Journal of Controlled Release 192 (2014) 167–173

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Suppression in mice of immunosurveillance against PEGylated liposomes by encapsulated doxorubicin

Jantana Yahuafai [a,b], Tomohiro Asai [a], Genki Nakamura [a], Tatsuya Fukuta [a], Pongpun Siripong [b], Kenji Hyodo [c], Hiroshi Ishihara [c], Hiroshi Kikuchi [c], Naoto Oku [a,*]

[a] Department of Medical Biochemistry, University of Shizuoka School of Pharmaceutical Sciences, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan
[b] Natural Products Research Section, Research Division, National Cancer Institute Thailand, 268/1 Rama 6, Rajthavee, Bangkok 10400, Thailand
[c] Global Formulation Research, Pharmaceutical Science & Technology Core Function Unit, Eisai Product Creation Systems, Eisai Co. Ltd., 5-1-3 Tokodai, Tsukuba 300-2635, Japan

ARTICLE INFO

Article history:
Received 28 February 2014
Accepted 10 July 2014
Available online 17 July 2014

Keywords:
PEGylated liposomes
Doxorubicin
Immunosurveillance
Repetitive administration
Biodistribution

ABSTRACT

PEGylated liposomes (PEG-lip) can escape from recognition by immune system and show a longer half-life in the blood than non-PEGylated liposomes. In this study, we investigated the influence of injected PEG-lip encapsulating doxorubicin (PEG-lip-DOX) on the biodistribution of subsequently injected PEG-lip in mice. PEG-lip-DOX, free doxorubicin or empty PEG-lip were initially injected into BALB/c mice via a tail vein, and 3 days later [^{3}H]-labeled PEG-lip ([^{3}H] PEG-lip) were injected into these same mice. At 24 h after the injection, the distribution of [^{3}H] PEG-lip in the liver and spleen was significantly reduced in the PEG-lip-DOX group compared with that in the free doxorubicin or PEG-lip group. Consequently, the plasma concentration of [^{3}H] PEG-lip was significantly elevated by the pretreatment with PEG-lip-DOX. Altered pharmacokinetics was observed at least until 72 h after the injection of [^{3}H] PEG-lip. The influence of the injected PEG-lip-DOX on the pharmacokinetics of the subsequently injected [^{3}H] PEG-lip was clearly observed from 1 to 14 days, and slightly observed on days 21 and 28, after the injection of the PEG-lip-DOX. Flow cytometric analysis showed that the number of liver Kupffer cells was significantly reduced after the treatment with PEG-lip-DOX. On the other hand, a similar alteration in the distribution of the subsequently injected [^{3}H] PEG-lip was observed in immunodeficient mice such as BALB/c nu/nu and severe combined immunodeficiency (SCID) mice. These findings suggest that immune cells including liver Kupffer cells responsible for recognizing PEG-lip were selectively damaged by the encapsulated doxorubicin in PEG-lip injected initially, which damage led to prolongation of the half-life of subsequently injected [^{3}H] PEG-lip in the blood.

1. Introduction

Two major goals of a liposomal drug delivery system are to achieve an adequate drug concentration at target disease sites and to reduce distribution of the drug in healthy tissues. In the systemic administration of liposomes, serum opsonins are adsorbed onto the liposomal surface in the blood, which molecules trigger recognition by the reticuloendothelial system (RES) [1]. Circulating liposomes are easily recognized by resident macrophages called Kupffer cells in the liver, which cells comprise the major population in the RES [2]. Consistent with this fact, the administration of liposomal doxorubicin depletes the liver macrophage population because of drug cytotoxicity [3]. Modification of the liposomal surface with hydrophilic polymers such as polyethyleneglycol (PEG) results in significantly reduced recognition by the host immune system and prolongation of the circulation time in the blood [4,5]. Long-circulating PEGylated liposomes (PEG-lip) can passively accumulate in tumors after intravenous injection via the enhanced permeability and retention (EPR) effect [1,6]. Thus, delivery of anticancer drugs via PEG-lip is a promising approach for the treatment of tumors. In fact, Doxil®, an approved PEG-lip encapsulating doxorubicin, is used to treat ovarian cancer, breast cancer, and AIDS-related Kaposi's sarcoma. The clinical advantage of Doxil® is demonstrated by a marked reduction in the side effects of doxorubicin, such as cardiotoxicity [7]. Prolonged exposure of tumors to Doxil® versus shorter exposure to doxorubicin is consistent with its higher antitumor activity in preclinical models and also with its clinical efficacy in patients with cancer [8,9].

