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Evaluation of intracellular trafficking and clearance from HeLa cells of doxorubicin-bound block copolymers

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A B S T R A C T

New technologies are needed to deliver medicines safely and effectively. Polymeric nanoparticulate carriers are one such technology under investigation. We examined the intracellular trafficking of doxorubicin-bound block copolymers quantitatively and by imaging doxorubicin-derived fluorescence using confocal microscopy. The polymers were internalized by endocytosis and distributed in endosomal/lysosomal compartments and the endoplasmic reticulum; unlike free doxorubicin, the polymers were not found in the nucleus. Moreover, the ATP-binding cassette protein B1 (ABCB1) transporter may be involved in the efflux of the polymer from cells. This drug delivery system is attractive because the endogenous transport system is used for the uptake and delivery of the artificial drug carrier to the target as well as for its efflux from cells to medium. Our results show that a drug delivery system strategy targeting this endogenous transport pathway may be useful for affecting specific molecular targets.

1. Introduction

Recently, genomic drug discovery techniques, organic synthesis, and screening technologies have been used to develop molecularly targeted medicines, some of which are already being used clinically (Hopkins and Groom, 2002; Hughes, 2009). However, these new technologies do not necessarily lead to the introduction of new treatments because even when promising compounds are discovered by genomic drug discovery techniques, they often have harmful properties or are difficult to deliver to the target because they are relatively insoluble (Hopkins and Groom, 2002; Lipinski et al., 2001). New formulation technologies are being developed to enhance the effectiveness and safety of pharmaceutical products by focusing on improving the release, targeting, and stability of drugs within the body, so that the location and timing of their action in the living body can be controlled.

Nanotechnological advances have contributed to the development of new drug delivery system (DDS) products such as polymeric micelles and liposomes that range in size from several tens of nanometers to 100 nm (Ferrari, 2005). Some of these DDS products are already being marketed as innovative medical treatments (O'Brien et al., 2004), and the number being used in clinical trials has risen impressively in recent years (Hamaguchi et al., 2007; Kuroda et al., 2009; Matsumura et al., 2004). These nanoparticulates possess several unique advantages for drug delivery, including high drug-loading capacity, controlled drug release, and small size, which allows the drug to accumulate in pathological tissues such as tumors, which have increased vascular permeability (Nishiyama and Kataoka, 2006).

Polymeric micelles have received considerable attention recently as promising macromolecular carrier systems (Allen et al., 1999; Kataoka et al., 1993, 2001; Lavasanifar et al., 2002; Torchilin, 2002; Torchilin et al., 2003). Polymeric micelles are amphipathic systems in which a hydrophobic core is covered with an outer

Abbreviations: DDS, drug delivery system; PEG, polyethyleneglycol; RES, reticuloenodothelial system; EPR, enhanced permeability and retention; Dox, doxorubicin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DLS, dynamic light scattering; AFM, atomic force microscopy; HBSS, Hank's balanced salt solution; ER, endoplasmic reticulum; ECFP, enhanced cyan fluorescent protein; Alexa-transferrin, Alexa Fluor 488 conjugate of transferrin; MTOC, microtubule-organizing center; ABCB1, ATP-binding cassette protein B1; MDR1, multidrug resistance 1; (PBS), phosphate-buffered saline; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene fluoride.

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shell consisting of hydrophilic macromolecules such as polyethylene glycol (PEG) chains. Polymeric micelles can both encapsulate medicine of high density and evade the foreign body recognition mechanism within the reticuloendothelial system (RES), and they show excellent retention in the blood (Illum et al., 1987). In addition, accurate size control of the nanoparticulates enables them to accumulate in cancerous tissue, owing to the increased permeability of tumor vessels due to the enhanced permeability and retention (EPR) effect (Matsumura and Maeda, 1986).

To maximize the efficacy and safety of DDS products, it is important to deliver these products to specific target cells and subcellular compartments. In the experiments reported here, we used confocal microscopy to study the intracellular trafficking of polymeric nanoparticulate carriers. The use of covalently bound fluorescent reagents as probes is gradually clarifying the internalization pathways and intracellular localizations of polymeric nanoparticulate carriers (Lee and Kim, 2005; Manunta et al., 2007; Murakami et al., 2011; Rejman et al., 2005; Richardson et al., 2008; Sahay et al., 2008; Savić et al., 2003). However, the excretion of the polymers from target cells after they have released the incorporated drugs has not yet been clarified in detail, although information about the clearance of carriers from cells is important from the perspective of safety. In this study, we examined the trafficking of a polymeric nanoparticulate carrier in detail, including the efflux of the polymers from cells to medium, by direct measurement of doxorubicin (Dox) covalently bound to the block copolymer. This technique avoids the necessity of considering the effects of exogenously tagged fluorescent probes on the intracellular trafficking.

