as 2D monolayer cell cultures to better understand the penetration mechanism in the mimicked solid tumor.

2. Materials & methods

2.1. Materials

Poly(lactide-*co*-glycolide)-*block*-poly(ethylene glycol) (PLGA-*b*-PEG-NH_2, PLGA *Mw* 12 kDa, PEG *Mw* 5 kDa) was purchased from Akina Inc.(West Lafayette, IN, USA). Poly(D,L-lactide-co-glycolide) [PLGA 36 kDa; Resomer® RG503H; lactide:glycolide = 1:1 (mole/mole); approximate *Mw* 36 kDa], branched polyethyleneimine (bPEI 25 kDa; *Mn* 10 kDa), dimethyl sulfoxide (DMSO), HEPES, McCoy's 5A medium, alpha modified Eagle's medium (αMEM), Collagen type I from calf skin, Hoechst 33258, FITC-dextran (FD70), chlorpromazine, methyl-β-cyclodextrin (MβCD), genistein and 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Exo1 was purchased from Enzo Life Sciences, Inc. Penicillin-streptomycin antibiotics, fetal bovine serum, and transferrin from Human Serum, Alexa Fluor® 633 Conjugate (T-23362) were purchased from Life Technologies (Carlsbad, CA, USA). Culture inserts (Costar® 12 mm Snapwell™ insert, 0.4 μm, polycarbonate membrane) were purchased from Corning Inc. (Corning, NY, USA). Dialysis membranes (Spectra/Por® dialysis membrane MWCO: 15 kDa and 3.5 kDa) were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA). Cy3-NHS ester and Cy5-NHS ester were purchased from Combinix, Inc. (Sunnyvale, CA, USA). Doxorubicin hydrochloride was purchased from Boryung pharmaceutical co., Ltd (Seoul, South Korea). Purified Mouse Anti-caveolin 1 antibody (610406) and FITC conjugated Goat Anti-Mouse Ig (554001) were purchased from BD Biosciences (San Jose, CA).

2.2. Preparation of micelles

2.2.1. DOX-loaded micelles

PLGA-*b*-bPEI-*b*-PLGA (PLGA *Mw* 36 kDa, bPEI *Mw* 25 kDa) polymer was synthesized and used for the preparation of the DOX-loaded cationic micelles as previously described with slight modification [16]. Briefly, PLGA-*b*-bPEI-*b*-PLGA was dissolved in DMSO at a concentration of 20 mg/mL and stirred for 4 h at room temperature. DOX was neutralized with triethylamine at a 1:2 molar ratio of DOX to triethylamine in DMSO. 2 mL of the DOX solution (10 mg/mL) was mixed with 2 mL of the PLGA-*b*-bPEI-*b*-PLGA/DMSO solution (20 mg/mL). Then, the mixture was injected into 4 mL of HEPES buffer (20 mM, pH 7.4) and transferred to a pre-swollen dialysis membrane tube (MWCO: 15 kDa) and dialyzed against distilled water for 72 h. The resulting solution was filtered through a 0.22 μm filter and subsequently lyophilized. Similarly, the DOX-loaded PLGA-*b*-PEG micelles were prepared with PLGA-*b*-PEG-NH_2 (PLGA *Mw* 12 kDa, PEG *Mw* 5 kDa) polymer. As necessary, these micelles were conjugated with Cy5-NHS to detect the polymer distribution in cells.

2.2.2. FRET micelles

The FRET micelles were prepared as previously described with slight modification [8]. Briefly, PLGA-*b*-bPEI-*b*-PLGA was respectively conjugated with Cy3-NHS ester and Cy5-NHS ester at a 2:1 molar ratio of PLGA-*b*-bPEI-*b*-PLGA to the dye in DMSO. The FRET micelles composed of Cy3-conjugated polymers and Cy5-conjugated polymers at a 1:1 molar ratio of Cy3 to Cy5 were prepared without a DOX solution according to the procedures for DOX-loaded micelles described above. Fluorescence spectrum was confirmed by a fluorescence spectrophotometer (SpectraMax M2/Molecular devices).

2.3. Characterization of micelles

The particle size and zeta potential were measured using a Malvern Zetasizer, NANO ZS (Malvern Instruments Limited, UK)