Although PEG-lip have significant potential to escape from the RES-trapping compared with non-PEGylated liposomes, they are also recognized by the host defense system. Daemen et al. [10] reported that 5 mg/kg doxorubicin encapsulated in PEG-lip was cytotoxic toward liver macrophages of rats. In these rats, the blood clearance of bacteria was significantly delayed, since the phagocytic activity and numbers of liver macrophages had been reduced. These data suggest that the

* Corresponding author. Tel.: +81 54 264 5701; fax: +81 54 264 5705.
E-mail address: oku@u-shizuoka-ken.ac.jp (N. Oku).

http://dx.doi.org/10.1016/j.jconrel.2014.07.010
0168-3659/

recovery of liver macrophages might be important to prevent infections and cancer metastasis. Robert et al. [11] determined the influence of repetitive administration of 5.67 mg/kg doxorubicin encapsulated in PEG-lip on the pharmacokinetics of doxorubicin in a rat brain tumor model. By the repetitive administration, an increase in doxorubicin concentration in the plasma and tumors and a decrease in that in the liver and spleen were observed after the second injection. Gabizon et al. [12] demonstrated in mice that the initial administration of Doxil® (4 mg/kg as doxorubicin) increased the plasma concentration of doxorubicin after a subsequent injection of it. In addition, Ohara et al. [13] demonstrated an increase in the plasma concentration of doxorubicin after Doxil® administration to mice that had been depleted of liver Kupffer cells by the pretreatment with clodronate liposomes. These previous findings indicate that liver Kupffer cells responsible for immunosurveillance against PEG-lip might be damaged by encapsulated doxorubicin.

On the other hand, we and Ishida et al. [14,15] previously showed that PEG-lip could be recognized by splenic marginal zone B cells as a thymus-independent type 2 (TI-2) antigen. These B cells recognize PEG-lip and secrete IgM antibody, resulting in the rapid elimination of subsequently injected PEG-lip from the blood [15]. This rapid elimination is called the accelerated blood clearance (ABC) phenomenon [16]. The ABC phenomenon can be observed when the first dose of PEG-lip is low (less than approximately 1 μmol phospholipids/kg) [17,18]. These findings indicate that splenic marginal zone B cells are also involved in host immunosurveillance against PEG-lip.

In the present study, we determined the influence of initially injected PEG-lip encapsulating doxorubicin on the biodistribution of subsequently injected PEG-lip in mice to explore the mechanism of immune responses. Although some previous studies monitored the concentration of doxorubicin in each organ and in the blood [11–13], the present study used [^{3}H]-labeled PEG-lip ([^{3}H] PEG-lip) to evaluate the distribution of PEG-lip themselves. By using [^{3}H] PEG-lip, the difference in doxorubicin metabolism between each injection can be ignored. Furthermore, we focused on the correlation between the depletion of immune cells and pharmacokinetic alteration of PEG-lip.

2. Materials and methods

2.1. Materials

Hydrogenated soybean phosphatidylcholine (HSPC), cholesterol, and distearoylphosphoethanolamine-*N*-[methoxy (polyethyleneglycol)-2000] (mPEG-DSPE) were the gifts of Nippon Fine Chemical, Co. Ltd (Takasago, Hyogo, Japan). [^{3}H] cholesterylhexadecyl ether was purchased from GE Healthcare (Little Chalfont, UK); and doxorubicin, from Kyowa Hakko Kirin (Tokyo, Japan).

2.2. Animals

Five-week-old male BALB/c mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Male BALB/c nu/nu and severe combined immunodeficiency (SCID) mice of the same age were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The animals were cared for according to the animal facility guidelines of the University of Shizuoka.