Dox is one of the most effective available anticancer drugs in spite of its severe toxic effects, especially cardiotoxicity (Olson et al., 1988). As the carrier we used a PEG-poly(aspartic acid) block copolymer with covalently bound Dox (Fig. 1) (Yokoyama et al., 1999), because Dox has relevant hydrophobicity to form globular micelles by means of the hydrophobic interactions, and inherent fluorescence to investigate the intracellular trafficking of the carrier itself. Dox is partially covalently bound to the side chain of the aspartic acid (about 45% of aspartic acids), so that prepared Dox-conjugated block copolymers show good Dox entrapment efficiency possibly due to the π–π interaction between conjugated and incorporated Dox molecules (Bae and Kataoka, 2009; Nakanishi et al., 2001). Therefore, in this carrier system, there are two kinds of Dox; one is Dox covalently bound to block copolymers, and the other is free Dox which is incorporated in the inner core and has a pharmacological activity by its release from the inner core. The inner core of the micelles is greatly hydrophobic owing to the conjugated Dox, while the PEG of the outer layer prevents uptake by the RES. The resulting micelle effectively accumulates in tumor tissue by the EPR effect and shows much stronger activity than free Dox (Nakanishi et al., 2001). Because the block copolymer can form globular micelles by means of hydrophobic interactions with the conjugated Dox, as shown in Section 3.1, we used a carrier without incorporated free Dox to investigate the intracellular trafficking of the carrier itself. Furthermore, by quantifying directly the amount of Dox covalently bound to the polymers, we could measure the intracellular amount of the polymers.

2. Materials and methods

2.1. Cells and micelles

HeLa cells (Health Science Research Resources Bank, Osaka, Japan) were kept in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences Inc., Tokyo, Japan) and 100 U/mL penicillin/streptomycin (Invitrogen). Cells were grown in a humidified incubator at 37 °C under 5% CO_2. Dox-bound polymeric micelles and fluorescent dye (DBD)-labeled PEG-polyaspartate block copolymers partially modified with 4-phenyl-1-butanol were synthesized by Nippon Kayaku Co. Ltd. (Tokyo, Japan) (Nakanishi et al., 2001).

2.2. Physicochemical data of Dox-bound micelles

The diameters and distribution of micelles were determined by using dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern, UK) at 25 °C. The micelles were dissolved in water and filtered through a 0.2-μm filter before measurement. Atomic force microscopy (AFM) measurements were conducted with a NanoWizard II (JPK Instruments, Berlin, Germany) at room temperature. Images were obtained in tapping mode using a commercial microcantilever with a spring constant of 150 N/m (Olympus Corporation, Tokyo, Japan). AFM images were processed with SPM image processing v. 3 software from JPK Instruments.

2.3. Quantitation of Dox-bound polymers in HeLa cells

The amounts of Dox-bound polymers in HeLa cells were determined by measuring the amount of doxorubicinone, which is released by acid hydrolysis of Dox-bound polymers (Fig. 1b). HeLa cells (1.5×10^5) were plated in 35-mm glass-bottom dishes coated with poly-L-lysine (Matsunami, Osaka, Japan) in DMEM containing 10% FBS and 100 U/mL penicillin/streptomycin. After incubation for two days (37 °C, 5% CO_2), the cells were exposed to 50 μg/mL Dox-bound polymers in culture medium. After the indicated durations, the cells were washed and kept in phosphate-buffered saline (PBS) or Hank's balanced salt solution (HBSS; Invitrogen). The cells were trypsinized with 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA) (Invitrogen) and collected. Cells were then washed with PBS three times, and a small part of the cell suspension was used for cell counting. After centrifugation at 1000 rpm for 5 min, cell pellets were resuspended in 100 μL PBS, and the suspension was divided into two parts (50 μL was used with acid hydrolysis and 50 μL without) and stored at −80 °C until analysis. After thawing, the cell suspensions were disrupted by ultrasonic liquid processor (ASTRASON 3000, Misonix, NY, USA) for 1 min. Then, 50 μL of suspension was hydrolyzed by 0.5 N HCl at 50 °C for 15 h. After hydrolysis, samples were deproteinized with methanol, followed by centrifugation at $15{,}000 \times g$ for 5 min at 4 °C. The supernatant was then neutralized with ammonium buffer, and evaporated to dryness under reduced pressure (Savant SpeedVac concentrator, Thermo Fisher Scientific, MA, USA). The residues were resuspended in 60% methanol, and the doxorubicinone released from the polymers by acid hydrolysis was quantified by ultra-high-performance liquid chromatography by using our previously reported method (Sakai-Kato et al., 2010) to determine the amount of intracellular Dox-bound polymers (Fig. 1b). The other 50 μL of cell suspension was treated in the same way but without the hydrolysis step to evaluate the amount of free doxorubicinone, that is, doxorubicinone not derived from Dox-bound polymers. The results of three independent experiments were averaged and analyzed statistically by *t*-test.

2.4. In vitro cytotoxicity

HeLa cell lines were evaluated in the present study. The HeLa cells were maintained in monolayer cultures in DMEM containing 10% FBS and 100 U/mL penicillin/streptomycin. WST-8 Cell Counting kit-8 (Dojindo, Kumamoto, Japan) was used for cell proliferation assay. 3000 cells of HeLa cell line in 100 μL of culture medium were plated in 96 well plates and were then incubated for 24 h at 37 °C. Serial dilutions of Dox-bound polymers, micelles incorporating free Dox or just free Dox were added, and the cells were incubated for 24