2.3. Preparation of PEGylated liposomes encapsulating doxorubicin

Liposomal doxorubicin was prepared with the same lipid components and concentrations as those in Doxil®. The final lipid contents in the preparation were HSPC (9.58 mg/mL), cholesterol (3.19 mg/mL), and mPEG-DSPE (3.19 mg/mL). At first, HSPC and cholesterol dissolved in chloroform were evaporated to obtain a thin lipid film. In the case of radiolabeling, a trace amount of [^{3}H] cholesteryl hexadecyl ether (74 kBq/mouse) was added to the initial chloroform solution. Then, liposomes were formed by hydration with 250 mM ammonium sulfate. This liposomal solution was frozen and thawed 3 cycles, sonicated for 10 min, and then extruded 5 times through polycarbonate membrane filters with pore sizes of 0.4 μm and 0.1 μm (Nuclepore, Cambridge, MA, USA). After ultracentrifugation at 453,000 ×*g* for 1 h at 4 °C (HITACHI, Tokyo, Japan), the liposomes were resuspended in PBS and then incubated with 3.3 mg/mL doxorubicin at 65 °C for 1 h. Subsequently, the liposomal surface was PEGylated by incubating the liposomes with MPEG-DSPE for 15 min at 65 °C. PEG-lip encapsulating doxorubicin (PEG-lip-DOX) were centrifuged at 453,000 ×*g* for 1 h at 4 °C (HITACHI) to remove unloaded doxorubicin and then resuspended in PBS. The concentration of doxorubicin was determined by its absorbance at 484 nm. The particle size and ζ-potential of liposomes were measured by use of a Zetasizer Nano ZS (MALVERN, Worcestershire, UK) after dilution of the liposomes with PBS (pH 7.4).

2.4. Biodistribution of subsequently injected PEGylated liposomes in mice

BALB/c male mice (5-week-old) were intravenously injected with different doses of PEG-lip-DOX (1.25, 2.5 or 5 mg/kg as doxorubicin), free doxorubicin (5 mg/kg), empty PEG-lip or PBS via a tail vein. At 1, 3, 5, 7, 14, 21 or 28 days after the injection, these mice were intravenously injected with [^{3}H] PEG-lip via a tail vein. The lipid dose of liposomes in each injection was 60 μmol phospholipids/kg. At 24, 48 or 72 hours after the injection of [^{3}H] PEG-lip, the mice were sacrificed under deep anesthesia for collection of the blood. The collected blood was centrifuged (700 ×*g*, 15 min, 4 °C) to obtain the plasma. Then, the heart, lungs, liver, spleen, and kidneys were removed, washed with PBS, and weighed. The radioactivity in the plasma and in each organ was determined with a liquid scintillation counter (LSC-7400, Hitachi Aloka Medical, Tokyo, Japan). Similar experiments were performed by using 5-week-old BALB/c nu/nu and SCID male mice. Distribution data were presented as % injected dose per tissue or per 100 mg tissue. The total amount in the plasma was calculated based on the body weight of mice, where the plasma volume was assumed to be 4.27% of the body weight based on the data of total blood volume.

2.5. Flow cytometric analysis of liver Kupffer cells

Mice were intravenously injected with PEG-lip-DOX (5 mg/kg) or PBS. After their livers had been dissected on day 3, 7 or 14 post-injection, a single-cell suspension was prepared as reported previously [19,20]. In brief, the mice were anesthetized by an intraperitoneal administration of 450 mg/kg trichloroacetaldehyde monohydrate (Wako, Osaka, Japan). Each liver was perfused with Hanks' balanced salt solution (HBSS) via the heart at 28 °C until the liver had become completely discolored and then with 0.16 mg/mL collagenase IV in HBSS for 10 min. Immediately after the perfusion, the liver was removed, minced with scissors, and incubated for 10 min at 37 °C with HBSS containing 0.16 mg/mL collagenase IV and 10 μg/mL DNaseI. The liver homogenates were filtered through nylon gauze (40-μm pore size, BD Biosciences, San Jose, CA, USA) to remove undigested tissue. The obtained single-cell suspensions were centrifuged at 50 ×*g* for 3 min at 4 °C to separate the parenchymal cells from nonparenchymal ones. Each supernatant (nonparenchymal cells), enriched in Kupffer cells, was centrifuged for 10 min at 500 ×*g*. The pellet was then resuspended in PBS containing 1% bovine serum albumin (BSA). Cell viability was assessed by trypan blue dye exclusion.

After total nonparenchymal cells in whole liver were counted, a part of them (1×10^6 cells/100 μL) were blocked with Fc-blocker for 10 min on ice. Thereafter, the cells were incubated with Kupffer cell-reactive anti-F4/80 antibody directly conjugated with phycoerythrin (PE; BioLegend, San Diego, CA, USA) in the dark for 30 min on ice, and then washed with PBS containing 1% BSA. Flow cytometric analysis was performed by using a BD FACS Canto II analyzer with FACS Diva software (BD Biosciences) to determine the proportion of Kupffer cells. The